VOLUME 4
NUMBER 1
1969

Acta biochimica et biophysica

ACADEMIAE SCIENTIARUM HUNGARICAE

AKADÉMIAI KIADÓ, BUDAPEST

Acta Biochimica et Biophysica

Academiae Scientiarum Hungaricae

Szerkeszti:

STRAUB F. BRUNÓ és ERNST JENŐ

Technikai szerkesztők:

SAJGÓ MIHÁLY és NIEDETZKY ANTAL

Szerkesztőség címe: Budapest XI., Karolina út 29. (biokémia);
Pécs, Rákóczi út 80. (biofizika)

Az *Acta Biochimica et Biophysica*, a Magyar Tudományos Akadémia idegen nyelvű folyóirata, amely angol nyelven (esetleg német, francia vagy orosz nyelven is) eredeti tanulmányokat közöl a biokémia és a biofizika — fehérjék (struktúra és szintézis), enzimek, nukleinsavak, szabályozó és transzport-folyamatok, bioenergetika, izom-összehúzódás, radiobiológia, biokibernetika, funkcionális és ultrastruktúra stb. — tárgyköréből.

A folyóirat negyedévenként jelenik meg, a négy füzet évente egy kb. 400 oldalas kötetet alkot. Kiadja az Akadémiai Kiadó.

Előfizetési díja belföldre 120,— Ft, külföldre 165,— Ft kötetenként. Megrendelhető az Akadémiai Kiadónál (Bp. V., Alkotmány u. 21), a külföld részére pedig a Kultúra Könyv és Hírlap Külkereskedelmi Vállalatnál (Budapest I., Fő u. 32).

The *Acta Biochimica et Biophysica*, a new periodical of the Hungarian Academy of Sciences, publishes original papers, in English, on biochemistry and biophysics. Its main topics are: proteins (structure and synthesis), enzymes, nucleic acids, regulatory and transport processes, bioenergetics, excitation, muscular contraction, radiobiology, biocybernetics, functional structure and ultrastructure.

The Acta Biochimica et Biophysica is a quarterly, the four issues make up a volume of some 400 pp, per annum. Manuscripts and correspondence with the editors and publishers should be sent to

Akadémiai Kiadó Budapest 24 P.O.B. 502.

The subscription rate is 165,— Ft per volume. Orders may be placed with *Kultúra* Trading Co. for Books and Newspapers (Budapest 62, P.O.B. 149) or with its representatives abroad, listed on p. 3 of the cover.

Acta Biochimica et Biophysica — новый журнал Академии Наук Венгрии, публикующий на английском языке (возможно и на немецком, французском и русском языках) оригинальные статьи по проблемам биохимии и биофизики — белков (структура и синтез), энзимов, нуклеиновых кислот, процессов регуляции и транспорта, биоэнергетики, мышечного сокращения, радиобиологии, биокибернетики, функциональной структуры и ультраструктуры и т. д.

Журнал выходит ежеквартально, выпуски каждого года составляют том объемом около 400 страниц. Журнал выпускает Издательство Академии Наук Венгрии.

Рукописи и корреспонденцию просим направлять по следующему адресу:

Akadémiai Kiadó Budapest 24 P.O.B. 502.

Подписная цена — 165 Фт за том. Заказы принимает:

Предприятие по внешней торговле книгами и газетами «Kultúra» (Budapest 62. P. O. B. 149) или его заграничные агентства.

Acta Biochimica et Biophysica

Academiae Scientiarum Hungaricae

Volume 4

Akadémiai Kiadó, Budapest
1969

Acta Biochimica et Biophysica

Volume 4

Contents

Author Index

Subject Index

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (1) pp. 1—13 (1969)

Protein Composition of the Messenger RNA-containing Ribonucleoprotein of the Nucleus

J. MOLNÁR

Institute of Physiology and Biology of the Medical University, Pécs

(Received January 24, 1968)

The protein constituent of the mRNA-containing ribonucleoprotein component of the nucleus, the informofer, was analyzed by polyacrylamide gel electrophoresis.

The protein composition of the informofer was found to be relatively simple: three components were detected, one of which contained about half of the informofer proteins. Two to five additional minor components were found which contained altogether not more than 10—15 per cent of the proteins of the informofer.

The mRNA-containing polysome-like complexes which were isolated from the nucleus by means of an RNase inhibitor contained the same protein components as the 30 *S* monomers. Consequently the polysome-like complexes of the nucleus contain nothing but informofers and mRNA.

The proteins of the informofer were found to differ considerably from the proteins of the deoxyribonucleoprotein complex, from the ribosomal proteins and from the proteins of the soluble fraction of the nuclear extract. They are characterized by a relatively low electrophoretic mobility at pH 4.5.

Samarina et al. (1967b, 1968) isolated from the nucleus a mRNA-containing, hitherto unknown ribonucleoprotein which, under physiological conditions, occurs in the nucleus in the form of a polysome-like complex. This ribonucleoprotein is a complex of globular protein particles, the informofers, and high molecular polycystronic mRNA. It performs presumably the transport of mRNA from the nucleus to the cytoplasm. The polysome-like complex is, however, highly sensitive to RNase and can therefore be isolated from the nucleus only in the presence of an RNase inhibitor. Without RNase inhibitor the polysome-like complex disintegrates into its monomers as a result of ribonuclease action during extraction and subsequent manipulations. This explains why in earlier works the mRNA-containing ribonucleoprotein of the nucleus became known under the name of 30 *S* particle (Samarina et al., 1965, 1966, 1967a) which may be considered as the monomer of the polysome-like complex consisting of a protein component – the informofer – and the pertaining mRNA molecule.

It is known from earlier studies that in salt solutions of high ionic strength (0.7 M – 2.5 M NaCl or KCl) or in 4 – 6 M urea the 30 *S* particles dissociate into mRNA and proteins and that this dissociation is reversible. The sedimentation constant of the dissociated proteins of the particles is 4 – 6 *S* (Samarina et al., 1967c, d).

In the present work the protein composition of the 30 *S* particles was studied and compared to the protein composition of other cellular components. It was further investigated whether the polysome-like complexes contain proteins other than those of the informofer.

These investigations confirmed the relatively simple protein composition of the informofer which differs considerably from that of other cellular components. It has also been shown that the polysome-like complexes contain no other protein component than the proteins of the 30 *S* particles.

Materials and Methods

The mRNA-containing ribonucleoprotein component of the nucleus was prepared from rat liver.

The 30 *S* particles were prepared from isolated nuclei in the usual way (Samarina et al., 1967a). The polysome-like complexes were extracted from the nuclei in the presence of an RNase inhibitor (Samarina et al., 1968).

In several experiments the 30 *S* particles were prepared by the following modified procedure: On top of 15 ml of a 15 to 25 per cent linear sucrose gradient 3 ml of the nuclear extract were layered and centrifuged in the rotor SW-25 of the Spinco L ultracentrifuge for 6 hours at 25 000 rpm. This modification led not only to a reduction in the time required for centrifugation, but also to a higher concentration of the particles in the 30 *S* zone than usual.

In certain cases the 30 *S* particles were purified by means of resedimentation in a sucrose density gradient. In these cases the solution containing the 30 *S* particles obtained by the above procedure was dialyzed against 0.1 M NaCl – 0.001 M $MgCl_2$ – 0.01 M tris–HCl (pH 7.2) for 6 hours. Three milliliters from the solution in the dialysis bag were then layered on top of a 25 ml of 15 to 30 per cent sucrose gradient and centrifuged for 12 to 14 hours as above.

Preparation of the proteins of the 30 *S particles for electrophoresis*

Method I: To the preparation of the 30 *S* particles obtained from the sucrose gradient urea was added to 6 M final concentration. The mixture was allowed to stand at 4 °C for 12–16 hours and dialyzed for 10–12 hours against a buffer of 0.06 M K-acetate (buffer U), pH 6.8 containing 6 M urea and 10 per cent sucrose.

Method II: The 30 *S* particles were dialyzed against a 6 M urea solution for 12–16 hours. The proteins were precipitated with trichloroacetic acid (TCA, 5% final concentration). The precipitate was washed twice with a 5% TCA solution, dissolved in buffer U and dialyzed against the same buffer for 10–12 hours. The protein concentration in the dialysate was 1–5 mg/ml.

Method III: To the solution containing the 30 *S* particles in 6 M urea commercial crystalline bovine pancreatic RNase was added in a concentration of 10 μg/ml. Before use the enzyme solution (1 mg/ml) was heated to 90 °C for 10 minutes. The mixture was then incubated at 20 °C for 1 hour. The next steps (precipitation and washing of the proteins with TCA) were the same as in Method II.

The proteins of the polysome-like complexes and that of the RNase inhibitor were prepared according to Method III.

Preparation of ribosomal proteins

Rat liver ribosomes isolated and purified according to Tashiro (Tashiro, Siekevitz, 1965) were obtained by the courtesy of Dr M. I. Lerman (Institut Biologicheskoy i meditsinskoy khimii, AMNSSR, Moscow). Ribosomal proteins were extracted at 4 °C for two days with a 3 M LiCl solution containing 4 M urea (Spitnik–Elson, 1965). The precipitate consisting of RNA was removed by centrifugation and after precipitation with TCA the proteins were treated as described in Method II.

The histone proteins were prepared from the residual nuclear fraction after the extraction of the 30 *S* particles. The nuclei were extracted with cold 0.25 M HCl three times for 15 minutes. The extract was centrifuged at 12 000 *g* for 90 minutes and the supernatant fluid dialyzed against buffer U for 10 to 12 hours.

The proteins of the deoxyribonucleoprotein complex were isolated by the following procedure: The residual nuclear fraction after the extraction of the 30 *S* particles was treated with a 0.4 M NaCl solution and after centrifugation the sediment was extracted with 0.8 M NaCl for 14 hours. The solution was centrifuged at 30 000 rpm (Spinco L ultracentrifuge, rotor No. 30) for 90 minutes to precipitate the nucleoli. To the supernatant fluid NaCl was added to 2.5 M final concentration. The mixture was allowed to stand for a few hours and chromatographed on a Sephadex G 200 column. The material of the protein peak was collected, the salt in it removed by dialysis, the residue liophylized and used to prepare a protein solution of a concentration of 1 mg/ml in buffer U.

The soluble fraction of the nuclear extract was prepared as follows: When the nuclear extract is centrifuged in sucrose gradient, the 4 *S* to 6 *S* zone will be fairly rich in protein. The middle fractions of this peak were collected and treated in the same way as the 30 *S* particles according to Method III.

Polyacrylamide gel electrophoresis

Electrophoresis was carried out according to Reisfeld et al. (1962) in the presence of 6 M urea (Leboy et al., 1964) in a discontinuous buffer system.

The large pore (spacer) gel contained 2.5% acrylamide and 0.6% methylene-bis-acrylamide (pH 6.8).

The small pore (running) gel contained 10.5% acrylamide and 0.12% methylene-bis-acrylamide (pH 4.3). The polymerization of the gel was initiated with a saturated $(NH_4)_2S_2O_8$ solution.

For electrophoresis 65 mm long glass tubes with 5 mm internal diameter were used. On top of 1 ml polymerized small pore gel 0.13 ml of the large pore gel

was layered. The large pore gel was polymerized in sunlight or in artificial light. 0.01 – 0.2 ml quantities of the samples in the U-buffer (20 – 200 μg of protein) were applied to the top of the gel.

The buffer for electrophoresis contained 6.24 g β-alanine and 360 g urea per 1000 ml. The pH was adjusted to 4.5 with acetic acid.

Electrophoresis was performed in the cold room by applying first a current of 3 mA/column for 20 minutes and then a current of 5 mA/column for 3 hours. The amperage was raised by increasing the voltage gradient; the output voltage was 60 – 150 V.

After the completion of the electrophoretic run the gel was removed from the glass tubes and stained for 3 – 6 minutes in a 0.8% amidoblack (Chemapol) solution (methanol–water–glacial acetic acid, 5 : 5 : 1). The excess stain was removed from the gels by serial transfers in the above mixture. The gel columns were stored in this methanol – water – glacial acetic acid solution.

The stained and differentiated preparations were photographed on hard gradation diapositive plates. During exposure the gel was placed in the washing solution. The photographic plates (twofold magnification) were evaluated by means of a densitometer (Joyce, Loebl et Co., Microdensitometer MK III C). As this instrument does not record the areas of the individual peaks their proportions were determined gravimetrically.

Results

Protein composition of the 30 S particles

Figure 1 shows a characteristic electrophoretogram (a) and its densitometric curve (b). Three main components and additional 3 – 6 minor components consistently appeared independently of the method applied for the preparation of the proteins and of the concentrations of acrylamide and methylene-bis-acrylamide. The three main components were given the symbols A, B and C, beginning with the most rapidly moving band nearest to the cathode. The minor components were given the symbols B′, C′, C″ etc. in the order of their location behind the three main fractions. The bands moving more rapidly than A were given the symbols O′, O″ etc. starting from the cathode.

Under the experimental pH conditions every protein component moved in the direction of the cathode. In some experiments the pH was changed to the alkaline range and other buffer systems were also tested (Williams, Reisfeld, 1964). Though no buffer with a pH higher than 6.8 was used in the electrophoretic runs, in all pH ranges investigated the protein moved in the direction of the cathode and no new fractions appeared.

In quite a high number of experiments the relative amounts of the main protein components were: B > A > C. Sometimes, however, C occurred in higher quantities than A, or the ratio of the sum of bands C and A to B shifted. This was mostly, though not always, the case when a comparatively large amount of pro-

tein was layered on the gel. In such cases more protein remained at the start than usual. A more detailed analysis of this phenomenon will be given below.

The extent of contamination with RNA or its degradation products of protein preparations obtained by different methods was investigated. In these experiments the RNA of the 30 *S* particles was labeled with ^{14}C originating from ^{14}C

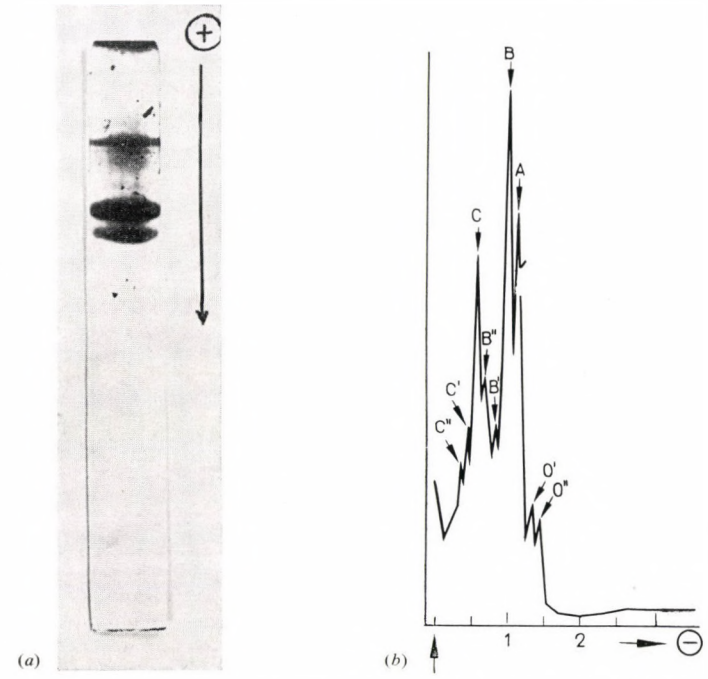


Fig. 1. Disc electrophoresis of the proteins of 30 *S* particles (*a*) and its densitogram (*b*). 100 μg of informofer protein washed with TCA (Method II). 3 hours, 5 mA/tube. Abscissa: displacement in cm. Ordinate: transparency of the photographed gel

labeled orotic acid. The data of Table 1 show that the RNA content of the preparation dropped by at least 85% even when the 30 *S* particles were dialyzed against urea only (Method I). Urea is known to activate latent RNase in the 30 *S* particles (Lukanidin et al., in preparation). If the proteins are subsequently washed with TCA, their nucleotide and RNA contamination will decrease further (Method II). Large doses of RNase in urea appeared to be the most effective (Method III).

These data indicate that our protein preparations may be considered practically free of RNA and nucleotides. Indeed irrespective of the method used for the preparation of the protein component of the 30 *S* particles no detectable difference was observed in the number of protein components or in their relative proportions or electrophoretic motilities.

Table 1

Changes in the RNA content of 30 S particles on various treatments

Expt. No.	Material	^{14}C radio-activity counts/min	Decrease in RNA content
1	Untreated 30 *S* particles	1200	—
	30 *S* particles dialyzed against 6 M urea at 4 °C for 14 hours	175	85.4
2	Untreated 30 *S* particles	1300	—
	Solution of the proteins of the 30 *S* material dialyzed against 6 M urea after 3 washings with TCA	65	95
3	Untreated 30 *S* particles	2200	—
	RNase treatment in 6 M urea (10 μg/ml RNase for 1 hour at room temperature according to Method III).	67	97

Larger quantities of RNase (Fig. 3, peak marked with an arrow) have no effect on the electrophoretic behaviour of the proteins of the 30 *S* particles.

The question arises whether the minor components always appear on the electrophoretogram, or they are only contaminating soluble proteins attached to the particles. It was namely observed that the relative proportion of the 4 *S* peak (which contains mostly protein) to the 30 *S* peak gradually decreased with repeated extractions. It may therefore be expected that the 30 *S* particles prepared from extracts No 2 and No 4, respectively, will have different protein compositions. However, with respect to the number of minor components there was no significant difference between the electrophoretograms, irrespective of the origin of the 30 *S* particles from extracts No 2, No 3 or No 4 (Figs. 2a, b and c). A certain shift appeared only in the relative proportion of the three main components. It should be mentioned, however, that in such cases the quantity of proteins subjected to electrophoresis was larger than usual in order to make the minor components clearly visible.

The protein composition of 30 *S* particles purified by resedimentation in a sucrose density gradient was also investigated. The minor components were just as much present in these preparations as in the proteins of the 30 *S* particles purified to a lesser degree (Fig. 3).

The experimental results suggest that the minor protein components are structural elements of the protein complex of the 30 *S* particles (informofer). Up to the present we have been unable to determine their number accurately. Their average quantity does not exceed 10–15 per cent of the overall protein content of the 30 *S* particles.

Comparison between the proteins of informofer and of other cellular components

The proteins of the 30 *S* particles were compared to other proteins under identical conditions of electrophoresis.

Because of their more basic character ribosomal proteins moved to a much greater distance than the informofer proteins (Fig. 4).

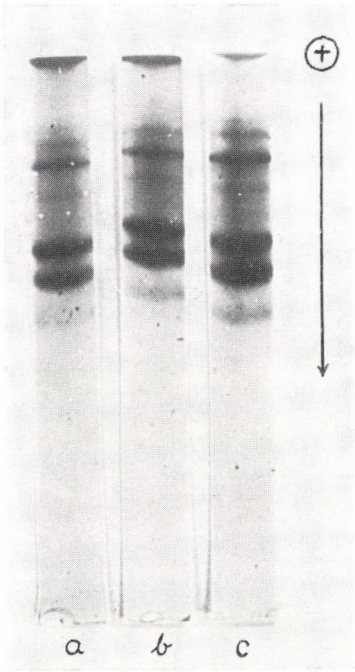


Fig. 2. Disc electrophoresis of the proteins of 30 *S* particles prepared with urea (Method I). The 30 *S* particles derived from the 2nd (a), 3rd (b) and 4th (c) nuclear extracts. 170 (a) and 200 (b) and (c) μg of informofer proteins were electrophoretized for 3 hours; 5 mA/tube

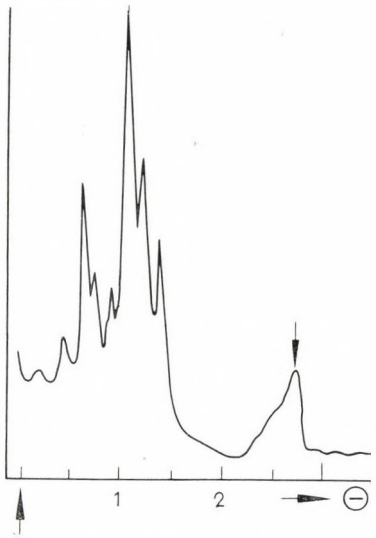


Fig. 3. Densitogram of the electrophoretogram of the proteins of 30 *S* particles purified by resedimentation in sucrose gradient. 100 μg of informofer protein prepared in the presence of RNase and washed with TCA (Method III). 3 hours, 5 mA/tube. (The band marked with an arrow is produced by RNase). Abscissa: displacement in cm. Ordinate: transparency of the photographed gel

Histones were also examined. On the electrophoretogram the two proteins are located separately (Fig. 5). The same is true for the proteins of the deoxyribonucleoprotein complex whose electrophoretogram is essentially identical with that of the histones.

Several components of the "light fraction" of the nuclear extract (4–6 *S* peak) were located in the same zone as the proteins of the 30 *S* particles. It is, however, clearly visible that none of the bands of the "light fraction" proteins

coincided with any of the main components of the informofer which was also subjected to electrophoresis as a control (Fig. 6). It should further be mentioned that the composition of the soluble proteins of extracts No 2, No 3 and No 4 were very similar to each other and no essential difference between them could be demonstrated. It was, however, observed that occasionally some of minor com-

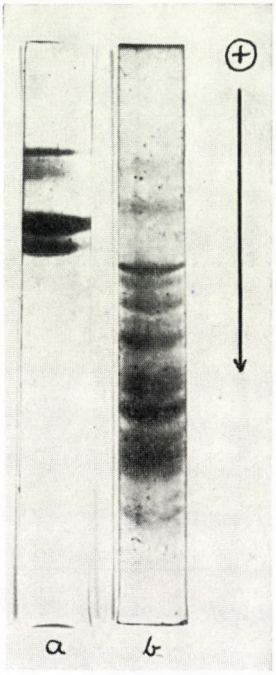


Fig. 4. Disc electrophoresis of the proteins of 30 *S* particles (a) and of ribosomal proteins (b). 90 μg of informofer proteins prepared according to Method III and 40 μg of ribosomal proteins were electrophoretized for 3 hours; 5 mA/tube

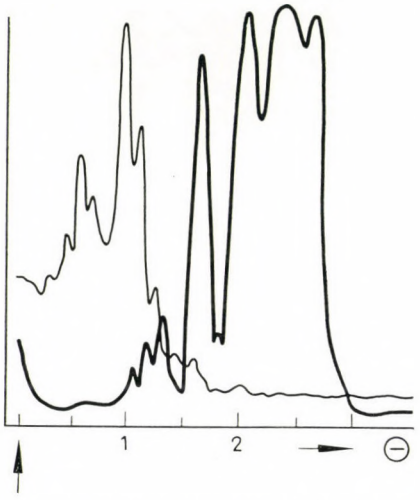


Fig. 5. Disc electrophoresis of informofer proteins (thin line) and of histone proteins (thick line). 100 μg of informofer proteins (washed with TCA according to Method II) and 40 μg of histone proteins were electrophoretized for 3 hours; 5 mA/tube. Abscissa: displacement in cm. Ordinate: transparency of the photographed gel

ponents in the protein preparation from the 30 *S* particles coincided with certain bands of the proteins of the soluble fraction.

The fact that the proteins of the 4–6 *S* peak are not the same as the proteins of the 30 *S* particles is in agreement with experimental results indicating that only the 30 *S* particles or their proteins are capable of binding specifically mRNA, while the proteins of the "light fraction" are devoid of this property (Samarina et al., 1966, 1967d).

The protein composition of the nuclear polysome-like complexes

The data reported so far are the results of gel electrophoretic studies of proteins prepared from 30 *S* particles which have been isolated by the standard method (Samarina et al., 1967 a). In the course of the experiments it became clear, however, that the 30 *S* particles were monomers of a more complicated polysome-like complex (Samarina et al., 1967b, 1968). Analysis of the proteins of this polysome-like complex was expected to answer the question whether proteins other than the informofer proteins were also present in these complexes.

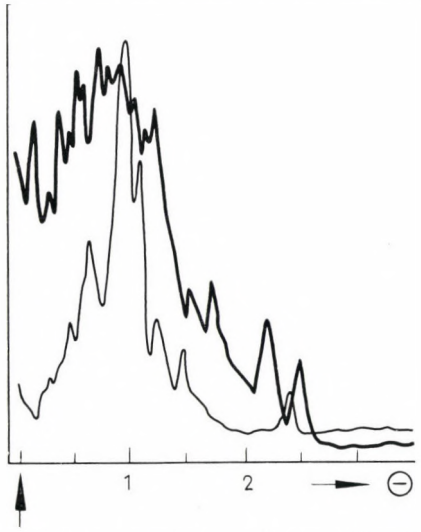


Fig. 6. Disc electrophoresis of the proteins of 30 *S* particles (thin line) and of the proteins of the soluble fraction (4—6 *S* peak) of the nuclear extract (thick line). 80 μg of informofer preparation (Method III, see Materials and Methods) and 200 μg of protein of the 4—6 *S* peak. 5 mA/tube, 3 hours. Abscissa: displacement in cm. Ordinate: transparency of the photographed gel

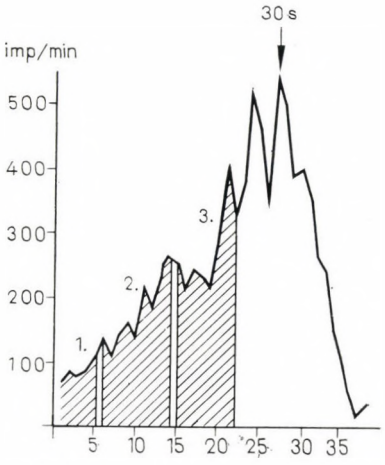


Fig. 7. Sedimentation diagram of mRNA-containing nuclear extract (45 min labeling with ^{14}C orotic acid). The nuclear extract was obtained by using an RNase inhibitor (Samarina et al., 1967b). Conditions of ultracentrifugation in the Spinco model L: 2 ml sample was layered on 25 ml of 15 to 30 per cent linear sucrose gradient; SW 25, 4 hours at 25 000 rev/min, 2 to 4 °C

Since the polysome-like complexes were extracted in the presence of RNase inhibitor which contained also insufficiently fractionated proteins of the 105 000 *g* supernatant fraction of the cytoplasm, the proteins of the inhibitor preparation were also analyzed. Upon electrophoresis the proteins of the inhibitor preparation occupied a common zone with the informofer proteins, but, with the exception of band A no other common component was found in any of the parallel experiments (Fig. 9).

Proteins were prepared from the polysome-like complexes of the nuclear extract by pooling the fractions of the sucrose gradient as indicated in Fig. 7

(hatched zones) and by isolating the proteins from them using Method III. Zone 1 contains the fractions with *S* values higher than 140, zone 2 the fractions with *S* values between 95 and 140 and zone 3 the fractions with *S* values between 55 and 95.

The fractions with *S* values higher than 140 contained the same protein components as the 30 *S* particles (Fig. 8; the band marked with an arrow is produced by RNase). The proteins in zone 2 of the polysome-like complex were also equivalent to the informofer proteins and their components were easy to separate

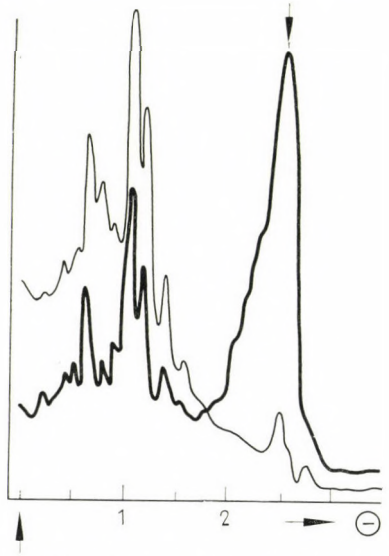


Fig. 8. Disc electrophoresis of the proteins of 30 *S* particles (thin line) and of the proteins prepared from the mRNA-containing polysome-like complexes of the nuclear extracts (thick line). Both proteins isolated by Method III (see Materials and Methods). 100 μg of informofer proteins and 35 μg of proteins prepared from particles with *S* values higher than 140 (Fig. 7, zone 1) were electrophoretized for 3 hours; 5 mA/tube. (The band marked with an arrow is produced by RNase). Abscissa: displacement in cm. Ordinate: transparency of the photographed gel

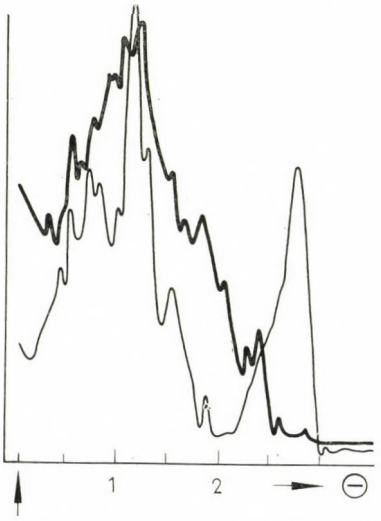


Fig. 9. Disc electrophoresis of the proteins of the mRNA-containing polysome-like complexes of the nuclear extract (thin line) and of the proteins of the RNase inhibitor (thick line). Proteins prepared from the zone of sucrose gradient with *S* values between 95 and 140 (Fig. 7, zone 2) and of the RNase inhibitor preparation by Method III (see Materials and Methods). 160 and 200 μg of proteins, 5 mA/tube, 3 hours. Abscissa: displacement in cm. Ordinate: transparency of the photographed gel

from the various fractions of the RNase inhibitor preparation (Fig. 9). The proteins of zone 3 gave a quite similar picture.

It was thus established that in the structure of the polysome-like "heavy" components besides mRNA only informofer proteins participate. Even in preparations which contained RNase inhibitor too (30–45 *S* zone) in addition to the

bands of the informofer proteins only components of the RNase inhibitor could be observed on the electrophoretograms, but in none of the cases was a different new component detected.

Discussion

Electrophoretic studies have pointed to a relatively simple protein composition of the informofer. About 50% of the proteins occur in one of the main components (B), while the other two main components (A and C) contain each 20% of the proteins. The quantity of the minor components amounts to 10% of the total proteins of the informofer.

As known from our earlier publications the molecular weight of the informofer is approximately 800 000 daltons. As component B represents 50% of the proteins, at least 400 000 units of the molecular weight of the proteins pertain to this component. It has also been demonstrated that the protein components of the dissociated 30 *S* particles upon centrifugation in a sucrose density gradient will be located in the 4–6 *S* zone, therefore their molecular weight is obviously lower than 400 000. This leads to the conclusion that a single informer is composed of several identical protein sub-units. Assuming an average molecular weight of 25 000 for these protein sub-units, a single informofer should contain 16 molecules of protein B and 7–8 molecules of proteins A and C. Under these conditions the minor components will have a molecular weight of about 15 to 20×10^3.

If the molecular weight assigned to the minor components is divided by the actual number of the latter, enough molecular weight units pertain to each individual minor component to justify their qualification as structural elements of the informofer. This assumption is supported by the fact that the number of minor components is the same in the electrophoretograms of 30 *S* particles irrespective of whether these particles have been prepared from Extract No 2, No 3 or No 4. Moreover, these minor components are also present in 30 *S* particles which have been subjected to additional purification by resedimentation in a sucrose density gradient. Nevertheless the possibility that the minor components are adsorbed on the particles as impurities originating e.g. from the deoxyribonucleoprotein complex cannot be excluded with absolute certainty.

Neither can an unequivocal answer be given to the question concerning occasional changes in the relative proportions of the three main protein components. This change consists of an increase in component C relative to component A, or in a simultaneous increase of both components A and C in relation to component B. This phenomenon may be due to aggregation resulting from the loss of some of the material during preparation, or to a mutual transformation of the individual protein components (aggregation–deaggregation). It may further be possible that the electrophoretogram contains only a single type of protein molecule in various stages of polymerization. The first assumption seems to be the most probable, namely that each band on the electrophoretogram represents an in-

dividual protein component with a defined primary structure. It was namely observed that precipitation and washing with TCA, or even treatment with hot TCA had no effect on the separation of ribosomal proteins by polyacrylamide gel electrophoresis (Traut, 1966). This assumption is supported by Traut's finding that during the electrophoresis of ribosomal proteins in polyacrylamide gel the 10 protein fractions investigated by him had different primary structures and it seems highly probable that the other fractions also consisted of individual proteins (Traut et al., 1967).

Our experiments clearly show that the composition of the informofer protein is relatively simple, considerably simpler than for example that of the ribosomal proteins (Waller, 1964; Kedes et al., 1966; Traut et al., 1967); further, that the individual informofer contains identical polypeptide chains. This simple structure explains the highly efficient self-assembly type reconstruction of the 30 *S* particles after dissociation (Samarina et al., 1967c, 1967d).

The results of electrophoresis yielded further evidence on the structural organization of nuclear polysome-like complexes. It has been confirmed that the latter contain indeed no other proteins than those of the informofer and that the polysome-like complexes are the complexes of mRNA with informofers.

The protein composition of the informofer may be considered typical and specific, as its proteins are not present among the ribosomal and histone proteins or in the soluble proteins of the nucleus. The isoelectric point of the proteins is nearly neutral or slightly basic under the given experimental conditons, but is definitely less basic than the isoelectric point of ribosomal proteins and even less so than that of histones.

The author wishes to thank Dr G. P. Georgiev, Head of the Virus and Cell Culture Laboratory of the Institute of Molecular Biology, Academy of Sciences, USSR and Dr Olga P. Samarina, senior research associate, for their valuable help and suggestions in the course of this work.

References

Kedes, L. H., Koegel, R. J., Kuff, E. L. (1966) J. Mol. Biol. *22* 359
Leboy, P. S., Cox, E. C., Flaks, J. G. (1964) Proc. Natl. Acad. Sci. US *52* 1367
Reisfeld, R. A., Lewis, U. J., Williams, D. E. (1962) Nature *195* 281
Spitnik-Elson, P. (1965) Biochem. Biophys. Res. Comm. *18* 557
Samarina, O. P., Asriyan, I. S., Georgiev, G. P. (1965) Dokl. Akad. Nauk *163* 1510
Samarina, O. P., Krichevskaya, A. A., Georgiev, G. P. (1966) Nature *210* 1319
Samarina, O. P., Krichevskaya, A. A., Molnar, J., Bruskov, V. I., Georgiev, G. P. (1967a) Mol. Biol. USSR *1* 129
Samarina, O. P., Lukanidin, E. M., Georgiev, G. P. (1967b) Biochim. Biophys. Acta *142* 561
Samarina, O. P., Molnar, J., Lukanidin, E. M., Bruskov, V. I., Krichevskaya, A. A., Georgiev, G. P. (1967c) J. Mol. Biol. *27* 187
Samarina O. P., Molnar, J., Krichevskaya, A. A., Lukanidin, E. M., Bruskov, V. I., Georgiev

G. P. (1967d) Mol. Biol. USSR *1* 648
Samarina, O. P., Lukanidin, E. M., Molnar, J., Georgiev, G. P. (1968) J. Mol. Biol. *33* 251
Tashiro, Y., Siekevitz, P. (1965) J. Mol. Biol. *11* 149
Traut, R. R. (1966) J. Mol. Biol. *21* 571
Traut, R. R., Moore, P. B., Delius, H., Noller, H., Tissieres, A. (1967) Proc. Nat. Acad. Sci. US *57* 1294
Waller, J. P. (1964) J. Mol. Biol. *10* 319
Williams, D., Reisfeld, R. (1964) Annals N. Y. Acad. Sci. *121* 373

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (1) pp. 15—25 (1969)

Electrolytic Reduction of Disulfide Bonds and Biological Activity of Some Proteins

JUDIT NAGY, F. B. STRAUB

Institute of Medical Chemistry, University Medical School, Budapest

(Received June 4, 1968)

1. Electrolytic reduction of several proteins containing disulfide bonds has been studied in dilute buffer solutions near pH 7.

2. Addition of ethanol facilitates in most cases the reduction of disulfide bonds in these proteins.

3. The maximum number of disulfide bonds which may be reduced under these conditions without loss of biological activity is: lysozyme 0, ribonuclease 2, chymotrypsin 2, trypsin 3, soy-bean trypsin-inhibitor 2, and pig pancreatic amylase either 1 or 4 (see below).

4. The availability of disulfide bonds to electrolytic reduction (in dilute buffer solution) appears to be proportional to the number of polar residues in the neighbourhood of the disulfide bond.

5. The relative importance of the disulfide bonds in maintaining native protein structure decreases according to the increasing ratio of the total number of amino acid residues to the number of disulfide bridges.

6. Two different types of pig pancreas amylase can be prepared. One of them may be completely reduced without loss of activity, whereas in the other type of preparation the reduction stops after the appearance of 2 sulfhydryl groups.

Disulfide bonds of proteins have been reduced by various methods in order to study the role of this bond in maintaining the structure and function of proteins. Most of these experiments were carried out using high concentration of thiols (thioglycollate or mercaptoethanol) in the presence of a denaturing agent, mostly concentrated urea solution. The addition of urea, the distortion of the protein structure, seems necessary to obtain full or even partial reduction of the disulfide bonds in proteins if thiol compounds are used as reducing agents. From these studies it was concluded that biological activity is lost upon reduction of the disulfide bonds, apparently justifying the emphasis of textbooks on the outstanding importance of these covalent bonds in maintaining protein structure.

The present studies were undertaken to establish whether reduction *per se*, without applying denaturing conditions, would lead to a similar loss of biological activity. Our results have justified the doubts: if electrolytic reduction is applied in dilute neutral salt solution, a varying number of bonds may be reduced without change in enzyme activity. This result, we believe, is due to the fact that protein as well as solute concentration and pH applied in our experiments correspond to those conditions which have been found to be optimal in the reoxidation and

reactivation of reduced proteins, i.e. these are the conditions which favour the native structure of the proteins.

While our work was in progress, some reports had appeared on the reduction of enzymes under non-denaturing conditions (Sela, 1967; Light, Sinha, 1967). Although different reductive methods have been used, these results agree well with the findings reported below.

Materials and Methods

Enzyme preparations

Lysozyme (hen egg white), 2× crystallized, was obtained from Worthington Co. Chymotrypsin (bovine) 2× crystallized, was purchases from Reanal (Budapest) and Calbiochem (Los Angeles, Calif.). Ribonuclease (bovine) 2× crystallized, was a product of Reanal (Budapest). Trypsin (bovine) crystalline, was a Reanal preparation. Soy-bean trypsin-inhibitor was purchased from Sigma (St. Louis, Missouri), 3× crystallized, and Serva (Heidelberg), 5× crystallized.

Pig pancreatic amylase, 2–3 times recrystallized, was prepared according to Hatfaludi et al. (1966).

Another type of pig pancreatic amylase was prepared by a "mild method" in the following way:

The organs were removed and packed in ice soon after slaughtering the animal. Preparations started not later than 1.5 hours afterwards. All manipulations were carried out in a cold room at 2 °C. Fat and connective tissue were removed as far as possible and the chopped tissue was homogenized in successive 150 g portions with 100 ml ice-cold solution of 0.1 M Tris-buffer, pH 7, containing 2.5×10^{-4} M diisopropylphosphofluoridate and 2×10^{-3} M $CaCl_2$. The homogenate was immediately cooled to −2 °C and acetone (−4 °C) was added to reach an acetone concentration of 41 per cent. After 15 minutes the precipitate was removed by centrifugation. To the supernatant further acetone was added to reach 67 per cent acetone concentration. The precipitate settled rapidly, was centrifuged and taken up in a 0.01 M Tris buffer solution, pH 7, containing 2×10^{-3} M $CaCl_2$. The resulting solution was dialyzed against the same buffer for 20 hours at 2 °C. If a precipitate was formed during dialysis, this was centrifuged and discarded. The supernatant contains about 3 per cent protein.

A neutral, ice-cold, saturated ammonium sulfate solution was added to the clear dialyzed solution to reach 0.35 saturation. The resulting precipitate was dissolved and dialyzed as above. The protein concentration of the dialyzed solution was around 15 mg/ml. A second acetate fractionation was performed as described above, with the difference that the fraction precipitating between 33 and 64 per cent (v/v) acetone was collected and dialyzed in the cold against 0.01 M Tris-buffer, pH 7, containing 2×10^{-3} M $CaCl_2$.

Lysozyme activity was followed by measuring the decrease of optical density at 420 mμ of a Micrococcus lysodeicticus suspension in 0.05 M phosphate

buffer, pH 6.5, incubated at 22 °C. The optical density was followed for 5 minutes, the enzyme activity was calculated from the changes in the first two minutes.

Ribonuclease activity was measured by the method of Klee and Richards (1957) with slight modifications.

Chymotripsin and *trypsin* were assayed simultaneously by the method of Anson and by the azocoll method.

Soy-bean trypsin-inhibitor. An aliquot to be tested (containing about 30–40 μg of the inhibitor) was incubated for 30 minutes at 37 °C with excess chymotrypsin in 0.05 M phosphate buffer, pH 7.5, in a total volume of 1.5 ml. Chymotrypsin was then assayed after addition of 1 ml of azocoll solution in 0.05 M phosphate buffer, pH 7.5.

Amylase was assayed by the method of Smith and Roe (1949) with slight modifications.

Electrolytic reduction was carried out as described by Markus (1964) using a cathode vessel immersed in an ice bath. CO_2 was bubbled through the solution at an appropriate rate to compensate for the increase in hydroxyl ion concentration. Current density was usually 5 mA per square cm. Protein concentration varied between 1–2 mg/ml.

Sulfhydryl groups were determined with the Ellman reagent (1958) immediately after withdrawal of a 50–100 μl sample which was diluted either with Tris-buffer, pH 8, or with a concentrated urea solution containing phosphate buffer, pH 8. In the latter case all the SH groups, including the masked ones, became available to the reagent.

In a number of cases electrolytic reduction was carried out at higher pH values (pH > 9). The maximum number of sulfhydryl groups measured in these experiments, in which the enzymes were denatured, agreed with that expected from the analytical data of the respective enzyme.

Results

I. Rate and level of reduction

The time curve of the appearance of SH groups during electroreduction is illustrated in Figs 1–5. The experimental conditions were very similar in all cases, as far as salt and protein concentration and pH were concerned.

In previous studies several investigators have tried to establish whether during reduction of disulfide bonds certain types are reduced before others (Lindley, 1955; Cecil, Loening, 1960; Cecil, Wake, 1962). As could be expected, the availability of a particular disulfide bond to reduction depends, among others, on the method of reduction and experimental conditions. According to Markus (1964) the electrolytic reduction of insulin leads to rapid reduction of the interchain disulfide bridges, leaving the interchain bridge intact for some time.

The shape of the curves in Figs 1–5 suggests that disulfide bridges are split stepwise, the rate of the reduction of different disulfide bridges within the same

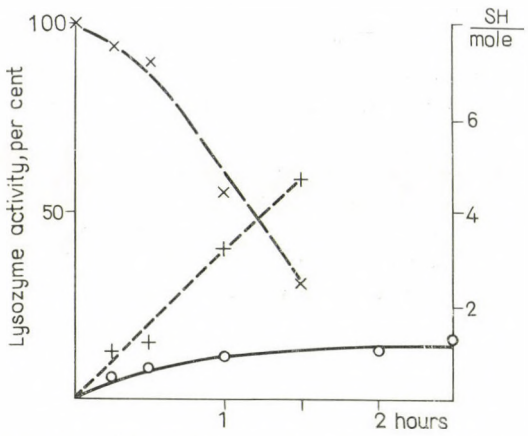


Fig. 1. Electrolytic reduction of lysozyme. ○ — ○: appearance of SH groups in 0.04 M phosphate buffer, pH 7.5; + — +: SH content during reduction in the presence of 30 per cent ethanol and 0.04 M phosphate buffer, pH 7.0; × — ×: enzyme activity during reduction in presence of alcohol

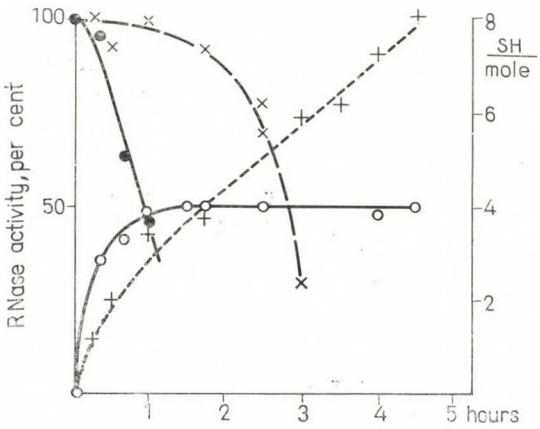


Fig. 2. Electrolytic reduction of ribonuclease. ○—○: SH content and ●—●: enzyme activity during reduction in 0.04 M phosphate buffer, pH 7.0. +—+: SH content and ×—×: enzyme activity during reduction in 30 volume per cent ethanol and 0.04 M phosphate buffer, pH 7.0

protein being different. When reduction is followed in time, it can be observed that two disulfide bonds of trypsin are reduced whithin one hour and the appearance of SH groups practically stops at these levels. The remaining disulfide bonds are reduced only in the later phase of the experiments, when the extensive loss of enzyme activity indicates the denaturation of the proteins (Figs 2–4). It may be mentioned that simple disulfides (e.g. cystine, oxidized glutathione) are reduced under such conditions at a much higher rate.

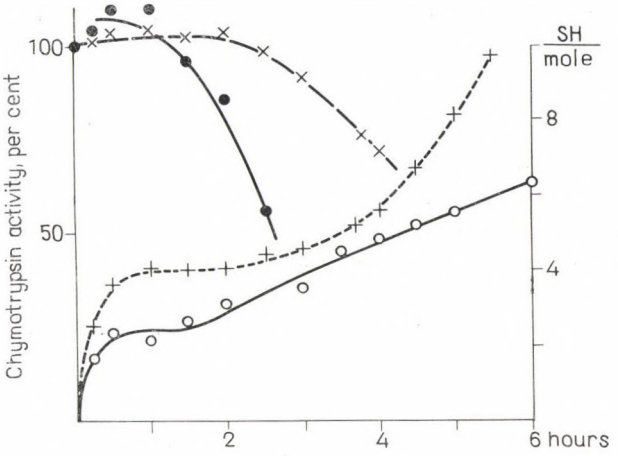


Fig. 3. Electrolytic reduction of chymotrypsin. ○—○: SH content and ●—●: enzyme activity during reduction in 0.04 M phosphate buffer, pH 7.0. +—+: SH content and ×—×: enzyme activity during reduction in 30 volume per cent ethanol and 0.05 M phosphate buffer, pH 7.5

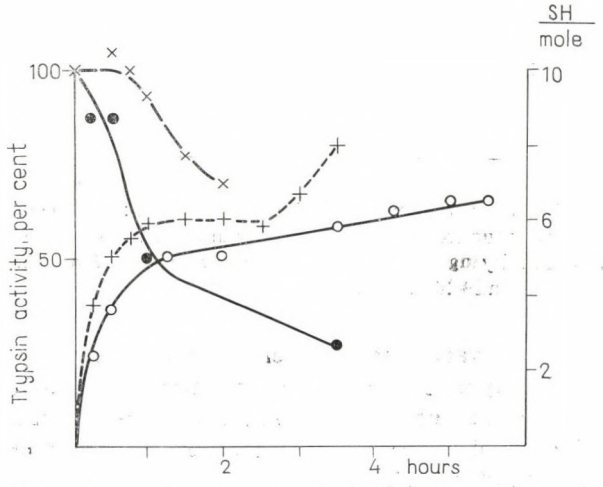


Fig. 4. Electrolytic reduction of trypsin. ○—○: SH content and ●—●: enzyme activity during reduction in 0.05 M phosphate buffer, pH 7.5. +—+: SH content and ×—×: enzyme activity in the presence of 30 volume per cent ethanol and 0.04 M phosphate buffer, pH 7.5

Addition of ethanol at concentrations between 10 and 45 per cent (v/v) has been tested in most cases. The results obtained using 30 per cent (v/v) ethanol are presented together with the experiments performed using buffer only (Figs 1–5). The presence of alcohol accelerates in many cases the reduction of those disulfide bonds which are less available to reduction in buffer solution. The effect of alcohol

is particularly interesting when the loss of enzyme activity is followed during reduction.

II. Enzyme activity after reduction of disulfide bonds

In the experiments described above the enzyme activity was determined on aliquots taken at the same intervals as the samples for SH determination. The changes in enzyme activity are shown in Figs 1 – 5.

In most cases we have regularly observed an increase in enzyme activity in the early phase of reduction. Whether the increase is due to a higher activity of the partially reduced enzymes or to removal of inhibitors, is not clear.

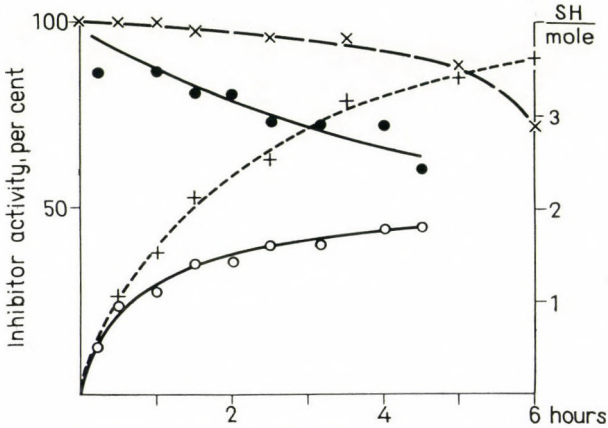


Fig. 5. Electroreduction of soy-bean trypsin-inhibitor. ○—○: SH content and ●—●: inhibitor activity during reduction in 0.04 M phosphate buffer, pH 7.5. +—+: SH content and ×—×: inhibitor activity during reduction in the presence of 30 volume per cent ethanol and 0.04 M phosphate buffer, pH 7.5

It would be difficult to evaluate the later phases of the reduction experiments, e.g. when half of the activity is lost and half of the disulfide bonds are reduced. We have therefore focussed our attention to that phase of the reduction, in which the enzyme activity did not decrease below 90 per cent of the original activity. These results are summarized in Table 1.

III. Influence of preparative methods on the reducibility of disulfide bonds in pig pancreatic amylase

During the past years pig pancreatic amylase has been studied in our laboratory from various points of view and several methods of preparation have been worked out. We have observed that different lots of amylase were reduced to a different extent. The variation has been traced to the method of preparation and two extremes are illustrated below.

Table 1

Reducible disulfide bonds of enzymes without loss of enzyme activity

Protein	Number of disulfide bridges	Total number of amino acid residues per number of SS bridges	Disulfide bridges reduced with less than 10% loss of activity	
			in buffer	in buffer + 30% ethanol
Lysozyme	4	31	0	0
Ribonuclease	4	31	0	2
Trypsin	6	37	2	3
Chymotrypsin	5	48	1	2
Soy-bean trypsin-inhibitor	2	103	—*	2
Pancreatic amylase	4	108	1 or 4**	—*

* not investigated
** according to method of preparation.

1. Amylase, pig pancreatic, recrystallized 2 – 3 times according to the method of Hatfaludi et al. (1966). During the course of the electrolytic reduction the protein SH content was determined both in Tris-buffer and in 8 M urea-containing phosphate buffer. It is known (Schramm, 1964) that the masked SH groups of amylase are measured only in the second case, whereas they do not react in Tris-buffer. Table 2 shows the results of such an experiment. Up to the reduction of one disulfide bridge (i.e. 2 SH measured in Tris-buffer and 4 SH in urea-phosphate) the activity does not diminish. Further reduction is very slow and if it occurs, it is connected with loss of enzyme activity.

2. *Amylase, pig pancreatic, prepared by a "mild method"* as described under Materials and Methods. In sharp contrast to the above preparation, the electrolytic reduction of amylase prepared by this "mild method" can be completely suppressed and the enzyme activity is retained until the appearance of all cysteine residues. As shown in Table 2, the masked SH groups remain masked during the course of the reduction. In the later phases of reduction part of the newly appearing SH groups are similarly not measured in Tris-buffer, only after addition of urea (8 M) and phosphate.

Discussion

Reduction of protein disulfide bridges is usually carried out using a great excess of a thiol compound in a solution of concentrated urea (Sela et al., 1959; Fleischman et al., 1963). Urea seems to be necessary to cause conformational changes which would make the disulfide bonds available to the thiol reagent. The resulting fully reduced protein is inactive. In many cases, however, it can be reoxidized to the original native enzyme containing the correct pairing of half cystine residues and regaining full enzyme activity. An early study of intermediate

Table 2

Electrolytic reduction of two different amylase preparations

(2 mg/ml amylase in both cases in 0.025 M phosphate buffer, pH 7.5. The molecular weight of amylase is assumed to be 50 000)

Time of reduction (hours)	Recrystallized amylase prepared according to Hatfaludi et al.			Amylase prepared by the "mild method" (see text)		
	SH groups/mole		enzyme activity Smith-Roe units/mg	SH groups/mole		enzyme activity Smith-Roe units/mg
	in Tris	in urea		in Tris	in urea	
0	0	1.5	3700	0.0	1.7	3800
0.5	1.1	2.5	4050	1.8	2.8	4000
1	1.2	3.2	3700	—	—	—
1.5	1.5	3.1	3500	3.4	5.2	4000
2	1.8	3.1	3500	—	—	—
2.5	1.9	3.5	—	4.4	7.3	4000
3	2.1	4.1	1800	—	—	—
3.5	3.8	4.6	1060	5.	10.0	4000
4	3.8	3.3	—	—	—	—
4.5	—	—	—	6.2	10.4	3800

changes during the process of reduction (White, Sandoval, 1962) suggested that a partially reduced RNase may still have full enzyme activity. This has been questioned (Asai, Morales, 1965).

Electrolytic reduction is a new approach to this problem, as the nascent hydrogen might be able to enter the fabric of the protein to such an extent that it is able to reduce disulfide bonds without permanent distortion of the protein structure. In some cases extensive reduction is observed with no loss of activity and it seems justified to assume that no great conformational changes follow the reduction of disulfide bridges. It is, therefore, assumed that the hydrogen bonds, hydrophobic interactions, electrostatic and van der Waals forces are able to hold together the molecule of such enzymes even when some of its disulfide bonds have been split.

It has been generally accepted that disulfide bonds are exceptionally important stabilizers of protein structure. Indeed, most pancreatic secretory enzymes are very stable under extreme conditions, e.g. high concentrations of urea, acid milieu, etc. It is particularly instructive also to compare the increasing stabilities of different lysozymes containing different numbers of disulfide bonds and nearly the same number of total amino acid residues (Jolles et al., 1967).

It may be admitted that disulfide bridges are important in the stabilization of the protein (or perhaps rather in facilitating its reversible renaturation) when it is exposed to such extreme environment. It does not follow, however, that they possess an equally predominant role at physiological pH values and solute concentrations. Our experiments show that their role is only relative: some, or even all of them may be broken with no change in enzyme (or inhibitor) activity.

We can compare the extent of reducibility which is compatible with biological activity, with the relative abundance of disulfide bridges in a protein. These data are found in Table 1 and suggest that the importance of disulfide bridges in maintaining the native structure of a protein decreases with inreasing ratios of total amino acid residues to half-cystines.

Addition of ethanol facilitated electrolytic reduction in most cases. Both the rate and the extent of the reduction could be increased by the addition of alcohol.

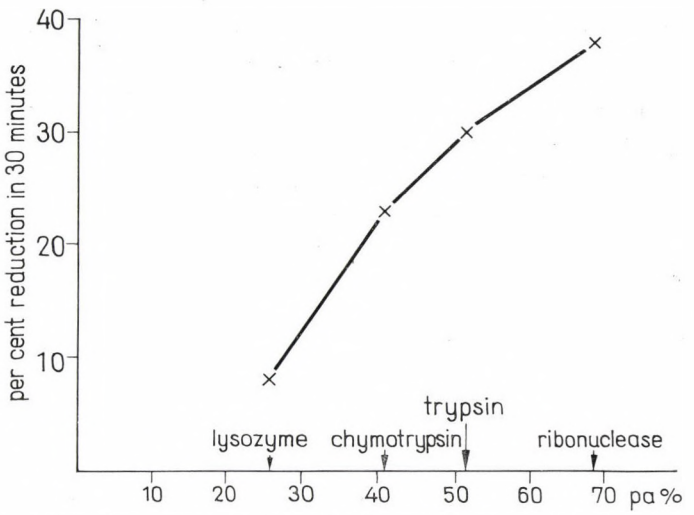


Fig. 6. Reducibility of some enzymes as a function of the percentage of polar amino acids present in their "disulfide containing dekapeptides" (= per cent *pa*)

Organic solvents are known to influence the conformation and enzyme activity (Előd i, 1961; Takemori et al., 1967). Our results which show an easier reducibility of disulfide bridges in some enzymes when 30 per cent (v/v) ethanol is present suggest a more flexible protein structure, a decrease in the interactions.

The effect of alcohol may be due to a number of different effects. It is known that in a mixture of alcohol and water the hydrophobic interactions and the dissociation of SH groups are weaker (Gorin, Clary, 1960) and the hydrogen bonds are stronger (Singer, 1962) in proteins if compared to the state of the protein in aqueous solution. A mixture of alcohol and water may in many respects simulate better the physiological environment than the dilute buffer, as under physiological conditions the protein molecule interacts with other proteins, lipids, etc. Therefore, the possibility that the behaviour of these enzymes in the presence of 30 per cent (v/v) alcohol is actually closer to their physiological behaviour, cannot be disregarded.

No attempt has been made in our work to determine the position of the disulfide bonds which are reduced without loss of activity. Although such studies

have been made by others during the time our work was in progress (Sela, Neumann, 1967; Light, Sinha, 1967), it is not possible nor advisable to assume simple analogies. When reducing agents like lithiumborohydride or phosphorothioate are used, the availability of disulfide bonds may be different from that observed with nascent hydrogen. We find some correlation between the composition of a protein and the reducibility of disulfide bonds in buffer solution. In Fig. 6 the initial rate of reduction of disulfide bonds of some proteins (the sequences of which are well established) is plotted against the percentage of polar amino acid residues which are found in the vicinity of the half-cystine residues. To do so, we define a "disulfide containing dekapeptide" as the sum of two pentapeptides which are joined by a disulfide bridge (two half-cystines and eight other amino acid residues which are the first and second neighbours of the half-cystines). The "dekapeptides" of lysozyme contain predominantly non-polar amino acid residues, whereas one can find increasingly more polar "dekapeptides "in chymotrypsin, trypsin and ribonuclease, corresponding to the reducibility of these enzymes. As the polar amino acid residues are found predominantly on the surface of the protein molecules, this correlation may simply mean that those disulfide bonds are first and more rapidly split which are found in the outer part of the protein.

The effect of ethanol agrees with this picture since in the presence of ethanol the non-polar interactions are weakened and disulfide bridges become more available.

Finally, we have to point out the differences in the reducibility of pig pancreatic amylase samples. It appears that the amylase preparation according to Hatfaludi et al. (1966) is rather resistant to electrolytic reduction. During the preparation the enzyme is brought in contact with denaturing agents (heating in the presence of butanol, dissolving the crystals in the urea solution) which, however, do not influence the activity of the enzyme and its ability to crystallize. On the other hand, the amylase prepared by the "mild method" – which has the same maximal specific enzyme activity – is fully reduced during electroreduction. Such a full reduction is observed with fresh samples of amylase prepared by the "mild method". The same preparation, if kept for some time in the cold, will become resistant against electrolytic reduction. Obviously, a subtle change in the conformation of the enzyme is enough to change its reducibility, while having no influence on the enzyme activity itself.

In this connection it has to be remembered that electrolytic reduction of the other enzymes used in this study was carried out using commercial preparations. We cannot exclude the possibility that e.g. trypsin prepared by a "mild method" would be reduced at a different rate and to a greater extent than that used in everyday practice.

The data and considerations presented in this paper have been used by one of us (Straub, 1967) to discuss the possibility that disulfide bonds may have *in vivo* a dynamic character, in this way contributing to the stability of the protein under physiological conditions.

References

Asai, H., Morales, M. F. (1965) Biochemistry *4* 830
Cecil, R., Loening, U. E. (1960) Biochem. J. *76* 146
Cecil, R., Wake, R. G. (1962) Biochem. J. *82* 401
Ellman, G. L. (1958) Arch. Biochem. Biophys. *74* 443
Előd i, P. (1961) Acta Physiol. Acad. Sci. Hung. *20* 311
Fleischman, J. B., Porter, R. R., Press, E. M. (1963) Biochem. J. *88* 220
Gorin, G., Clary, C. W. (1960) Arch. Biochem. Biophys. *90* 40
Hatfaludi, F., Strashilov, T., Straub, F. B. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 39
Jolles, J., Dianoux, A.-C., Hermann, J., Niemann, B., Jolles, P. (1967) Biochim. Biophys. Acta *128* 568
Klee, W. A., Richards, F. M. (1957) J. Biol. Chem. *229* 489
Light, A., Sinha, N. (1967) J. Biol. Chem. *242* 1358
Lindley, H. (1955) J. Am. Chem. Soc. *77* 492
Markus, G. (1964) J. Biol. Chem. *239* 4162
Schramm, M. (1964) Biochemistry *3* 1231
Sela, M., Neumann, H., (1967) Europ. J. Biochem. *3* 171
Sela, M., White, F. H., Anfinsen, C. B. (1959) Biochim. Biophys. Acta *31* 417
Singer, S. J. (1962) Advanc. Prot. Chem. *17* 1
Smith, B. W., Roe, J. H. (1949) J. Biol. Chem. *179* 53
Straub, F. B. (1967) Proc. 7th Int. Congr. Biochem. (IUB) Tokyo, vol. *36* 41
Takemori, S., Furuya, E., Suzuki, H., Katagiri, M. (1967) Nature *215* 417
White, F. H., Sandoval, A. (1962) Biochemistry *1* 983

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (1) pp. 27—36 (1969)

Location of Prolactin Activity in the Polyacrylamide Gel Electrophoretograms Prepared from the Adenohypophyses of Some Species

I. NAGY, M. KURCZ, P. BARANYAI

Central Laboratory of the "Heim Pál" Hospital for Children, Budapest and the Departments of Biology and Biological Chemistry, National Institute of Health, Budapest

(Received August 1, 1968)

Adenohypophyses from Syrian gold hamster, rat, mouse, guinea-pig, rabbit pig, cattle, monkey (M. Rhesus) and human as well as some prolactin preparations were investigated by polyacrylamide gel electrophoresis. The electrophoretograms showed great differences with different species even in the case of close relationship. Albumin, haemoglobin and prolactin were identified. The electrophoretic mobilities of the fractions prepared from various species and exhibiting prolactin activity were dissimilar. This phenomenon is explained by physico-chemical and chemical differences and is supported by data on the species specificity and physico-chemical properties of prolactin. The activity of prolactin was associated with a single fraction with one intensively and several slightly staining components. The possible reasons of the latter phenomena are discussed in detail.

The experimental results reported suggest that polyacrylamide gel electrophoresis may be a suitable method for the quantitative estimation of prolactin content in the adenohypophyses of some species.

Starch-gel electrophoresis as described by Smithies (1955) and Poulik (1957) proved to be a suitable method for studying the purity and homogenity of proteohormones as well as the protein composition of hypophysis extracts (Papkoff, Li, 1958; Carsten, Pierce, 1960; Ferguson, Wallace, 1961a, 1961b; Papkoff et al., 1962; Li, 1962; Barrett et al. 1962; Lloyd, Meares, 1962, 1964; Ferguson, Wallace, 1963; Baker et al., 1963; Catt, Moffat, 1965; Baranyai et al., 1968; Kurcz et al., 1968). However, these investigations can be carried out in a more precise, simpler and quicker way by using acrylamide gel electrophoresis introduced by Ornstein (1964) and Davis (1964). Changes due to various effects in the prolactin and somatotrophin content of rat hypophysis can be measured semiquantitatively by these two methods (Vanderlaan et al., 1964; Reisfeld et al., 1964a; Lewis et al., 1965; Jones et al., 1965; Catt, Moffat, 1965, 1967; Baker, Zanotti, 1966; Kragt, Meites, 1966; Baranyai et al., 1967; Lewis, Cheever, 1967; Kurcz et al., 1968, 1969). Our recent studies have shown that the P* content of the rat adenohypophysis can be determined quantitatively by acrylamide gel electrophoresis (Nagy et al., 1968).

The aim of the present paper is to answer the following questions:

* Abbreviations: P, prolactin; STH, somatotrophin

1. What kind of differences are there in the electrophoretograms of homogenates prepared from the adenohypophyses of commonly used laboratory animals?

2. Is there any difference in the electrophoretic mobility and localization of the fraction or fractions with P activity?

3. Is there any possibility with the method used for a quantitative determination of the P content of adenohypophyses from the species studied?

Table 1

Number of the species studied, preparations made and biological determinations carried out

Species	Weight (mg) of adenohypophyses subjected to electrophoresis in gel		Number of electrophoretic runs	Number of gel segments used for prolactin determination
	Analytical	Preparative		
Syrian gold hamster	3.0	17.0	16	33
Rat	1.0 2.0 3.0 4.0	22.6	250	100
Mouse	3.0	9.0 15.0	33	45
Guinea-pig	3.0	9.0 15.0	27	28
Rabbit	3.0	5.0 10.0 15.0 24.0	22	63
Pig	3.0	5.0 23.0	30	69
Cattle	3.0	5.0 15.0	12	43
Monkey (M. Rhesus)	3.0	11.8 14.5 16.5	19	36
Human	3.0	20.9	6	14

Materials and Methods

The hypophyses of the following species were used for the studies: Syrian gold hamster, rat, mouse, cattle, rabbit, pig, guinea-pig, monkey (M. Rhesus) and man (Table 1). The hypophyses of the laboratory animals were removed within 2–3 minutes after decapitation in deep ether narcosis. The adeno- and neurohypophyses were separated and an aliquot of the adenohypophysis was subjected to electrophoresis. In some cases the hypophyses were stored in a deep-

frozen state for a short time. The hypophyses from cattle and pig were also removed immediately after killing the animals. Human hypophyses were of sectional origin. Part of these were stored in acetone, and extracts from the hypophyses dehydrated in this way were subjected to electrophoresis.

In addition to the adenohypophyses of various species purified P preparations from sheep (Panlitar–USA–NIH) and pig as well as human P isolated by us according to Apostolakis (1965) were studied.

Aliquots of adenohypophyses were homogenized in 0.02 M phosphate buffer, pH 7.0. The volume of the buffer used for homogenization did not exceed the half-volume of the sample gel. After homogenization the large-pore gel solution was added to the homogenate, and mixed with it. Tissue fragments were centrifuged off and the supernatant fluid was subjected to electrophoresis. 1–4 mg wet tissue of the hypophysis were applied to the analytical column, and 5–24 mg to the preparative column. Various quantities of sheep P were dissolved in the above buffer, while pig and human P was dissolved in 0.05 N NaOH. After neutralization with HCl the samples were mixed with the large-pore gel solution. The preparations went well into solution and no centrifugation was necessary before electrophoresis.

The electrophoresis was carried out according to Ornstein (1964) and Davis (1964), only the dimensions of the gel were altered. The volume of the small-pore gel in the analytical column was 2.0 ml, and its length 66 cm, onto which 0.2 ml of the "spacer" gel and 0.2 ml of the sample gel were layered. Electrophoresis was continued for 120 minutes. A voltage of 300 V and a current of 46–48 mA were applied. The length of the preparative columns was 140 mm with a volume of 10.0 ml for the small-pore gel and a volume of 1.0 ml for the large-pore gel. Preparative electrophoresis lasted for 360 minutes with a voltage of 500 V and a current of 40 mA. After electrophoresis analytical gels were stained for 60 minutes and preparative gels for 90 minutes with 1 per cent naphtalene black 10 B (C. I. 20470 Reanal, Budapest) in 7 per cent acetic acid. The excess dye was removed from analytical gels with 7 per cent acetic acid. The preparative gels were discharged electrophoretically (Davis, 1964).

The number of analysis carried out in various gels is summarized in Table 1. It should be mentioned that a great number of experiments always gave very similar results with respect to the mobility and number of fractions as well as the distribution of prolactin activity.

In our present investigations we did not take into consideration individual differences within the same species. With the rat it has been demonstrated in our previous paper (Kurcz et al., 1968) that the intensity of the band exhibiting P and STH activities depends on the age, the sex, the physiological and pathophysiological state of the animal. This phenomenon was observed in the case of other species as well.

The P activity of the individual fractions was determined by a modification (Kurcz, 1966) of the "pigeon-crop" micromethod (Grosvenor, Turner, 1958). The unstained gel columns were cut to small pieces according to their stained

parallels and the gel discs were homogenized in 1.0–2.5 ml distilled water depending on their size. The homogenates were stored at 4 °C for 24 hours, and then centrifuged in the cold. The full quantity of the supernatant was intradermally injected above the sensitive part of the pigeon's crop. The number of activity measurements with the individual species is shown in Table 1.

Results

Considerable differences were found between the electrophoretograms of the adenohypophysis homogenates obtained from various, even closely related species. This is well demonstrated in the case of rodents (Fig. 1, a–g). The fraction with P activity from the Syrian gold hamster was of the most acidic character (Fig. 1a) as revealed by its greatest mobility (Fig. 3) among the corresponding fractions isolated from the other species studied. Slight activity could be detected in albumin, the other fraction with medium mobility and some components which were stained to a lesser degree and which migrated in the post-albumin region. However, these activities were insignificant as compared to the fractions marked with P. It should be mentioned that albumin isolated from hypophyses was identified by the albumin band of serum electrophoretograms of the adequate species. In all cases albumin was stained intensively.

In the rat the fraction moving in the prealbumin region showed a high P activity. This is in good agreement with the findings of Jones et al. (1965), Lewis et al. (1965) as well as Kragt and Meites (1966) and with previous data on starch gel electrophoresis (Baker et al., 1963; Catt, Moffat, 1965). The authors mentioned above could, in the case of rat, detect STH in the post-albumin region in a basic protein fraction with higher ability for staining. When the amount of rat adenohypophysis subjected to electrophoresis exceeded 20.0 mg on the preparative column, a slight P activity was observed in the fraction identified as STH as well. When 3.0 mg were subjected to electrophoresis on the analytical column, a slight P activity could be detected with STH and albumin only when a homogenate of high P activity was used and the activity of the extracts of some combined albumin or STH segments of such electrophoretograms was studied. Kragt and Meites (1966) and Baker et al. (1963) reported similar results.

The specific P activity of the hypophysis of the *mouse* is small (Hurst, Turner, 1942). Upon the electrophoretic separation of 15.0 mg adenohypophysis the corresponding fraction exhibited considerably smaller activity with the mouse than with the other species studied. There was no P activity in other parts of the gel (Fig. 1c). The active band was composed of three, poorly separating fractions. As serum albumin was located at the same place, for the time being we cannot say which band was responsible for the P activity. According to Kwa et al. (1967) the purified mouse P moves faster towards the anode than the homologous albumin.

The P from *rabbit* proved to have a medium mobility in the system used (Fig. 1 d). When hypophyses of high specific activity (e. g. from gravid and nursing

rabbits) were subjected to electrophoresis, some more acidic and more basic fractions showed activities, too.

The electrophoretogram from the adenohypophysis of the *cattle* is characterized by four well discernible discs (Fig. 1f). Albumin has the greatest mobility, it is followed by P, haemoglobin and a fraction which can be identified on the basis of literary data as STH (Furth, Moy, 1967). The hypophysis separated

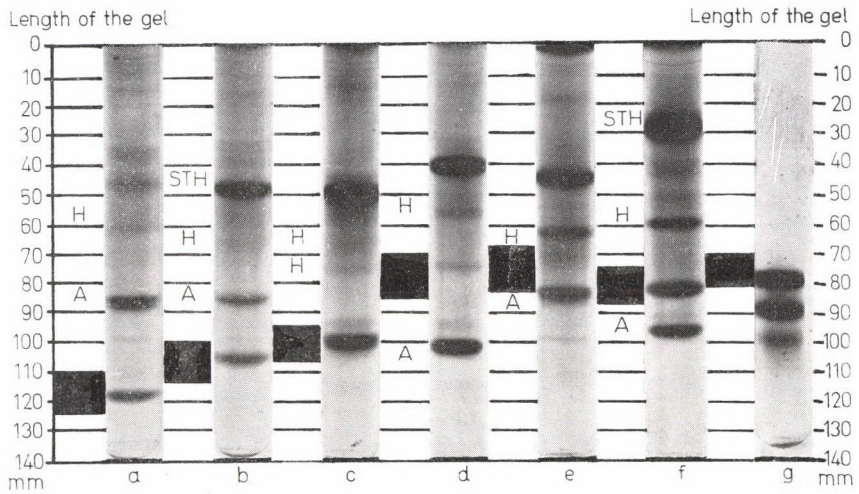


Fig. 1. Electrophoretograms of homogenates prepared from 3.0 mg adenohypophysis of Syrian gold hamster (a), rat (b), mouse (c), rabbit (d), guinea-pig (e), cattle (f). The scale hatched with black next to the electrophoretograms shows maximum prolactin activity. A, albumin; STH, somatotrophin; H, haemoglobin; g: 0.20 mg prolactin preparation from sheep (Panlitar, NIH)

from cattle has a great specific P activity (Hurst, Turner, 1942). This has been also confirmed by our investigations. When 15.0 mg hypophysis were separated electrophoretically, a slight activity could be detected in all fractions in addition to the P fraction. When 5.0 mg were used the activity disappeared from the post-prolactin fractions, but it remained in albumin.

The purified P of the *sheep* consisted of four fractions (Fig. 1g). Both the ability for staining and biological activity of these fractions decreased towards the anode (Pierce, Carsten, 1958; Cole, Li, 1959; Ferguson, Wallace, 1961b; Emmart et al., 1963; Reisfeld et al., 1964a).

In electrophoretograms (Fig. 2h) of homogenates prepared from *pig* hypophysis the most intensively stained fractions appeared in the post-albumin region. Maximum P activity was observed in the gel in a position between haemoglobin and albumin. Pig hypophysis, too, exhibited a high specific P activity. In the case of a 23.0 mg sample the fractions in the region between 30 and 90 mm from the

start showed significant activities even compared to peak P. When a 5.0 mg quantity was separated by electrophoresis the activity – which appeared in the post-prolactin fraction – disappeared and in addition to fraction P only that fraction showed a slight activity which was located between P and albumin. The position of the fraction with P activity in the electrophoretogram of pig adenohypophysis agreed with the location of the purified main fraction of pig P (Fig. 2i, j, k).

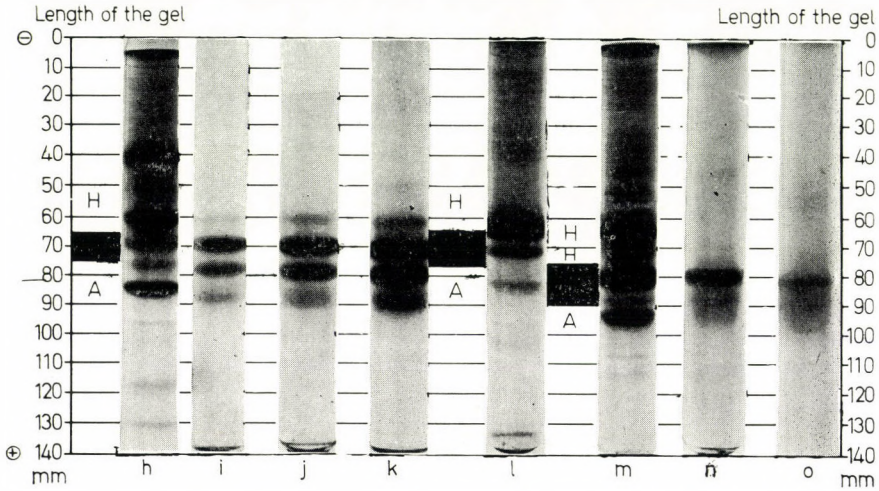


Fig. 2. Electrophoretograms of homogenates prepared from pig, monkey and human adenohypophyses and of prolactin preparations. h: 3.0 mg pig adenohypophysis; i: 0.05 mg; j: 0.10 mg; k: 0.20 mg pig prolactin (Dr Gráf); l: 3.0 mg monkey hypophysis; m: 3.0 mg human hypophysis; n: human hypophysis dehydrated with acetone; o: 0.40 mg „human prolactin". The scale hatched with black next to the electrophoretograms shows maximum prolactin activity. A, albumin; H, haemoglobin

In the *monkey* (M. Rhesus) hypophysis (Fig. 2l) seemed to be an insignificant fraction. In mobility it was followed by a fraction with P activity which could not be separated from haemoglobin. Haemoglobin, on the other hand, was very close to P, and consequently it was very difficult to separate these two fractions. Perhaps this is the explanation of the slight P activity of that fraction.

When 23.0 mg of *human* hypophysis extract were separated by electrophoresis there was a slight P activity in each fraction (Fig. 2m). The maximum was observed between albumin and a poorly separated component which contained haemoglobin. With respect to its location this fraction corresponded to the well discernible band produced by alkaline extracts of hypophyses dehydrated by acetone (Fig. 2n) as well as to the main component (Fig. 2o) of "human P" prepared by us according to Apostolakis (1965).

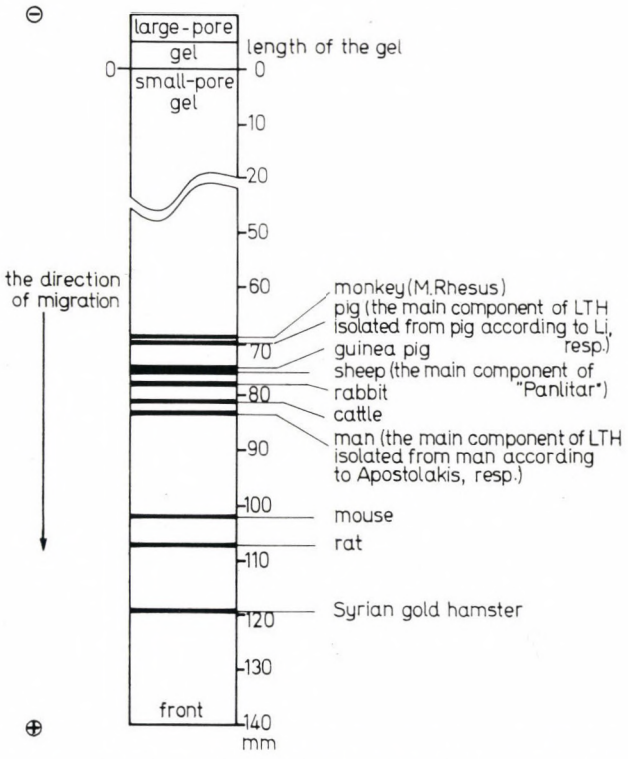


Fig. 3. The mobility and location of the fractions with maximum prolactin activity. The fractions were separated electrophoretically from the adenohypophyses of various species

Discussion

The results of our experiments show that the electrophoretograms are characteristic of the individual species studied. The actual electrophoretic pattern is mainly brought about by differences in the location of the 3–4 well discernible fractions. Of these fractions albumin and P were identified. In most cases haemoglobin also moves in the form of a characteristic band which is visible even in unstained gels. The albumin fraction of the various species studied does not show great differences in mobility. On the basis of its location the gel can be divided into a pre- and a post-albumin region.

Among the species studied the P of Syrian gold hamster and rat has a great mobility and is located in the pre-albumin region. The next in the sequence of decreasing mobility is the mouse P. The P-s of the other species studied occur as post-albumin fractions with mobilities characteristic of the individual species, and about half that observed in the case of the above mentioned three rodents (Fig. 3). If we take into consideration that the mobility which is stable in a given electrophoretic system, is the function of the charge, molecular weight, size and

structure of the protein molecule, we can draw the conclusion that there are chemical and structural differences in the P-s of various species. This difference might be the basis of the species specificity of P. The anti-sheep P serum precipitates only the cattle and sheep P, but it does not react with P from man, dog, pig, rabbit, rat, guinea-pig and pigeon (Baranyai et al., 1966; Kurcz et al., 1967). Likewise anti-sheep and antihuman P sera do not react with extracts from tumorous hypophyses of the rat known to produce P (Levy, Sampliner, 1962). These results led to the immunological determination of P. This was carried out in an elegant way with rat and mouse by Kwa et al. (1967a, b, c) and with sheep by Arai and Lee (1967). Differences in mobility and location (even in the case of close relationship) are fully compatible with the results of Kwa et al. (1967b) indicating that P-s from mouse and rat do not show complete antigenic identity. Li (1961) has shown that there is a difference in solubility and in tyrosine content between sheep and cattle P. Ferguson and Wallace (1961a) gave an account on the differences between cattle and sheep P on the basis of electrophoretic studies.

According to literary data the most slowly moving, intensively staining fraction in the electrophoretograms of homogenates prepared from rat and cattle adenohypophyses is STH. In the electrophoretogram of rat hypophysis the STH fraction moved to the same distance as greatly purified rat STH isolated by us according to Reisfeld et al. (1964b). The location of this slowly moving component in the rodents studied is practically the same as that of rat STH. We suggest that this is also STH.

As mentioned above, in addition to the fraction with a high P acitivity some smaller bands also showed slight activities, when too large amounts of homogenate or homogenates with high specific activity were separated by electrophoresis. It is evident that the resolving capacity of acrylamide gel is limited. It is influenced not only by the total volume of protein applied to the column but also by the ratio of the components (Davis, 1964). In some other experiments we have observed that when relatively large amounts of sheep P were subjected to electrophoresis, activity could be detected only in the stained bands. When STH (CHOAY) was subjected to electrophoresis, no P activity could be detected in the gel. However, when both preparations were used together for electrophoresis a slight P activity could be observed in STH, too. This means that excessive amounts of several components may influence separation in such a way that although there will be no difference in the location of the components, protein bands will form perfectly, at the same time, however, the components will be cross-contaminated (Nagy et al., 1968). The probable reason of the presence of P activity in more than one fraction can be explained by such a mechanism. The above results, however, do not exclude the possibility that the hypophysis may contain more than one component with P activity. At any rate the fact remains that a significant proportion of the activity was observed in a single fraction, and that this fraction proved to be homogeneous in the system used.

It is known, that purified sheep P preparations contain several fractions (Fig. 1g). The most slowly moving component has the greatest activity (Ferguson,

Wallace, 1961b; Reisfeld et al., 1964a). As this particular fraction contains most of the protein content of the preparation (Emmart et al., 1963; Reisfeld et. al., 1964a), even in the case of a preparate, the greatest specific activity and the bulk of the absolute activity can be measured in a single fraction. Lewis (1952; 1963, 1964) has proved that under various enzymatic or chemical effects P is fragmented. The data of Reisfeld et al. (1964a) suggest that the isolated main component of P is degraded under effects which may well occur during preparation. In all probability these effects also contribute to the characteristic electrophoretic inhomogeneity of purified sheep (Fig. 1 g) and pig (Fig. 2 i, j, k) P. Though the effect of proteolytic enzymes during the time of electrophoresis cannot be excluded, in our opinion this separation technique is significantly more gentle than any of the generally used methods of P preparation (Lyons, 1936; Cole, Li, 1955; Apostolakis, 1965; Jiang, Wilhelmi, 1965). The Syrian gold hamster and rat P do not show any degradation, but with other species we could not observe either such a phenomenon which occurred with purified preparations. Enzymatic rather than chemical effects may play a role in the occurrence of slight activities outside the band with maximum P activity.

Our results suggest that the P content of the adenohypophysis of several species can be determined quantitatively by polyacrylamde gel electrophoresis. To achieve satisfactory separation it is important to know with each species the optimum quantity of hypophysis to be applied to the column.

Thanks are due to the National Institute of Health (Bethesda, Md.) for providing us with purified sheep prolactin and to Dr L. Gráf (Pharmacological Research Institute Budapest) for the pig prolactin preparation.

The skillful technical assistance of Mrs L. Hejtejer, Miss A. Szulinszky, Mr M. Lazarits, Mrs I. Káldi and Miss I. Szarka is gratefully acknowledged.

References

Apostolakis, M. (1965) Acta endocr. (Kbh.) *49* 1
Arai, Y., Lee, T. H. (1967) Endocrinology *81* 1041
Baker, B. L., Clark, R. H., Hunter, R. L. (1963) Proc. Soc. Exp. Biol. *114* 251
Baker, B. L., Zanotti, D. B. (1966) Endocrinology *78* 1037
Baranyai, P., Nagy, I., Kurcz, M., Orosz, A. (1966) Nature *212* 1255
Baranyai, P., Nagy, I., Kurcz, M. (1967) Clin. Chim. Acta *16* 448
Barrett, R. J., Friesen, H., Astwood, E. B. (1962) J. Biol. Chem. *237* 432
Carsten, M. E., Pierce, J. G. (1960) J. Biol. Chem. *235* 78
Catt, K., Moffat, B. (1965) Endocrinology *76* 678
Catt, K., Moffat, B. (1967) Endocrinology *80* 324
Cole, R. D., Li, C. H. (1955) J. Biol. Chem. *213* 197
Cole, R. D., Li, C. H. (1959) Biochim. Biophys. Acta *33* 563
Davis, B. J. (1964) Ann. N. Y. Acad. Sci. *121* 404
Emmart, E. W., Bates, R. W., Condliffe, P. G., Turner, W. A. (1963) Proc. Soc. Exp. Biol. *114* 754
Ferguson, K. A., Wallace, A. L. C. (1961a) Nature *190* 629
Ferguson, K. A., Wallace, A. L. C. (1961b) Nature *190* 632

Ferguson, K. A., Wallace, A. L. C. (1963) Rec. Progr. Horm. Res. *19* 1
Furth, J. ,Moy, P. (1967) Endocrinology *80* 435
Grosvenor, C. E., Turner, C. W. (1958) Endocrinology *63* 530
Hurst, V., Turner, C. W. (1942) Endocrinology *31* 334
Jiang, N., Wilhelmi, A. E. (1965) Endocrinology *77* 150
Jones, A. E., Fisher, J. N., Lewis, U. J., Vanderlaan, W. P. (1965) Endocrinology *76* 578
Kragt, C. L., Meites, J. (1966) Proc. Soc. Exp. Biol. *121* 805
Kurcz, M. (1966) Ann. Univ. Sci. Budapest de Rolando Eötvös, Sect. Biol. *8* 149
Kurcz, M., Nagy, I., Baranyai, P., Orosz, A., Ajtai, K. (1967) Endokrinologie *51* 308
Kurcz, M., Nagy, I., Gerhardt, V. J., Baranyai, P. (1968) Acta Biol. Acad. Sci. Hung. *19* 123
Kurcz, M., Nagy, I., Baranyai, P., Gerhardt, V. J. (1969) Acta Biol. Acad. Sci. Hung. in press
Kwa, H. G., Verhofstad, F. (1967a) Biochim. Biophys. Acta *133* 186
Kwa, H. G., Van der Bent, E. M., Prop, F. J. A. (1967b) Biochim. Biophys. Acta *133* 301
Kwa, H. G., Verhofstad, F. (1967c) J. Endocr. *38* 81
Kwa, H. G., Verhofstad, F., Van der Bent, E. M. (1967d) Acta Physiol. Pharmacol. Neerl. *14* 514
Levy, R. P., Sampliner, J. (1962) Proc. Soc. Exp. Biol. *109* 672
Lewis, U. J. (1962) J. Biol. Chem. *237* 3141
Lewis, U. J. (1963) J. Biol. Chem. *238* 3330
Lewis, U. J. (1964) J. Biol. Chem. *240* 247
Lewis, U. J., Cheever, E. V., Vanderlaan, W. P. (1965) Endocrinology *76* 362
Lewis, U. J., Cheever, E. V. (1967) Endocrinology *81* 1338
Li, C. H. (1961) Postgrad. Med. *29* 13
Li, C. H. (1962) J. Gen. Physiol. *45* 169
Lloyd, H. M., Meares, J. D. (1962) Acta endocr. (Kbh.) *39* 163
Lloyd, H. M., Meares, J. D. (1964) Clin. Chim. Acta *11* 238
Lyons, W. R. (1936) Proc. Soc. Exp. Biol. *35* 645
Nagy, I., Kurcz, M., Baranyai, P. (1968) Magyar Élettani Társaság XXX. Vándorgyűlése, Debrecen (Abstracts, p. 114)
Ornstein, L. (1964) Ann. N. Y. Acad. Sci. *121* 321
Papkoff, H., Li, C. H. (1958) Biochim. Biophys. Acta *29* 145
Papkoff, H., Li, C. H., Liu, W. K. (1962) Arch. Biochem. Biophys. *96* 216
Pierce, J. G., Carsten, M. E. (1958) J. Am. Chem. Soc. *80* 3482
Poulik, M. D. (1957) Nature *180* 1477
Reisfeld, R. A., Williams, D. E., Cirillo, V. J., Tong, G. L., Brink, N. G. (1964a) J. Biol. Chem. *239* 1777
Reisfeld, R. A., Muccilli, A. S., Williams, D. E., Steelman, S. L. (1964b) Nature *201* 821
Smithies, O. (1955) Biochem. J. *61* 629
Vanderlaan, W. P., Lewis, U. J., Jones, A. E., Fisher, J. N. (1964) Calif. Med. *100* 175

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (1) pp. 37—46 (1969)

Effect of Tropomyosin on the Aggregation of Myosin

VERONIKA HARSÁNYI,* F. GUBA

Chemical Structure Research Laboratory of the Hungarian Academy of Sciences, Budapest
and Institute of Biochemistry, Medical University, Szeged

(Received November 8, 1968)

1. The size and shape of myosin aggregates obtained at low ionic strength and containing different amounts of protein contamination were investigated by electron microscopy.

2. Both myosin and tropomyosin were extracted from myofibrils by a high ionic strength solution. By a further extraction of the residue with an ATP containing low ionic strength solution additional amounts of myosin and tropomyosin were obtained.

3. Electron microscopic examinations have indicated an interaction between myosin and tropomyosin.

4. It is concluded that tropomyosin or native tropomyosin may play a controlling role in the aggregation of myosin.

Introduction

Under the ionic conditions of muscle most myofibrillar proteins exist in an aggregated form. Myofibrillar function is closely related to aggregational changes of the molecules and complexes of the structural proteins (Szent-Györgyi, 1947; Ikemoto et al., 1966; Guba, 1966).

Investigations of isolated thick and thin filaments obtained from myofibrils by mechanical treatment and their similarity to the synthetic protein filaments under the electron microscope (Huxley, 1963; Hanson, Lowy, 1963) have presented a direct evidence for the location of myosin and actin.

Recently a physiological role for native tropomyosin, which proved to be a complex of Bailey's classical tropomyosin and troponin, has been described by Ebashi et al. (Ebashi, Ebashi, 1964; Ebashi et al., 1967). Several investigations have been published on the interaction of F-actin with the components of native tropomyosin (e.g. Drabikowski, Nonomura, 1968).

However, the location of tropomyosin in the myofibrillar system has remained to be cleared. According to recent data the thin filaments contain native tropomyosin or tropomyosin besides F-actin (Ebashi, Kodama, 1966; Drabikowski, Nonomura, 1968; Hanson, 1968). On the other hand, some ultrastructural

* Present address: Biochemistry Group, L. Eötvös University, Budapest

evidence has been presented on the existence of tropomyosin or native tropomyosin in the thick, myosin-containing filaments (Gilev, 1966; Corsi et al., 1967).

Recent investigations on myosin ATPase (ATP phosphohydrolase-muscle E.C.3.6.1.3.) by Chaplain (1967, 1968, 1968a) have shown that tropomyosin plays a role in the aggregation of the controllable unit of the allosteric enzyme.

During a systematical investigation on the aggregation of the structural proteins under different ionic conditions and in the presence of other protein components it was observed that tropomyosin affected the size and shape of the myosin aggregates formed.

Materials and Methods

Myosin was prepared from rabbit skeletal muscle as described by Mommaerts and Parrish (1951) and by Finck (1965).

Tropomyosin was prepared according to Bailey (1948) but EDTA and β-mercapto ethanol were present in all steps and a low temperature was maintained.

Protein fractions containing both myosin and tropomyosin were obtained either by the extraction of purified myofibrils prepared according to Perry (1952) in a medium containing 10^{-3} M $Na_4P_2O_7$ and 10^{-4} M EDTA at pH 7.0 ("natural system") or by mixing the two proteins at different weight ratios (reconstituted system).

Solutions:

ATP-ascorbic acid solution:
2×10^{-4}M ATP
2×10^{-4}M ascorbic acid
pH adjusted to 7.2–7.4

Bailey's solution:
10^{-2}M acetate buffer
0.12 M $(NH_4)_2SO_4$
pH 5.4

Hasselbach–Schneider solution:
0.47 M KCl
10^{-2}M $Na_4P_2O_7$
10^{-2}M phosphate buffer
pH adjusted to 6.1–6.3

Standard salt solution:
0.1 M KCl
10^{-3}M $MgCl_2$
pH adjusted to 7.0–7.2

Chemicals of analytical grade were purchased from the firm Reanal (Budapest). Protein concentration was determined by the biuret method (Gornall et al., 1949).

Sedimentation experiments were carried out in a Spinco Model–E analytical ultracentrifuge with a Philpot–Svenson optic, bar angle 70° and a final speed of 50 740 rpm.

Relative viscosity of the protein solutions was measured in an Ostwald type viscometer at 0 °C or 25 °C. The protein aggregates formed by dialysis against different salt solutions were examined by the negative staining technique (Huxley, 1963) under a Hitachi HU–10 electron microscope at an accelerating voltage of 75 kV. Diameter measurements were performed on the central part of the filaments. Magnification was checked by polystyrene particles of 1880 Å.

Results

1. *Myosin*

The myosin prepared according to Finck showed the following characteristics in 0.5 M KCl, pH 7.0: a single hypersharp peak in the ultracentrifuge with an intrinsic sedimentation coefficient of 6.3 *S* and an intrinsic viscosity of 2.1 dl/g. The aggregation of the Mommaerts type and Finck type myosins was compaired in the standard salt solution.

Fig. 1 shows a micrograph of the Mommaerts type myosin. Fig. 2 represents a histogram on the distribution of the diameter values. The filaments have an average length of 0.5 μ and an average diameter of 168 Å.

Fig. 3 shows a micrograph of the Finck type myosin forming much longer filaments. The average length of the filaments could not be determined because of the lack of sufficient number of measurable values. Fig. 4 represents a histogram on the distribution of the filament diameters. The average value is 200 Å.

2. *Tropomyosin*

Tropomyosin in standard salt solution sedimented as a single component with an intrinsic sedimentation coefficient of 3.1 *S*. Instrinsic viscosity was 0.3 d^1/g. Besides crystals tropomyosin appeared also in a fibrillar form in Bailey's solution. Fig. 5 shows tropomyosin filaments with an average diameter of 260 Å and sometimes a cross periodicity of about 60 Å.

3. *Myosin and tropomyosin containing "natural systems"*

a) The suspension containing relaxed myofibrils was extracted four times with 3 volumes of Hasselbach–Schneider solution. 54 per cent of the myofibrillar proteins were extracted.

All four protein extracts showed a single peak in the ultracentrifuge. The intrinsic sedimentation coefficients of the extracts were lower than that of pure myosin. Under the electron microscope myosin-like filaments, sometimes with a periodicity, and some crystalline formations were observed when dialysis was carried out against standard salt solution, as shown in Fig. 6. The myosin-like

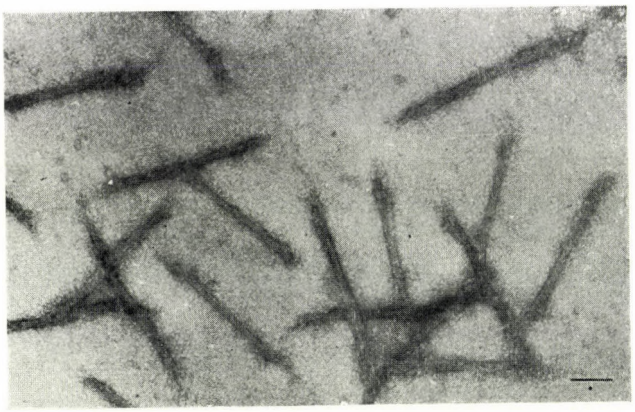


Fig. 1. Micrograph of filaments obtained from myosin prepared according to Mommaerts and Parrish

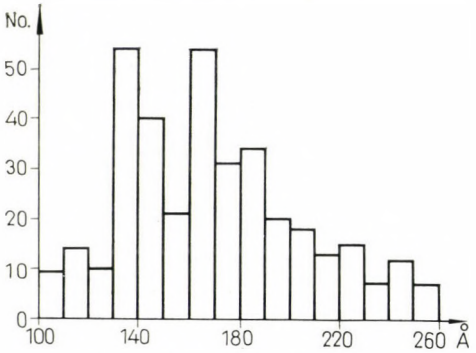


Fig. 2. Histogram on the distribution of the diameter values of the Mommaerts type-myosin aggregates

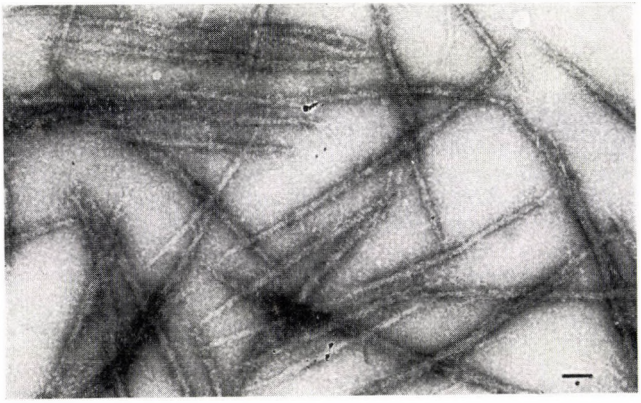


Fig. 3. Micrograph of filaments obtained from myosin prepared according to Finck

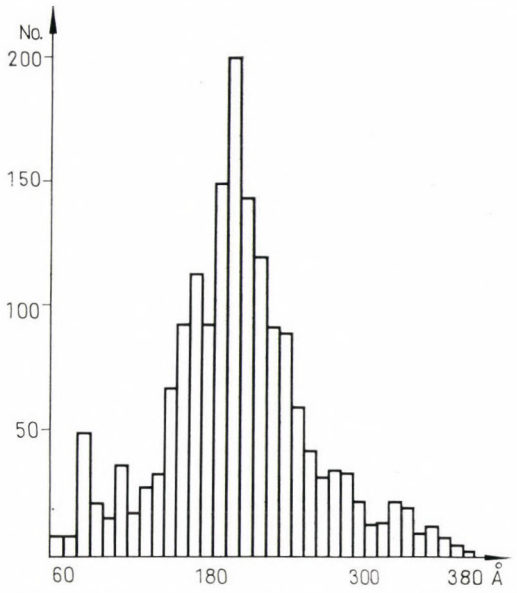


Fig. 4. Histogram on the distribution of the diameter values of the Finck-type myosin aggregates

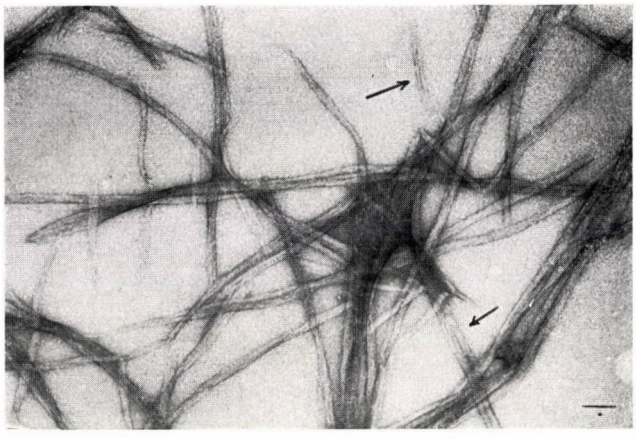


Fig. 5. Micrograph of tropomyosin in Bailey's solution

filaments had an average diameter value of 260 Å and a filament length of 0.7 μ.

b) By further extraction of the myofibrillar residue with a low ionic strength, ATP-containing solution an additional 7–8 per cent of myofibrillar proteins could be extracted.

This extract sedimented also as a single peak in the ultracentrifuge. The protein fraction showed a large viscosity drop on adding salt in a final concentration

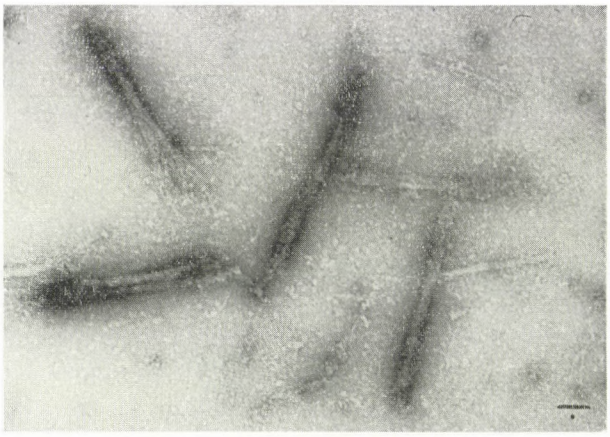


Fig. 6. Micrograph of a Hasselbach—Schneider extract of myofibrils in standard salt solution

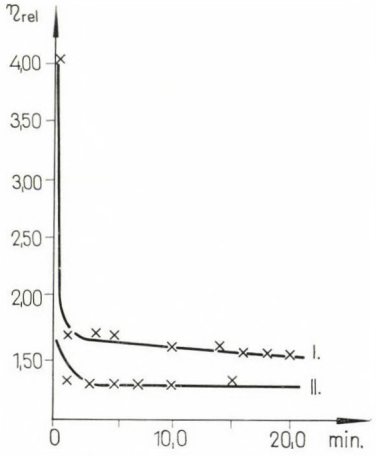


Fig. 7. Relative viscosity changes of ATP extracts of the myofibrillar residue on adding salt in a final concentration of 0.1 M KCl, 10^{-3} M $MgCl_2$. I. c = 3.0 mg/ml (first extract); II. c = 1.7 mg/ml (second extract)

corresponding to that of the standard salt solution (Fig. 7) indicating the presence of tropomyosin in a considerable amount. By free boundary and agar gel electrophoresis three protein components were observed. After heat treatment (in the presence of salt, for 1 minute at 100 °C) one single component remained. This corresponds to tropomyosin which is known to be heat-stable under these conditions. Tropomyosin, the main protein component of the ATP extract was also isolated by $(NH_4)_2SO_4$ fractionation. By chromatography on DEAE-cellulose with a NaCl gradient elution the presence of myosin was also proved.

In the electron microscope the protein fraction dialysed against Bailey's solution showed filaments similar to tropomyosin under the same conditions, except a cross periodicity of about 400 Å (Fig. 8).

4. *Myosin and tropomyosin-containing reconstituted systems*

The interaction of myosin and tropomyosin was investigated by mixing the two proteins in different weight ratios at high and low ionic strength.

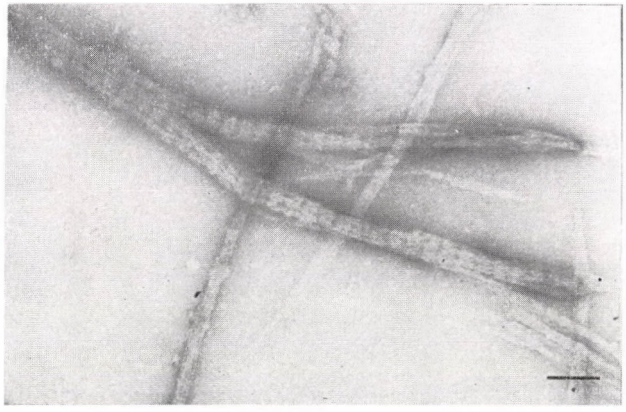


Fig. 8. Micrograph of an ATP extract of the myofibrillar residue in Bailey's solution showing a 400 Å periodicity

a) At 0.6 ionic strength myosin and tropomyosin were mixed in weight ratios of 4 : 1, 3 : 1 and 1 : 1. According to physical-chemical measurements no indication of complex formation was observed. In the ultracentrifuge two peaks appeared (Fig. 9) with 3.1 *S* and 6.3 *S* intrinsic sedimentation coefficients corresponding to tropomyosin and to myosin, respectively. The viscosity of the components calculated according to the formula:

$$[\eta]_{\text{mixture}} = \frac{[\eta]_{\text{M}} \cdot c_{\text{M}}}{c_{\text{M}} + c_{\text{TM}}} + \frac{[\eta]_{\text{TM}} \cdot c_{\text{TM}}}{c_{\text{M}} + c_{\text{TM}}}$$

$[\eta]_{\text{M}} = 2.1$ dl/g
$[\eta]_{\text{TM}} = 0.3$ dl/g
c_{M} and c_{TM} are the protein concentrations of myosin and tropomyosin expressed in g/dl.

b) Ultracentrifugation in the ATP-containing low ionic strength solution with different myosin and tropomyosin ratios revealed two peaks. The sedimentation coefficients indicated the presence of aggregates.

On the other hand when myosin and tropomyosin were mixed in a high ionic strength solution and dialysed against standard salt solution, a similar

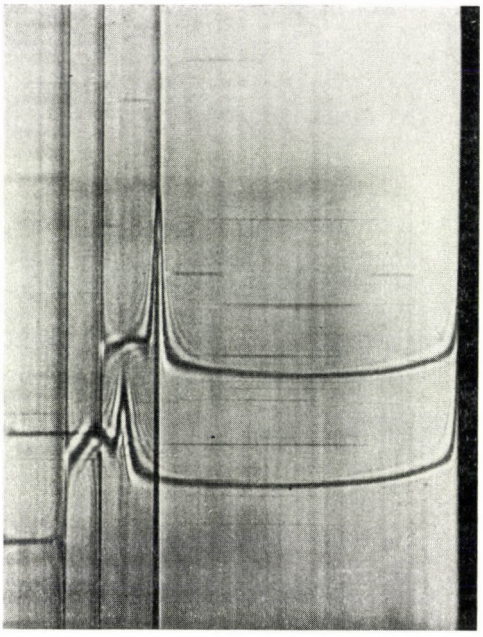


Fig. 9. Ultracentrifuge pattern of myosin and tropomyosin in 0.5 M KCl, pH 7.0. Upper curve: weight ratio 4 : 1; lower curve: weight ratio 1 : 1 24 min. after reaching full speed

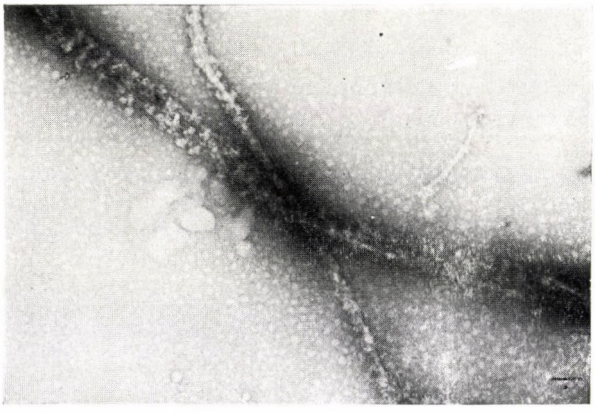


Fig. 10. Micrograph of myosin and tropomyosin containing filaments with a 400 Å periodicity in standard salt solution. The sign on the micrographs indicates 0.1 μ

electron microscopic structure was observed as with the ATP extract of the myofibrillar residue when dialysed against Bailey's solution. A cross periodicity was present in the filaments (Fig. 10).

Discussion

According to Mommaerts and Parrish their myosin preparation containing 10–15 per cent contamination, as shown by electrophoresis, had an intrinsic sedimentation coefficient of 6.7 *S* (Mommaerts, Parrish, 1951). On the other hand the Finck type myosin contains only about 5 per cent contamination which is considered to be a tropomyosin-like protein (Finck, 1965). Our investigations have also supported the finding that myosin prepared according to Finck shows a higher purity. It is to be mentioned that when Finck type myosin was treated on DEAE-cellulose or agarose gel columns both the E_{278}/E_{260} ratio and ATPase activity of the preparation indicated higher purity but the degree of aggregation as revealed by electron microscopy seemed to remain unchanged.

Comparing the size and shape of the Mommaerts type and Finck type myosin filaments it can be seen that these are the myosin filaments obtained from the less pure protein preparations which show a similarity to the isolated thick filaments investigated by Huxley (1963). From the diameter and length of the filaments measured it may be concluded that the aggregation of myosin in vivo is affected by a contaminating protein which is different from actin. We have tried to identify this contaminating protein as tropomyosin or native tropomyosin.

In accordance with some literary data (e.g. Corsi, 1957) both myosin and tropomyosin are extracted by a KCl and ATP or $Na_4P_2O_7$ containing solution. Kominz (1966) has described myosin preparations to contain 2–3 per cent native tropomyosin.

In the Hasselbach–Schneider extract of myofibrils the presence of tropomyosin besides myosin is indicated by electron microscopy (Fig. 6). Besides myosin filaments showing a row surface (periodicity) the crystalline formation observed originates very likely from tropomyosin. The average filament length is similar to that of the isolated thick filaments (Huxley, 1963). The lower sedimentation coefficient obtained (as compared to the s°_{20} value of pure myosin) may indicate an interaction between myosin and tropomyosin molecules (end-to-end aggregation?).

The presence of ATP or pyrophosphate is known to increase the hydration of the myosin molecules and to make myosin soluble also at a low ionic strength. In the ATP-containing low ionic strength extract of the myofibrillar residue the presence of myosin besides the native tropomyosin complex was detected by viscosity, sedimentation, chromatographic and electrophoretic measurements.

Electron microscopic examinations have indicated the presence of filaments containing native tropomyosin and myosin with a 400 Å periodicity in Bailey's solution (Fig. 8). We attribute this periodicity to the interaction of myosin with the native tropomyosin complex. This is in line with Drabikowski's (1968) observation. He has found that troponin binds at every 400 Å to tropomyosin molecules resulting in a cross periodicity and that this can be seen when native tropomyosin paracrystals are examined under the electron microscope.

In systems reconstituted from myosin and tropomyosin no complex formation has been detected by physical-chemical methods. On the other hand electron microscopic examinations have indicated an interaction between the proteins, i.e. a 400 Å cross periodicity was detected in the filaments formed when the mixture was dialysed from a high ionic strength solution against standard salt solution (Fig. 10).

Cohen and Longley (1966) have observed that tropomyosin in the presence of Mg ions formed paracrystals with a 400 Å periodicity.

The 400 Å periodicity observed with the myosin and tropomyosin-containing filaments may be due to tropomyosin because of the presence of Mg^{2+} ions or to troponin traces of which might be present as a result of the mild conditions during tropomyosin preparation. Tropomyosin alone under similar ionic conditions disaggregates into monomers and is not detectable by this method under the electron microscope.

From the experimental results we conclude that tropomyosin or native tropomyosin may play a controlling role in the aggregation of myosin, forming the thick filaments of the myofibrils.

The authors wish to express their thanks to Mr P. Kovács for carrying out sedimentation experiments, to Mrs E. Vajda for her continuous interest in the present work and to Prof. Dr N. A. Bíró for helpful discussion.

References

Bailey, K. (1948) Biochem. J. *43* 271
Chaplain, R. A. (1967) Arch. Biochem. Biophys. *121* 154
Chaplain, R. A. (1968) FEBS Fifth Meeting, Prague, 1968, Abstracts p. 184
Chaplain, R. A. (1968a) Symposium on Functional and Structural Aspects of the Myofibrillar Proteins, Balatonboglár, 1968, Abstracts 11
Cohen, C., Longley, W. (1966) Science *152* 794
Corsi, A. (1957) Biochim. Biophys. Acta *25* 640
Corsi, A., Muscatello, U., Ronchetti, I. (1967) J. Ultrastructure Res. *19* 260
Drabikowski, W. (1968) personal comm.
Drabikowski, W., Nonomura, Y. (1968) Biochim. Biophys. Acta *160* 129
Ebashi, S., Ebashi, F. (1964) J. Biochem. *55* 609
Ebashi, S., Kodama, A. (1966) J. Biochem. *59* 425
Ebashi, S., Ebashi, F., Kodama, A. (1967) J. Biochem. *62* 137
Finck, H. (1965) Biochim. Biophys. Acta *111* 208
Gilev, V. P. (1966) in Sixth Intern. Conf. for Electron Microscopy, Kyoto, 1966, Maruzen Ltd., Tokyo, 1966, p. 689
Gornall, A. G., Bardawill, C. S., David, M. M. (1949) J. Biol. Chem. *177* 751
Guba, F. (1966) D. Biol. Sci. Thesis, Budapest
Hanson, J. (1968) in Symposium on Muscle, Budapest 1966 (E. Ernst, F. B. Straub, eds.) Akadémiai Kiadó, Budapest, 1968, p. 99
Hanson, J., Lowy, J. (1963) J. Mol. Biol. *6* 46
Huxley, H. E. (1963) J. Mol. Biol. *7* 281
Ikemoto, N., Kitagawa, S., Gergely, J. (1966) Biochem. Z. *345* 410
Kominz, D. R. (1966) Arch. Biochem. Biophys. *115* 583
Mommaerts, W. F. H. M., Parrish, R. G. (1951) J. Biol. Chem. *192* 545
Perry, S. V. (1952) Biochem. J. *51* 495
Szent-Györgyi, A. (1947) in Chemistry of Muscular Contraction, Acad. Press, New York—London, 1947, pp. 3 37

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (1) pp. 47—55 (1969)

Studies on the Structure of Human Embryonic Haemoglobin

JUDIT G. SZELÉNYI, SUSAN R. HOLLÁN

Central Institute, National Blood Transfusion Service, Budapest

(Received November 11, 1968)

The polypeptide chain structure and amino acid composition of human embryonic haemoglobin are compared to those of foetal and adult haemoglobins. It is shown that the electrophoretic mobility of the non-α chain of embryonic haemoglobin is different from that of both the β and the γ chains. Especially the high Ileu content of embryonic haemoglobin indicates that embryonic haemoglobin is more closely related to foetal, than to adult haemoglobin.

On a fingerprint of the tryptic peptides of embryonic haemoglobin the peptides which contain Arg, His, Try, Tyr and S-containing amino acids, respectively, have been identified by specific colour reagents. The amino acid composition of two of the tryptic peptides is given. These amino acid compositions support the idea that the amino acid sequence of the ε chain is different from that of either chain β or chain γ.

As early as 1954 it was suggested by Drescher and Künzer (1954) that there existed a so-called embryonic haemoglobin (Hb U)*, the synthesis of which precedes the biosynthesis of human foetal haemoglobin (Hb F). The properties of embryonic haemoglobin were first studied by Huehns et al. (1961). They have shown that embryonic haemoglobin is different from the known human haemoglobins. Huehns et al. (1961) described two new haemoglobins, Hb Gower–1 and Hb Gower–2, and demonstrated that these haemoglobins were characteristic of early embryonic life. The structure of Hb Gower–1 and Hb Gower–2 were shown to be ε_4, and $\alpha_2\varepsilon_2$, respectively, where ε was a new polypeptide chain synthetized exclusively in the course of embryonic development.

Due to difficulties in obtaining a sufficient amount of starting material, information on the structure and properties of embryonic haemoglobin is still limited. Our present studies were aimed at demonstrating that embryonic haemoglobin is distinct from both foetal and adult haemoglobins by characterizing its subunit structure and amino acid composition. Our first results obtained in the fingerprint analysis of embryonic haemoglobin are also presented.

* Hb A is the main component of normal adult haemoglobin; Hb F is the main component of foetal haemoglobin; and Hb U (haemoglobin uterine) is used to denote embryonic haemoglobin. — The standard abbreviations are used for the amino acids.

Materials and Methods

Human embryonic haemoglobin was obtained from 6 – 10 week old embryos. Embryos of healthy mothers were used and these were obtained 2 – 24 hours following the artificial interruption of pregnancy.

The blood was washed from the tissues with physiological saline. Blood obtained from different tissues was never mixed. Blood cells were first washed with five changes of physiological saline and then haemolyzed by the addition of 1 volume of distilled water plus 0.4 volume of toluene. The haemolysate was centrifuged and filtered, and each preparation was checked by starch gel electrophoresis.

Starch gel electrophoresis was done in tris-EDTA-borate buffer, pH 8.6 (Huehns, Shooter, 1965). In case the electrophoretogram showed the presence of a considerable amount of haemoglobin the mobility of which was slower than that of Hb F, the haemolysate was further purified by chromatography on CM Sephadex 50 (Huehns et al., 1961). The Hb U content of the chromatographic fractions was checked by starch gel electrophoresis.

The polypeptide chain composition of purified embryonic haemoglobin was established by starch gel electrophoresis in the presence of 6 M urea in tris-borate buffer, pH 8.0 (Chernoff, Pettit, 1964).

The amino acid composition of globin and of the isolated peptides was determined with a Beckman Unichrom amino acid analyzer from hydrochloric acid hydrolysates.

Tryptic digest of the globin was used for fingerprint analysis. The digest was run on Whatman 3MM paper, first with pyridine-acetic acid buffer, pH 6.5 (100 ml pyridine and 4 ml acetic acid to a final volume of 1000 ml), and then with pyridine–isoamyl alcohol–water = 35 : 35 : 30. The fingerprints were developed with diazotized sulphanylic acid for histidine (Baldrige, Lewis, 1953); α-nitroso-β-naphthol for tyrosine (Acher, Crocker, 1952); p-dimethylaminobenzaldehyde for tryptophan (Smith, 1953); K_2PtCl_6 for sulphur containing amino acids (Tiennis, Kolb, 1951); and by a modified Sakaguchi reaction for arginine (cf. Hais, Macek, 1958). Peptides were eluted from the fingerprints following the development of the fingerprints with 0.01 per cent ninhydrin.

Results

Sufficient amount of embryonic haemoglobin was found in 74 embryos out of a total of 196. The material obtained from different embryos was very similar, in spite of some variation in their age.

We found that the non-α chain of embryonic haemoglobin had a slower anodic mobility upon starch gel electrophoresis at pH 8.0 in the presence of 6 M urea than the γ and β chains (Fig. 1). Even though denaturation might have occurred to some extent during preparation and electrophoresis, this should have been similar with similarly treated foetal and adult haemoglobins. Therefore, it is

noteworthy that the ε chain had a slower anodic mobility than either the β chain, or the γ chain.

The amino acid composition of embryonic globin is shown in Table 1, together with data on haemoglobins A and F. It is clear that the histidine content of Hb U is markedly lower, than that of either Hb F or Hb A. Hb U is further characterized by a lower Lys and Tyr content and by a higher Glu and Asp content. In respect to this latter finding, it is important to note that the values for glutamine and asparagine are included into those for Glu, and Asp, respectively. It is also seen from Table 1 that the isoleucine content of Hb U is more than two times higher than that of Hb F. Some further minor differences in the amino acid composition of the three haemoglobin chains are also apparent from Table 1, and these

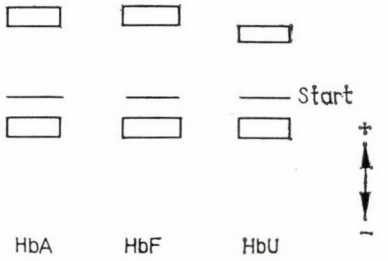


Fig. 1. Electrophoretogram of Hb A, Hb F and Hb U. Starch gel electrophoresis at pH 8.0 in the presence of 6 M urea. α-chain moves toward the cathode

Table 1

Amino acid composition of HCl hydrolysates of Hb U, Hb F and Hb A

	Hb U per cent	Hb F per cent	Hb A per cent
Lys	8.6	9.8	9.2
His	5.7	7.7	8.4
Arg	3.0	3.0	3.0
$CySO_3H$	(2.5)	0.7	1.0
Asp	10.5	9.6	9.5
Thr	5.7	6.6	5.4
Ser	5.1	6.7	4.8
Glu	9.6	7.2	6.7
Pro	4.3	3.7	4.6
Gly	4.1	4.3	4.3
Ala	8.4	8.3	9.1
Val	8.6	8.5	10.0
Ile	1.7	0.8	—
Leu	12.6	13.3	13.5
Tyr	1.6	2.6	3.1
Phe	7.1	7.2	7.1

indicate that the difference between Hb U and Hb F is less, than the difference between Hb U and Hb A.

The ninhydrin-positive peptides of the fingerprint of embryonic haemoglobin are shown in Fig. 2. Fig. 3 gives a schematic drawing of Fig. 2, together with a schematic fingerprint of Hb A. It is seen from Fig. 3 that all peptides of the α chain are present in embryonic haemoglobin, while six peptides of the β chain are missing from it (β_2, β_4, β_{50A}, $Lys\beta_7$, $Lys\beta_9$ and β_{13}). It is further seen that at least ten

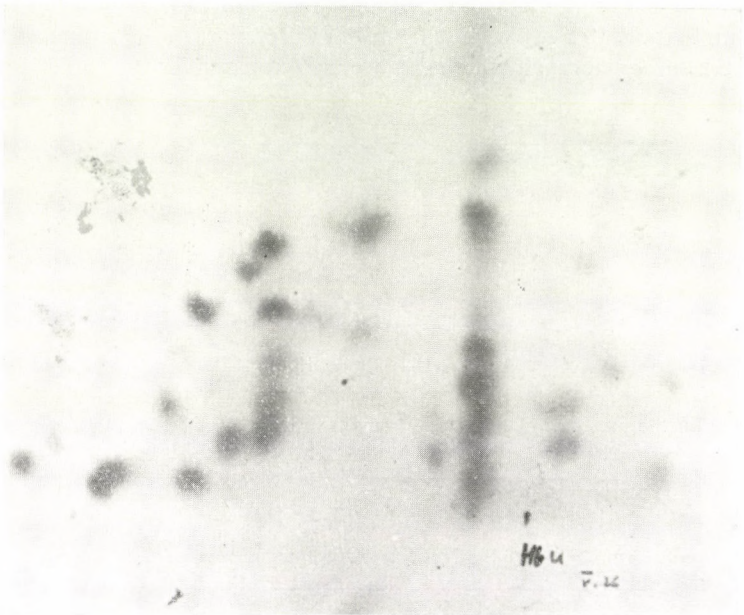


Fig. 2. Fingerprint of the tryptic peptides of embryonic haemoglobin (Hb U)

peptides of the non-α chain of embryonic haemoglobin are different from any peptide of Hb A (black spots in Fig. 3, numbered from the cathode).

Some further differences between the β chain and the non-α chain of embryonic haemoglobin could be demonstrated by colour reagents specific for certain amino acids. Results obtained in this way are summarized in Figs 4 and 5. It is seen that four His-positive peptides of Hb A (β_1, $Lys\beta_7$, β_9 and $Lys\beta_9$) are missing from Hb U, and there is only one new His-positive peptide in Hb U(6). This observation is in agreement with the finding that the His-content of Hb U is lower than that of Hb A.

A comparison of the tyrosine containing peptides indicates that two Tyr-positive peptides (β_4 and β_{13}) are missing from Hb U, while a new peptide (7) is present in it.

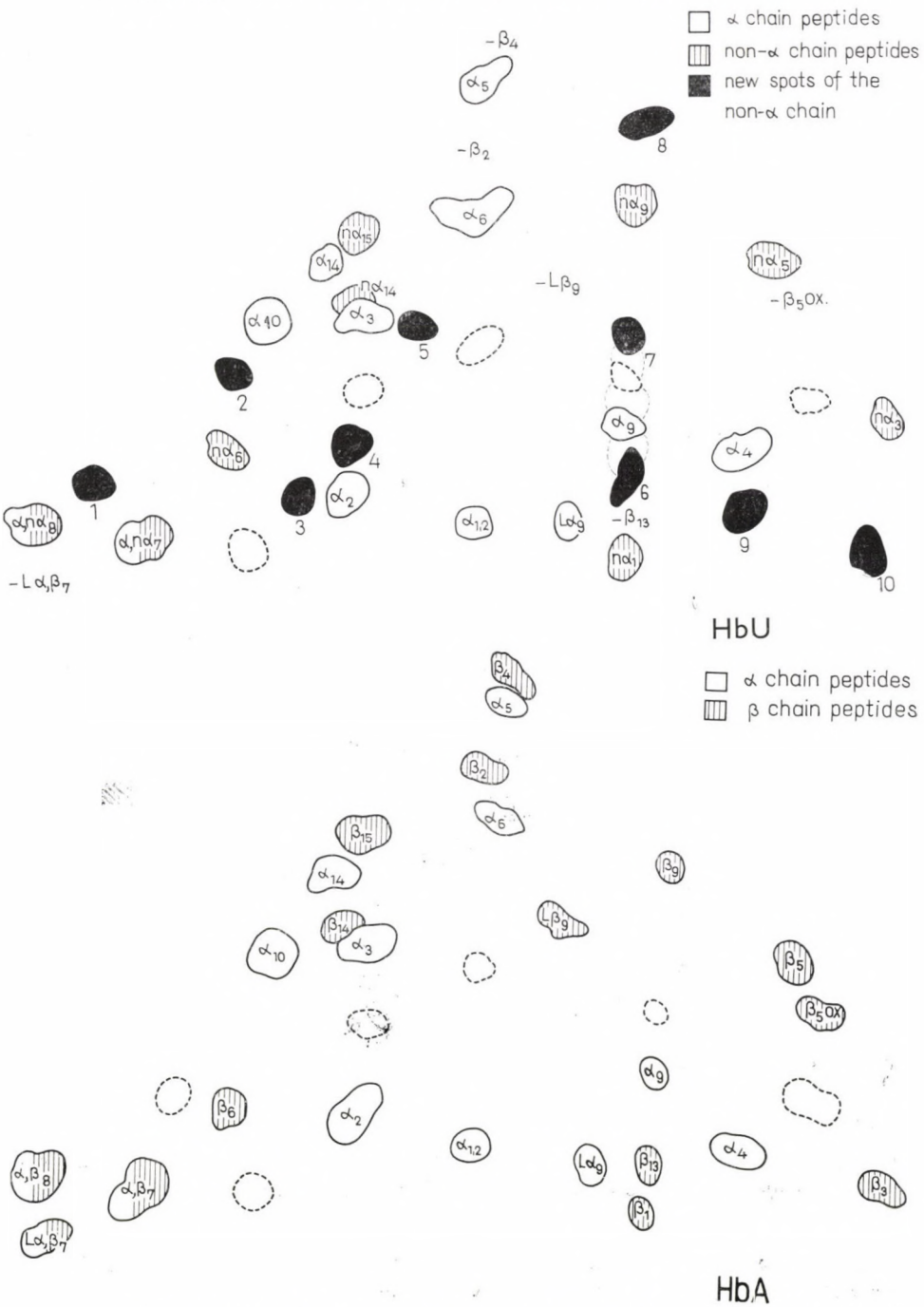


Fig. 3. Schematic fingerprint of the tryptic peptides of Hb A and Hb U

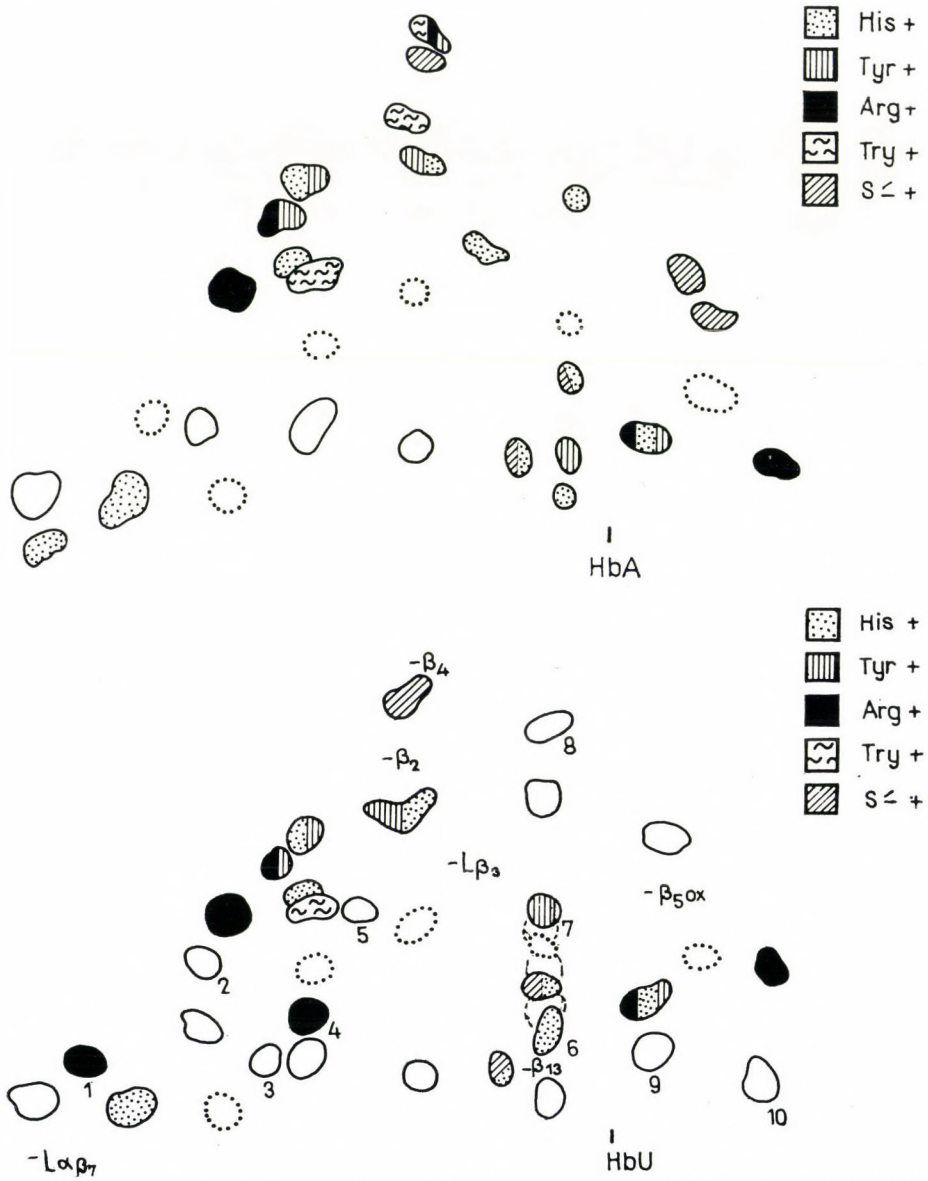


Fig. 4. Summary of the findings with specific colour reagents for His, Tyr, Try, Arg and for sulphur-containing amino acids. Tryptic peptides of Hb A and Hb U

None of the tryptophan-containing peptides of the chain (β_2 and β_4) are present in Hb U, in which the only Try-positive peptide is identical with the one in the α chain (α_3).

Similarly, no sulphur-containing peptide can be seen in the fingerprint of Hb U except those present in the α chain (α_5, α_9 and $Lys\alpha_0$).

Two Arg-positive peptides can be demonstrated in the non-α chain of Hb U (1 and 4). The Arg-containing peptide of the β chain (β_4) is apparently missing from it.

Table 2

Amino acid composition of peptides 1 and 6 of Hb U

For the sake of comparison, the amino acid composition of peptides β_1 and β_{13} of Hb A is also shown

	Hb U basic (1)	Hb U neutr. (6)	Hb A β_1	Hb A β_{13}
Lys	—	1	1	1
His	—	1	1	—
Arg	1	—	—	—
Asp	—	2	—	—
Thr	—	1	1	1
Ser	—	1	—	—
Glu	—	3	2	3
Pro	—	1	1	2
Gly	1	2	—	—
Ala	—	2	—	2
Val	—	1	1	1
Ile	—	1	—	—
Leu	—	1	1	—
Tyr	—	—	—	1
Phe	—	1	—	—

It can be concluded from the above results that the peptides of the non-α chain of Hb U are different from the peptides of the β chain of Hb A, even if they cannot be distinguished on the basis of their position in the peptide map. This is clearly the case with peptides 1 and 9 of the non-α chain, which do not contain histidin and with peptide 5, from which sulphur-containing amino acids are absent. This is confirmed by the absence of peptide β_{50x} from Hb U.

From the available material we succeeded in the isolation and analysis of two peptides of the non-α chain of Hb U. The amino acid composition of these two peptides is shown in Table 2. Peptide 1 is shown to be a dipeptide with a composition of Gly-Arg, while peptide 6 is seen to contain 18 amino acid residues. It is seen that peptide 6 has a high Ile content, and that its amino acid composition is markedly different from the composition of the two peptides of Hb A (β_1 and β_{13}) with a similar β position.

Discussion

Human embryonic haemoglobin is only present in very young embryos; it can no longer be detected when the embryos reach an age of about 3 months

(Huehns et al., 1964a, b). For this reason, there is only preliminary evidence on its properties, except for information on its electrophoretic mobility, resistance to alkaline pH and its absorbance spectrum. It has been the main purpose of these preliminary investigations to decide whether the non-α chain of embryonic haemoglobin is identical with one of the known haemoglobin chains (Huehns et al., 1961, 1964a, b). Huehns and coworkers (Huehns et al., 1964b; Huehns, Shooter, 1965) have shown in hybridization experiments and also by fingerprint analysis that the ε chain is different by more than one amino acid from all known normal and pathological haemoglobin chains. However, they have published no further information on the nature of the existing differences.

Our observation that the non-α chain of embryonic haemoglobin has a slower anodic mobility than the β and the γ chains upon starch gel electrophoresis in 6 M urea supports the conclusion mentioned above. It is interesting to note that both the native haemoglobin and the non-α chain have slower anodic mobilities. At the same time they have a lower His and Lys content than either Hb A or Hb F. Since the amount of Glu and Gln was determined together, likewise Asp and Asn, the possibility of a decreased ratio of amino-dicarbonic acids to their amides exists, which may explain the above discrepancy. It is also possible that the polypeptide chain conformation alters the electrophoretic mobility expected on the basis of its amino acid composition. Similar unexpected results in the case of Hb Köln, Hb New York and Hb Wien were explained on this basis (Carrel et al., 1966; Perutz, Lehmann, 1968).

There is no published information on the amino acid composition of embryonic haemoglobin. It is an interesting feature of our results that the difference between the amino acid composition of Hb U and Hb F is less marked than the corresponding difference between Hb U and Hb A. It would appear that Hb F is an intermediate between Hb U and Hb A both ontogenetically and with respect to the evolution of the molecule. This is especially apparent in the Ile content, since this amino acid is absent from Hb A and is present in a two times higher amount in Hb U, than in Hb F. A similar finding has been obtained with His and with Tyr, while with respect to Thr, Ser and Pro Hb A appears to be intermediate between Hb U and Hb F. Therefore, it seems that for an unequivocal conclusion to be drawn further results of a more detailed structural analysis of Hb U are necessary.

Our fingerprint analyses support the conclusion of Huehns and coworkers that the tryptic peptides of the ε chain of Hb U are different from those of both foetal and adult haemoglobins. We have shown that even some of those peptides which cannot be distinguished from a similar peptide of the β chain on the basis of their position in the peptide map, can still be differentiated on the basis of their amino acid composition. This conclusion is further supported by the amino acid analysis of two peptides of the ε chain. The sequence Gly-Arg is absent from the γ and the β chains, while it is present in peptide 1 of the ε chain. Similarly, the composition of peptide 6 cannot be derived from the known sequence of chains β and γ.

We may conclude that the non-α chain of embryonic haemoglobin (chain ε) is identical with neither the β nor the γ chain. Some of the existing differences have

been more closely specified in these investigations. In current studies we should like to establish the evolutionary sequence of chains ε, γ and β by a more detailed characterization of the structure of the ε chain.

We want to thank Dr E. Czeizel and the 2. Dept. of Obstetrics of the János Hospital for making available the embryos. Our thanks are due to Mrs J. Báti (Institute of Biochemistry, Hungarian Academy of Sciences) for performing the amino acid analyses, and to Miss M. Hasitz for her valuable technical assistance.

References

Acher, R., Crocker, C. (1952) Biochim. Biophys. Acta *9* 704
Baldridge, R. C., Lewis, H. B. (1953) J. Biol. Chem. *202* 169
Carrel, R. W., Lehmann, H., Hutchinson, H. E. (1966) Nature *210* 915
Chernoff, A. I., Pettit, N. M. (1964) Blood *24* 750
Drescher, H., Künzer, W. (1954) Klin. Wschr. *32* 92
Hais, I. M., Macek, K. (1958) Handbuch der Papierchromatographie, Vol. 1. VEB Fischer Verl. Jena, p. 752
Huehns, E. R., Flynn, F. V., Butler, E. A., Beaven, G. H. (1961) Nature *189* 496
Huehns, E. R., Dance, N., Beaven, G. H., Hecht, F., Motulsky, A. G. (1964a) Cold Spring Harbor Symp. on Quant. Biol. Vol. *29* 327
Huehns, E. R., Hecht, F., Keil, J. V., Motulsky, A. G. (1964b) Proc. Natl. Acad. Sci. US *51* 89
Huehns, E. R., Shooter, E. M. (1965) J. Med. Genet. *2* 48
Perutz, M. F., Lehmann, H. (1968) Nature *219* 902
Smith, I. (1953) Nature *171* 43
Toennies, G., Kolb, J. J. (1951) Anal. Chem. *23* 823

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (1) pp. 57—69 (1969)

Secondary SH-Disulfide Exchange Reactions Following the Modification of Nucleophilic SH-Groups in D-glyceraldehyde-3-phosphate Dehydrogenase with DTNB*

L. Boross

Institute of Biochemistry, Hungarian Academy of Sciences, Budapest, Hungary

(Received November 21, 1968)

The nucleophilic SH-groups of D-glyceraldehyde-3-phosphate dehydrogenase react very rapidly with DTNB. The resulting enzyme containing GAPD-mercapto-nitrobenzoate mixed disulfide is labile. It is transformed *via* secondary SH-disulfide exchange reactions partly into the original active enzyme which contains only free SH-groups and partly into inactive enzyme containing only disulfide bonds.

The rate of the secondary SH-disulfide exchange reactions depends upon the pH and salt concentration of the solution. Increase in enzyme concentration results in a decrease of the relative rate of the splitting of mixed disulfide.

A hypothesis is set forth for the mechanism of the secondary reaction.

Introduction

D-glyceraldehyde-3-phosphate dehydrogenase contains SH-groups of different reactivities. One SH-group per subunit (active center), essential for enzymic activity, is of strong nucleophilic character, its reactivity is similar to that of mercaptide ions even in neutral solution (Racker, Krimsky, 1952; Genevois, Larrouquere, 1962; Harris, Perham, 1963; Polgár, 1964; Boross, Cseke, 1967). Two SH-groups per subunit, i.e. half of the total amount of SH-groups, react rapidly with PMB, the remaining two groups react only after prolonged incubation, after the disintegration of native protein structure (Szabolcsi et al., 1960; Friedrich, Szabolcsi, 1967).

In order to extend the physical-chemical characterization of the SH-groups of GAPD, we have undertaken experiments with several SH-reagents. We studied the kinetics of modification with DTNB, a reagent introduced by Ellman (1959) for the quantitative determination of SH-groups. DTNB seems to be a promising reagent for the characterization of the reactivity of SH-groups, since it forms

* Abbreviations: GAPD, D-glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD oxidoreductase (phosphorylating) E.C.1.2.1.12.); DTNB, 5,5'-dithio-bis(2-nitrobenzoate); PMB, p-chloromercuribenzoate; MNB, 5-mercapto-2-nitrobenzoate; GAP, D-glyceraldehyde-3-phosphate; GA, D-glyceraldehyde.

enzyme – MNB disulfide with the SH-groups of the enzyme *via* SH-disulfide exchange reactions with the concomitant release of MNB and such reactions are faster with mercaptide ions of nucleophilic character than with un-dissociated mercaptanes.

Indeed we have found that the nucleophilic SH-groups of GAPD react faster than mercaptanes with DTNB. GAPD containing the mixed disulfide is labile, secondary reactions take place which lead to the formation of reactivated enzyme molecules and enzyme molecules containing only disulfide bonds.*

Out of the 4 different SH-groups of GAPD per subunit, the nucleophilic one will be referred to as SH_I, the second fast-reacting one as SH_{II}, while the two buried ones as SH_{III} and SH_{IV}.

Materials and Methods

GAPD was isolated from pig muscle according to the method of Előd i and Szörényi (1956). Four times recrystallized enzyme preparations were used. A 6–8 per cent solution was made of the enzyme and was gel-filtered on a Sephadex G–100 column, washed previously with Tris buffer, pH 7, of 0.05 ionic strength, to remove myoglobin contaminations present in the enzyme preparation and the ammonium sulphate. The removal of myoglobin seemed to be desirable, since the reaction with DTNB was followed spectrophotometrically at 412 mμ, where myoglobin has appreciable light absorption. The light absorption of the effluent was measured at 360 and 410 mμ, and the fractions showing an E_{360}/E_{410} ratio equal to or higher than 2, were combined.

The experiments were carried out with GAPD free of bound NAD. The removal of bound NAD by charcoal treatment, the assay of protein concentration and enzymic activity were performed as described earlier (Boross, 1965; Boross, Cseke, 1967). The molecular weight of GAPD was taken as 140 000.

A 3×10^{-3} M DTNB stock solution was prepared and carefully neutralized with 1 M Tris solution to avoid even a temporary rise of pH over 8, as the reagent is transformed in alkaline medium. The concentration of the reagent was determined with excess 2-mercaptoethanol. The calculations were based on a molar extinction coefficient for mercaptonitrobenzoate of 13 600 (Ellman, 1959).

All reaction mixtures contained 10^{-3} M EDTA. GAP for the enzymic assay was prepared according to the method of Szewczuk et al. (1961). NAD was a Reanal, GA a Fluka, DTNB an Aldrich preparation. The other chemicals were commercial preparations of analytical grade.

The spectrophotometric measurements were carried out in Unicam SP 500 and SP 700A spectrophotometers equipped with thermostated cell house.

* Parts of this work were presented at the 4th and 5th FEBS Meetings (Boross, 1967, 1968).

Results

Reaction of GAPD with 1 to 4 mole equivalents of DTNB

DTNB, 1 to 3 moles per mole of enzyme, was added to a 1.4 mg/ml coenzyme-free GAPD solution, previously gel-filtered in 0.05 ionic strength Tris buffer, pH 7, and the change in light absorption at 412 mμ was measured. The optical density increased very rapidly following the addition of the reagent and in about 1 minute reached a value which corresponded to an MNB concentration equivalent to that of the added reagent (Fig. 1). When 4 moles of DTNB per mole of

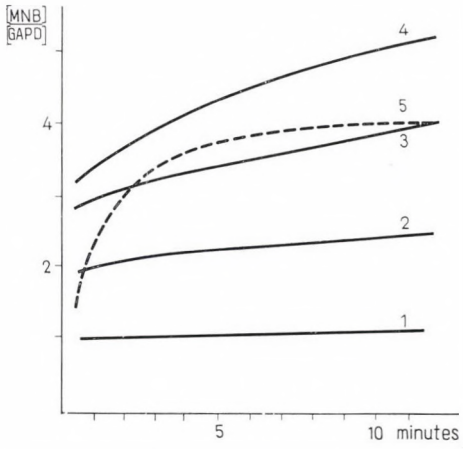


Fig. 1. Reaction of the nucleophilic SH-groups of GAPD with DTNB. Curves 1—4 show the amount of MNB released after the addition of 1—4 mole equivalents of DTNB to GAPD at 20 °C. The reaction mixtures contained 1.4 mg/ml GAPD, 10^{-3} M EDTA, and 1, 2, 3 and 4×10^{-5} M DTNB in 0.05 ionic strength Tris buffer, pH 7. Curve 5: time course of the reaction of 4×10^{-5} M 2-mercaptoethanol with 4×10^{-5} M DTNB, reaction conditions as above

enzyme were added 3.5—4.0 moles of MNB per mole of GAPD were formed within one minute. The same amount of nucleophilic SH-groups was found also by other methods (Cseke, Boross, 1967; Boross, 1967).

The reaction of 2-mercaptoethanol with DTNB was also studied in solutions of the same concentration and ionic strength (Fig. 1). It can be seen that GAPD reacts with DTNB faster than 2-mercaptoethanol under the experimental conditions. To test whether it is the nucleophilic SH_I groups of the enzyme which react with DTNB, activity was measured after the addition of 4 moles of DTNB per mole of enzyme. Within 30 seconds following the addition of the reagent enzymic activity was almost completely inhibited with both GAP and GA as substrates. These results confirm that SH_I, essential for enzymic activity, did react with DTNB.

On further incubation following the rapid reaction after the addition of 4 moles of DTNB per mole of GAPD, the light absorption at 412 mμ of the reaction mixture slowly increased. After 1 hour the light absorption approached the value corresponding to 8 moles of MNB per mole of GAPD. The absorption

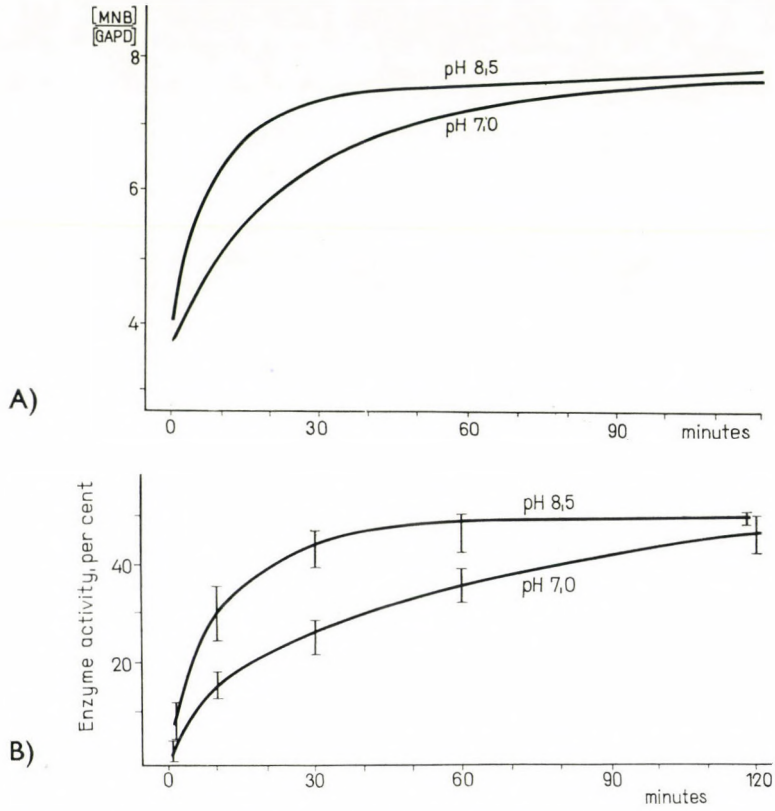


Fig. 2. pH-dependence of the decomposition of GAPD–MNB mixed disulfide and of the reactivation of the enzyme. A) Rate of release of MNB in reaction mixtures containing 10^{-5} M GAPD, 4×10^{-5} M DTNB, 10^{-3} M EDTA, in 0.05 ionic strength Tris buffer, pH 7 and 8.5, respectively, at 20 °C. B) Changes in enzyme activity at pH 7 and 8.5, respectively

spectrum of the solution in the near ultraviolet region agreed with that of MNB, i.e. the increase in absorption was actually due to the liberation of further MNB molecules. The second 4 moles of MNB per mole of enzyme could be released only from the enzyme-MNB mixed disulfide, *via* an SH-disulfide exchange reaction between an SH-group of the enzyme and the mixed disulfide. This means that two cysteinyl side chains of the enzyme, probably SH_I and SH_{II} were bonded together through a disulfide linkage. This product will be referred to as "disulfide-GAPD".

In reaction mixtures of higher pH the liberation of 8 moles of MNB was faster, the SH_{II} groups reacted with the enzyme-MNB mixed disulfide more rapidly (Fig. 2A).

During the course of incubation samples were taken from the reaction mixture and assayed for enzymic activity. A partial reactivation of the enzyme was observed. At 20 °C after 1 hour of incubation with 1.4 mg/ml enzyme concentration the recovery of enzymic activity was about 40 per cent, while after 2 hours of incubation 50 per cent of the untreated control (Fig. 2B). On further incubation, up to 48 hours, there was no change in enzymic activity, though half of the protein precipitated meanwhile from the solution.

The partial reactivation of the enzyme during incubation cannot be explained with the formation of "disulfide–GAPD" from GAPD–MNB mixed disulfide, since this species contains the nucleophilic SH_I group, essential for enzymic activity, in a disulfide bond. Therefore it had to be assumed that the originally buried SH_{III} and SH_{IV} groups of the protein became, at least partly, exposed due to a structural change of the molecule. Thus further SH-disulfide exchange reactions could take place that resulted in the recovery of free nucleophilic SH_I groups in certain enzyme molecules.

The precipitate, separated from the above reaction mixture after two days of incubation at room temperature was collected by centrifugation, washed three times in Tris buffer, then dissolved in 8 M urea and the SH-content measured. No free SH-groups could be detected in the precipitated protein or only in negligible amounts (0 – 0.5 SH per mole of enzyme). The supernatant contained about half of the initial amount of protein, its protein concentration was 0.7 ± 0.03 mg/ml, and half of the initial enzyme activity. This shows that about 50 per cent of the protein molecules regained full enzymic activity, while in the other 50 per cent all SH-groups were oxidized to disulfides and these molecules precipitated from the reaction mixture. Accordingly, the supernatant contained 15 – 16 SH-groups per enzyme molecule which could be titrated with DTNB in 8 M urea.

The experiments were repeated in reaction mixtures of various pH-values. Similarly to the decomposition of the mixed disulfide, the recovery of enzyme activity was faster at higher pH (Fig. 2B).

Effect of salt concentration on the secondary SH-disulfide exchange reactions

Ellman has observed (Ellman, 1959) that SH-groups react faster with DTNB in solutions of higher salt concentration. In the reaction of mercaptoethanol with DTNB we also found the acceleration of the reaction in the presence of sodium chloride or ammonium sulfate. We have examined therefore in detail the effect of salt concentration on the reaction of GAPD with DTNB and on the secondary SH-disulfide's exchange reactions. As before, the experiments were carried out at 1.4 mg/ml protein concentration at pH 7.

With 4 moles of DTNB per mole of GAPD in the presence of 0.1 – 1.5 M sodium chloride, the reaction with the nucleophilic SH-groups took place very

rapidly, its time course could not be followed in an ordinary spectrophotometer. Not only the liberation of the first MNB molecules, equivalent to the amount of nucleophilic SH-groups, was faster, but salt concentration affected also the splitting of GAPD–MNB mixed disulfide (Fig. 3). On increasing salt concentration from 0.1 M to 0.25 M, the rate of liberation of the second 4 equivalents of MNB became gradually higher. On further increase of salt concentration the effect exerted on the decomposition of mixed disulfide was ambivalent: the first part of the reaction was accelerated by high salt concentration, while from the appearance of 5–5.5 moles of MNB per mole of enzyme the reaction slowed down. Thus in solutions with sodium chloride concentrations higher than 0.25 M, liber-

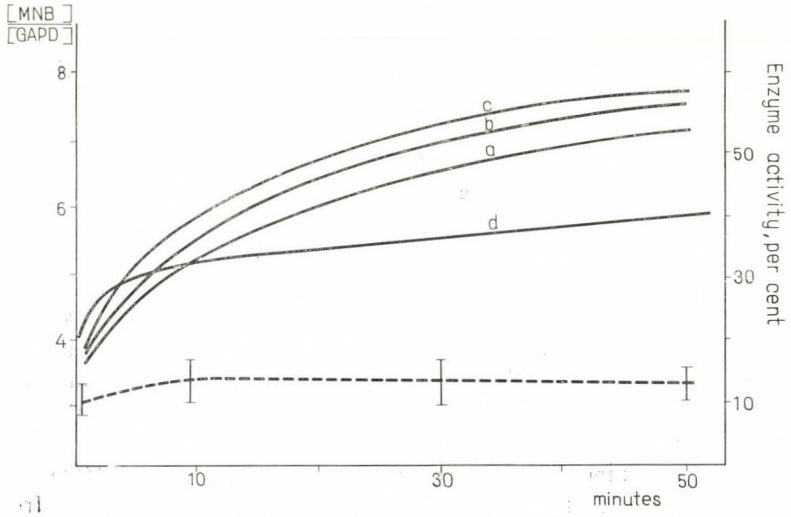


Fig. 3. Effect of salt concentration on the decomposition of GAPD–MNB mixed disulfide. Reaction mixture contained: *a)* 1.4 mg/ml GAPD, 4×10^{-3} M DTNB, 10^{-5} M EDTA in 0.05 ionic strength Tris buffer, *b)* The same as *a* plus 0.1 M sodium chloride, *c)* The same as *a* plus 0.25 M sodium chloride, *d)* The same as *a* plus 1.5 M sodium chloride. Dotted line: enzyme activity in the reaction mixture *d*

ation of 8 moles of MNB per mole of enzyme, i.e. splitting of the total amount of mixed disulfide, required a longer time than in reaction mixtures of lower salt concentration.

Since the free SH-groups of the enzyme are needed for splitting the GAPD–MNB mixed disulfide, we examined, whether the non-nucleophilic SH_{II} groups of the enzyme became buried in concentrated sodium chloride solution. DTNB was added to the enzyme, 8, 12, and 16 moles per mole of enzyme, respectively, in Tris buffer and in 1.5 M sodium chloride solution and the time course of the reaction was followed (Fig. 4). In Tris buffer all SH-groups of the enzyme could react with DTNB after prolonged incubation, though the reaction was considerably slower than with aliphatic SH-compounds, e.g. mercaptoethanol. On the other hand, in 1.5 M sodium chloride solution half of the total amount of SH-groups, i.e. 8 per

molecule, reacted very rapidly, within 2–3 minutes, with DTNB. Apparently the reactivity of SH_{II} was increased. After this stage the reaction very much slowed down indicating that 8 SH-groups became much less accessible to the reagent. This finding, however, does not explain the decreased rate of splitting of the GAPD–MNB mixed disulfide (Fig. 3, curve d), as the reactivity of the 4 surface-exposed SH_{II} groups of the enzyme increased rather than decreased.

In accordance with the foregoing, the restoration of enzymic activity after the addition of 4 moles of DTNB per mole of GAPD was slower in 1.5 M sodium chloride than in 0.05 ionic strength Tris buffer. After one day of incubation, when

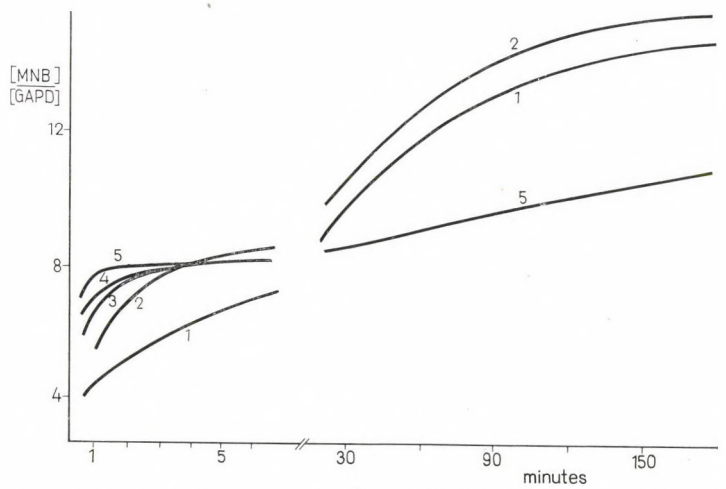


Fig. 4. Reaction of GAPD with various amounts of DTNB in 0.05 ionic strength Tris buffer and in 1.5 M sodium chloride. Reaction mixtures contained 10^{-5} M GAPD. Curves *1* and *2*: 0.05 ionic strength Tris buffer, pH 7; Curves *3*, *4* and *5*: 1.5 M sodium chloride. Temperature: 20 °C. DTNB concentration: *1* and *3*, 8×10^{-5} M; *4*, 1.2×10^{-5} M; *2* and *5*: 1.6×10^{-4} M

the amount of liberated MNB approached the value corresponding to 8 moles per mole of GAPD, the enzymic activity was completely lost rather than recovered. It appears that in reaction mixtures of high salt concentration the SH-disulfide exchange reactions, required for the restoration of the active center of the enzyme, take place at a lower rate than in reaction mixtures of low ionic strength.

Similar effect was observed when ammonium sulfate was used instead of sodium chloride. This result indicates that we are dealing with a general salt-effect rather than a specific one.

Effect of enzyme concentration on the secondary SH-disulfide exchange reactions

The secondary reactions which follow the modification of nucleophilic SH-groups with DTNB are partly intramolecular, partly intermolecular SH-disulfide exchange reactions. For this reason we examined the effect of GAPD concentration on the rate of these reactions.

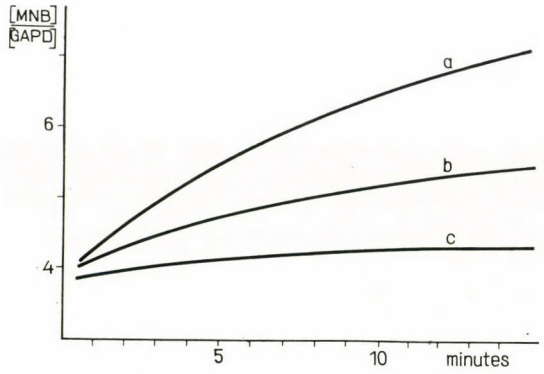


Fig. 5. Effect of enzyme concentration on the decomposition of GAPD–MNB mixed disulfide. All reaction mixtures contained 4 moles of DTNB per mole of enzyme. GAPD concentrations: *a*, 0.35 mg/ml; *b* 2.8 mg/ml; *c* 14 mg/ml. Reaction conditions as in Fig. 1

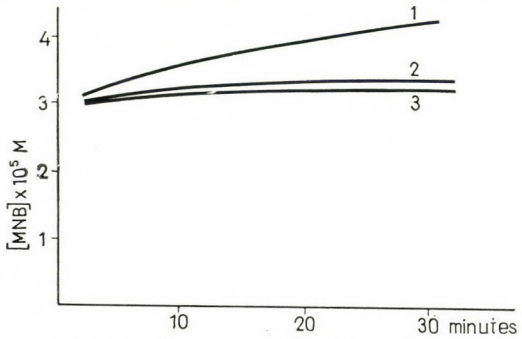


Fig. 6. Decomposition of GAPD–MNB mixed disulfide in the presence of free nucleophilic SH-groups. Reaction mixtures contained 3×10^{-5} M DTNB. Enzyme concentrations: *1.* 10^{-5} M; *2.* 2×10^{-5} M; *3.* 3×10^{-5} M, in 0.05 ionic strength Tris buffer, pH 7, containing 10^{-3} M EDTA

In one series of experiments with a constant ratio of 4 moles of DTNB per mole of GAPD the enzyme concentration was varied between 0.35 and 14.0 mg/ml. Strikingly enough, increase in enzyme concentration significantly reduced the relative rate of decomposition of the mixed disulfide (Fig. 5). Within the above concentration range the time course of the reaction could not be described by first order kinetics. In reaction mixtures of higher enzyme concentrations (7 and 14 mg/ml), the recovery of enzyme activity was slower than in 1.4 mg/ml concentration (Table 1). This is in agreement with the fact that under such conditions the GAPD–MNB mixed disulfide is transformed at a lower rate.

In another series of experiments DTNB concentration was kept constant and GAPD concentration was varied (Fig. 6). When the amount of the nucleophilic SH_I groups of the enzyme exceeded that of DTNB, the decomposition of the mixed disulfide was slower than in the case of a 1 : 1 reagent : nucleophilic SH_I ratio showing that the nucleophilic SH-groups of the enzyme are unable to attack a GAPD–MNB mixed disulfide bond present in another enzyme molecule

Table 1

Effect of enzyme concentration on the reactivation of GAPD–MNB mixed disulfide

Enzyme concentration mg/ml	Activity per cent			
	30 sec	30 min	60 min	120 min
1.4	0—6	22—28	34—46	42—50
7.0	0—9	15—19	20—22	22—25
14.0	0—4	10—15	13—25	18—26

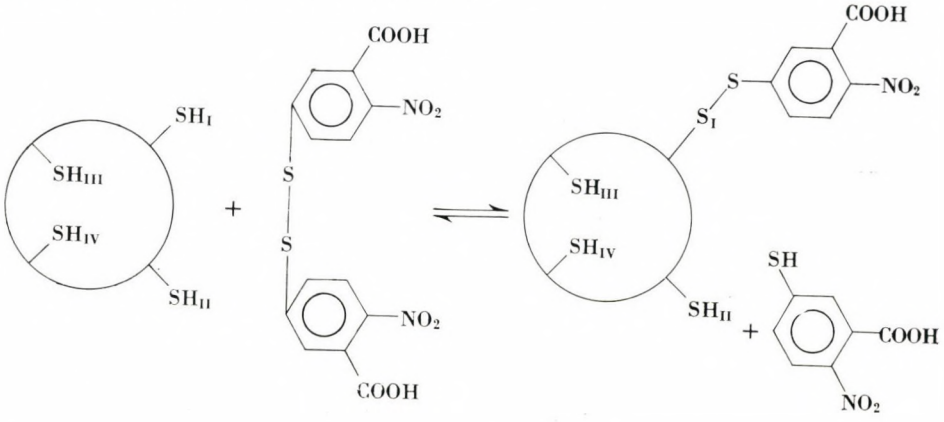
and to form, with concomitant release of MNB, GAPD–GAPD dimer molecules held together by disulfide bonds. On the other hand, free nucleophilic SH_I groups inhibit the GAPD–MNB mixed disulfide → disulfide–GAPD transformation.

Discussion

The experiments presented above show that modification of the active, nucleophilic SH-groups of GAPD with DTNB elicits secondary reactions. The end-products of these reactions are inactive enzyme molecules containing disulfide bonds and reactivated enzyme molecules containing SH-groups. On the basis of our results four steps can be distinguished in the overall process:

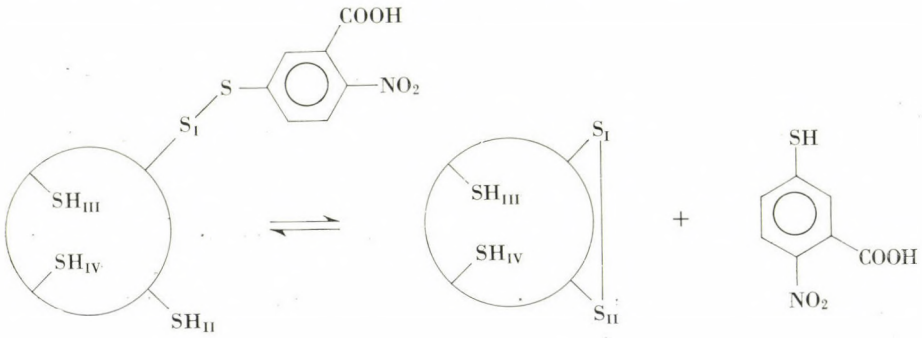
1. The nucleophilic SH_I groups of GAPD react with equivalent amounts of DTNB very rapidly to form GAPD–MNB mixed disulfide, with the simultaneous release of an equivalent amount of MNB. The fast reaction is in accordance with earlier observations according to which these active SH-groups behave as mercaptide ions in neutral solution as well. The rapid reaction of DTNB with with the reactive SH groups of yeast GAPD was also observed by Kirschner (1967).

Considering that GAPD contains one nucleophilic SH-group (SH_I), one surface-exposed but less reactive SH-group (SH_{II}) (Vas, Boross, 1969), and two buried SH-groups (SH_{III} and SH_{IV}) per subunit, the scheme of the reaction for one subunit can be delineated as follows:



The above reaction scheme is supported by the findings, that after the addition of 4 equivalents of DTNB, GAPD becomes completely inactive, and furthermore, that SH_{II} groups form mixed disulfide much more slowly after the addition of 8 equivalents of DTNB, than SH_{I} groups.

2. The GAPD–MNB mixed disulfide formed is split by the SH_{II} groups of the enzyme with further release of MNB. Thus two SH-groups of the enzyme become involved in a disulfide bond, "disulfide–GAPD" is formed:

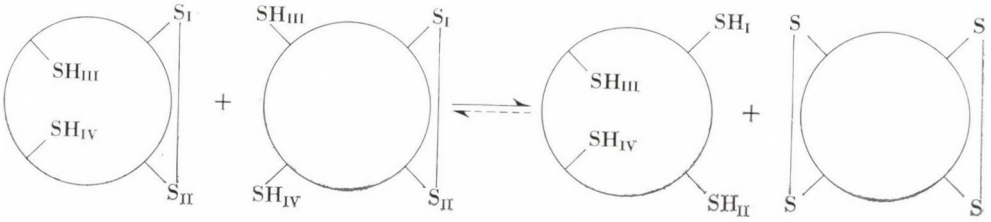


This product is enzymically inactive, since it does not contain any free nucleophilic SH-groups. Reaction (2) commences as soon as GAPD–MNB mixed disulfide is formed and its rate depends on the pH and ionic strength of the reaction mixture. The lower rate of the reaction in solutions containing high concentration of sodium chloride or ammonium sulfate suggests that under such conditions it is more difficult for SH_{II} to approach the mixed disulfide bond and thus the probability of the reaction is reduced. Under the same conditions SH_{II} groups react with DTNB faster than in solutions of lower salt concentration, i.e. they are not rendered buried (inaccessible) SH-groups. At the beginning reaction (2) is faster at high than at low salt concentrations, and slows down only after the appearance of about 5 moles of MNB per mole of GAPD. Presumably a complex redox equilibrium is set up at this stage and the solution comprises, in addition to MNB and disulfide–GAPD, GAPD–S_I–MNB and GAPD–S_{II}–MNB mixed disulfides as well as free GAPD molecules and DTNB in small amounts.

3. The labile structure of "disulfide–GAPD" is altered while the buried SH_{III} and SH_{IV} groups come to the surface:

The rate of structural change depends on the ionic composition of the solution. In neutral solution of high salt concentration the enzyme molecule is more compact than in a dilute buffer, and therefore the structural change takes place slowly. This assumption is corroborated by the finding that in concentrated salt solutions SH_{III} and SH_{IV} can react with DTNB on very prolonged incubation only. At this step the whole process slows down, the structural change becomes the rate-limiting step of the reaction.

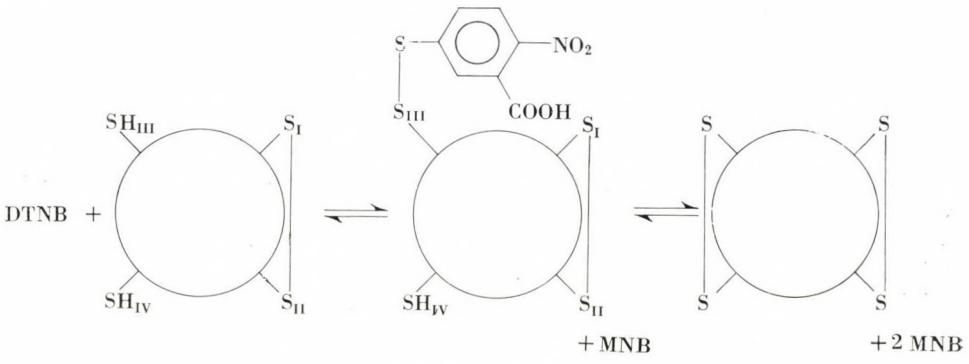
4. SH-groups rendered accessible by the structural change can react with the "disulfide-GAPD". The reaction scheme can be visualized as follows:



During the course of the reaction enzyme molecules containing only disulfide bonds are formed, while the same amount of enzyme molecules are reduced, reactivated. In the ideal case half of the enzyme molecules are reactivated while the other half denatured as a result of the process described above. Molecules containing only disulfide bonds precipitate from the solution.

The validity of reaction (4) is supported by the experiments of Móra et al. (1969) according to which oxidation of the SH-groups of GAPD with iodine yields a product which contains two disulfide bonds within each subunit. At any rate, the above experiments give no answer to the question whether in reaction (4) inter- or intra-subunit disulfide bonds are formed.

The collision of two enzyme molecules is not indispensable for the disulfide translocation which takes place in reaction (4). Reactions (1) and (2) are in principle reversible, thus DTNB present in small amount according to the equilibrium position, may react with SH_{III}, and SH_{IV} may in turn split the GAPD–S_{III}–MNB mixed disulfide:



The end-product is the same, an inactive species containing two disulfide bonds, while the active enzyme is formed through the reversal of step (1) due to the removal of DTNB.

The above schemes describe the process for a single subunit. The whole process, however, also includes interactions between the subunits: the end products are molecules containing only reactivated subunits or only inactive subunits, respectively. If interactions between subunits did not operate, enzyme molecules containing active and inactive subunits in a 1 : 1 ratio would be formed with greatest probability.

The anomalic dependence of the reaction rate on protein concentration suggests that there are still unknown factors that influence the process. Reaction (2) is relatively slower at high enzyme concentration than in a more dilute solution. This phenomenon could be explained by the presence of an inhibitor substance, since the dissociation of such an inhibitor–GAPD complex would be greater at low enzyme concentration. However, there is no experimental evidence for such an "inhibitor".

When reaction (2) is slowed down by the presence of free nucleophilic SH-groups, the inhibition can be interpreted not only in terms of the repression of dissociation of an hypothetical GAPD–inhibitor complex. In fact, it seems very probable that in such reaction mixtures the mixed disulfide bond "migrates" over several SH-groups *via* repeated SH-disulfide exchange reactions. If, for example, the number of nucleophilic SH-groups in the solution is 4 times greater than that of DTNB molecules, i.e. on the average only one mixed disulfide can be formed per enzyme molecule, this disulfide bond presumably exists in one subunit for only a very short time, it keeps moving around to other subunits. An SH_{II} group of the enzyme can react with the mixed disulfide if it is sterically close to SH_I. If juxtaposition is brought about by a reversible structural change of the enzyme, induced by the formation of a mixed disulfide bond, the time required for this structural change may well be longer than the time spent by the mixed disulfide bond in the same subunit. This would explain the lower rate of reaction (2).

Moreover, if this assumption is valid, the deviation of reaction (2) from monomolecular pattern also becomes conceivable: during the course of the reaction more and more nucleophilic SH-groups are set free, as indicated by the recovery of enzymic activity.

The secondary process which follows the modification of GAPD with 4 equivalents of DTNB is reminiscent of the process observed by Szabolcsi et al. (1960) after modifying the enzyme with PMB. In the latter case, PMB first binds to SH-groups located in the active center of the enzyme, then *via* secondary reactions fully active enzyme molecules containing no PMB and inactive enzyme molecules fully blocked with PMB are formed. A similar phenomenon can be observed when 8 equivalents of Ag^+ ion are added to the enzyme, with the difference that in this case a ternary Ag^+-enzyme–coenzyme complex and enzyme molecules containing 12 Ag^+ ions are finally produced (Boross, Keleti, 1965). Our present findings with DTNB serve as one example of these "disproportionation-like"

processes. The driving force of these reactions is the different stabilities of native, modified and denatured enzyme species. The stability of enzyme molecules, which was reduced by modification, will be regained by either denaturation or reactivation. If the modifying agent is bound to the enzyme reversibly and the buried groups which become exposed during denaturation can remove the reagent from other molecules, "disproportionation" takes place.

The author wishes to express his thanks to Prof. F. B. Straub for helpful discussion and to Miss É. Farkas for skillful technical assistance.

References

Boross, L. (1965) Biochim. Biophys. Acta *96* 52
Boross, L. (1967) 4th FEBS Meeting, Oslo, Abstract No. 74
Boross, L. (1968) 5th FEBS Meeting, Prague, Abstract No. 735
Boross, L., Keleti, T. (1965) Acta Physiol. Acad. Sci. Hung. *27* 297
Boross, L., Cseke, E. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 47
Cseke, E., Boross, L. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 39
Ellman, G. L. (1959) Arch. Biochem. Biophys. *82* 70
Elődi, P., Szörényi, E. (1956) Acta Physiol. Acad. Sci. Hung. *9* 339
Friedrich, P., Szabolcsi, G. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 59
Genevois, L., Larrouquere, J. (1962) Compt. Rend. Acad. Sci. France *255* 2532
Harris, J. I., Perham, R. N. (1963) Biochem. J. *89* 600
Kirschner, K. (1967) 4th FEBS Meeting, Oslo, Abstract No. 72
Móra, S., Hüvös, P., Libor, S., Elődi, P. (1969) Acta Biochim. Biophys. Acad. Sci. Hung. in press.
Polgár, L. (1964) Experientia *20* 408
Racker, E., Krimsky, I. (1952) J. Biol. Chem. *198* 731
Szabolcsi, G., Biszku, E., Sajgó, M. (1960) Acta Physiol. Acad. Sci. Hung. *17* 183
Szewczuk, A., Wolny, E., Wolny, M., Baranowski, T. (1961) Acta Biochim. Polon. *8* 201
Vas, M., Boross, L. (1969) in preparation

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (1) pp. 71—78 (1969)

Distribution of Intrafibrillar K and Na and Autoradiographic Investigation of the K–Na Exchange due to Direct Stimulation

N. KÁLLAY, A. TIGYI-SEBES

Biophysical Institute, Medical University, Pécs

(Received August 17, 1968)

Experiments have been performed with light microscopic and electron microscopic autoradiographic methods in order to determine the intrafibrillar distribution of K and Na exchanged through perfusion in the striated muscle. Parallel with the examination concerning localization data have been obtained as to the part played in the K–Na exchange by direct stimulation.

It was found that about 2/3 of the grains coming from ^{42}K was located in the A band and 1/3 of them in the I band of the muscle fibril the latter band also including the Z-line. The distribution of grains in experiments performed with ^{24}Na was similar to this but with a greater density of grains.

In experiments performed with the two isotopes in order to elucidate the role played in the ion exchange by direct stimulation an increase of the grain density was found over the muscles stimulated directly during perfusion when compared with non-stimulated muscles. In this case we found the grain density to be the same in experiments with ^{42}K and ^{24}Na, and also the distribution was equal.

From this increase of the number of grains and from the equal distribution of grain density we conclude that, under the effect of direct stimulation, an increase occurs in the K–Na exchange.

Introduction

One of the aims of the research work going on in this Institute is to clarify the submicro localization of K and Na, and the changes of these substances during muscular activity (Ernst, 1963; Tigyi, 1968; Tigyi-Sebes, 1962; Nesterov, Tigyi-Sebes, 1965). To fulfill this aim we performed investigations with autoradiographic methods into the localization of inorganic elements at fibrillar level. After determining the hystological localization of the PO_4 described (Kállay, Tigyi-Sebes, 1967, 1967a), we examined K and Na ions occurring in the muscle in a relatively high concentration (Ernst, 1966).

Beside hystological localization it was the purpose of our examinations to obtain some further data on the role of these elements in muscle excitation (Ernst, 1963; Tigyi, 1960).

Methods

Sartorius muscles of the frog (Rana esculenta) were used in our experiments. Läwen–Trendelenburg preparations were made from two frogs at the same time.

The hind limbs of the two frogs were perfused from a vessel with 600 ml of a Ringer solution containing ^{42}K or ^{24}Na with an average specific activity of 25 μCi/ml each. The common iliac artery leading to the right hind limb was ligated before beginning the perfusion. The rate of perfusion was regulated to 20 to 25 drops/min in both preparations so that the perfusions lasted 2 to 2.5 hours. The perfused hind limb of one of the frogs was stimulated with a stepped down alternate main current increasing from 2 to 30 Volts during the perfusion. The time duration of one stimulus was 0.5 sec and stimulation was repeated at 2 second intervals. The muscle was resting for about 5 minutes before increasing voltage.

The sartorius muscle was excised from the perfused limb at the end of the perfusion. An inactive sartorius was also excised in each experiment. For the light-microscopic experiments the slightly stretched sartorius muscles were fixed in the vapour of 35 per cent formol for 15 minutes. Fibril preparations were made from the fixed muscles with the aid of two needles on a microscopic slide. We made preparations from all the three materials (perfused, perfused and stimulated, and control muscles, resp.), two pieces from each in an experiment. The slides were covered with a twice-diluted solution of Ilford L–4 liquid emulsion according to Leblond's (1948) plunging method; thereafter they were dried in a cold air stream and then exposed at 2 °C in a lightproof box for 72 hours. After the exposure finished the slides were developed photographically (in Ansco 47/A developer for 7 minutes) and after fixing and rinsing, they were examined under light-microscope. The single fibrils found in the area of one visual field and the grains placed over them were counted using a 640-fold magnification.

We had to follow a cytological treatment slightly different from the traditional one in the electron microscopic experiments because of the water solubility and short half-life of the isotopes used here. The slightly flexed sartorius muscle was fixed in OsO_4 vapour for 1 hour. After the short dehydrating process we embedded the muscle into a 1 to 4 mixture of methylmetacrylate and butylmetacrylate using 2,4-dichlorobenzoic acid to accelerate polymerization. 5–6 hours after the beginning of embedding we obtained blocks suitable for cutting from which we made sections of about 300–500 Å in thickness with the aid of a Porter–Blum ultramicrotome (grey interference colour on water). After staining the grids with phosphotungstic acid in alcohol for 1 minute we placed them on a microscopic slide, and fastened with a formwar film of about 100 Å in thickness. (We made 8 to 10 pieces of perfused, directly stimulated and control grids resp. in each experiment.)

The preparations made in the above way were covered with an emulsion film from a twice diluted solution of Ilford L–4 emulsion. (See Fig. 1.) For preparing the emulsion film we used the loop method of Caro and van Tubergen (1962) slightly modified by us. The whole process was performed in a dark room, 1.5 to 2 m far from a 15 W green lamp in a green bulb. After a 70 to 72 hour exposure at 2 °C the preparations were developed in an Ansco 47/A developer (at 20 °C for 5 minutes), fixed (for 4 minutes) and rinsed in twice-distilled water (for 9–10 minutes). After drying in the air the grids were carefully removed from the slides and examined in an electron microscope at an accelaretor voltage of 60 kV.

Our experiments at electron microscopic level served for the determination of the intrafibrillar distribution of the exchanged ^{42}K and ^{24}Na. 20 photographs of the same magnification were made from each of the three kinds of materials during each experiment. The grains found over the A and I bands of the myofibril (considering the Z-line as belonging to the I band) were counted from the photograms made from these photographs. We calculated the density of grains from their number, taking the areas of different bands into account.

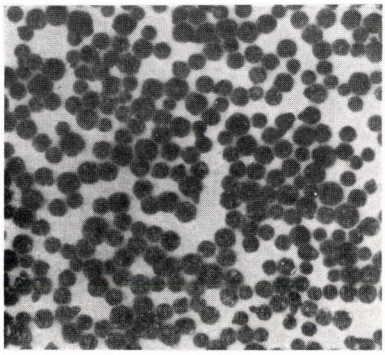


Fig. 1. Electron microscopic picture of an emulsion film consisting of monolayer silver-haloid grains obtained from the twice diluted solution of Ilford L–4 emulsion. (M = 25,000)

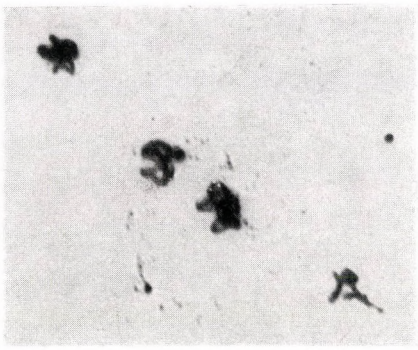


Fig. 2. Electron microscopic picture after developing of Ilford L–4 emulsion-grains exposed with ^{42}K. (M = 30,000)

Exposure trials were performed in our experiments as to the applicability of ^{42}K and of ^{24}Na for autoradiographic purpose at electron microscopic level. It was the short half-life and the great beta-energy of these two isotopes what raised the need of the investigation. The picture of the exposed grains obtained after developing (Fig. 2) does not differ from grains exposed by beta emitters of smaller energy.

Results

Comparison of the pictures with those found in inactive control experiments revealed an intrafibrillar ^{42}K and ^{24}Na incorporation to occur owing to the perfusion, as indicated by the 5 to 6 times greater density of grains over the active muscle. This can well be seen in the electromicrograms of Figs 3 and 4. Fig. 3 shows a muscle perfused with ^{42}K (a), one directly stimulated during perfusion (b) and a control photograph (c). We can see similar photographs in Fig. 4 showing experiments performed with ^{24}Na.

Table 1 shows the average density of grains in 9 repeated experiments performed with ^{42}K. The grain densities originating from ^{42}K exchanged through perfusion can be seen in the first line of Table 1. The density of grains

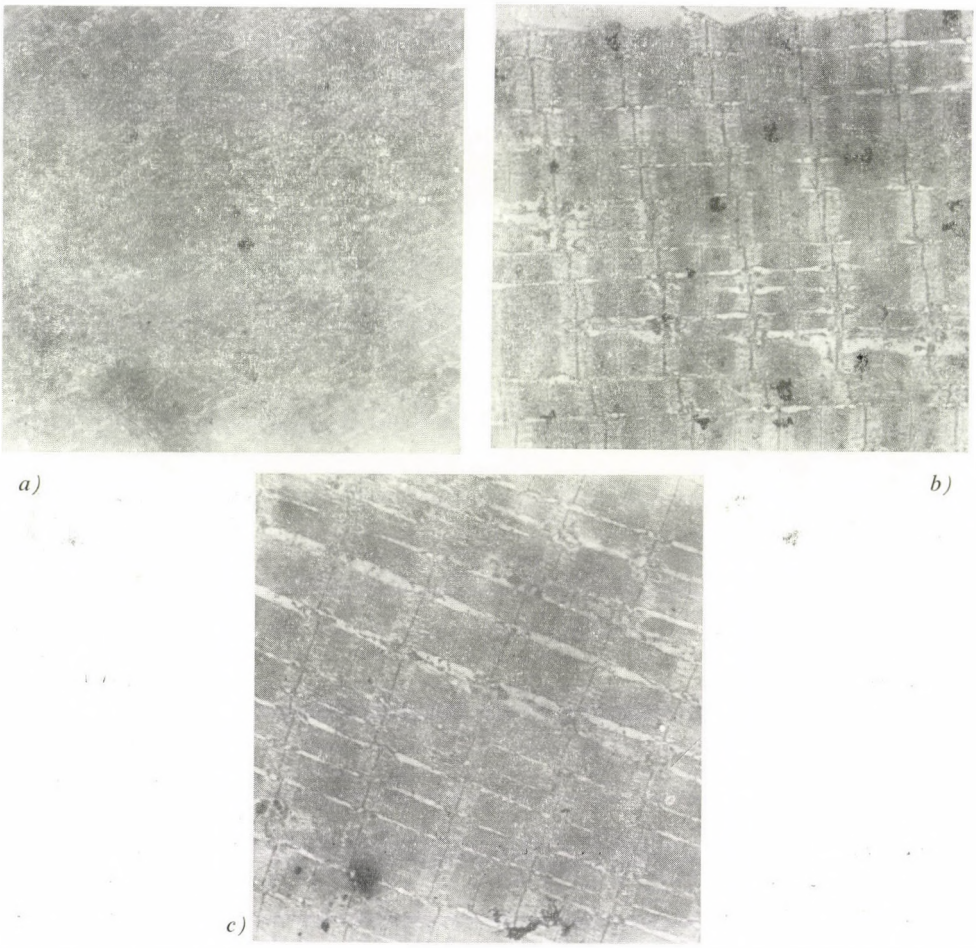


Fig. 3. Electron microscopic autoradiogram of ultrathin sections from the sartorius muscle of a frog (Rana esculenta) treated with ^{42}K. *a)* perfused, *b)* stimulated during perfusion, *c)* inactive control. (M = 6000)

Table 1

Distributions of the density of grains over the muscle fibre in autoradiographic experiments performed with ^{42}K at electron microscopic level

Treatment of the muscle	Density of grains $\frac{\text{grain}}{100\,\mu^2}$	
	over the A-band	over the I-band
perfused	7.82±1.34	5.41±0.83
perfused and stimulated	13.02±1.34	9.52±1.66
control	2.16±0.46	2.15±0.42

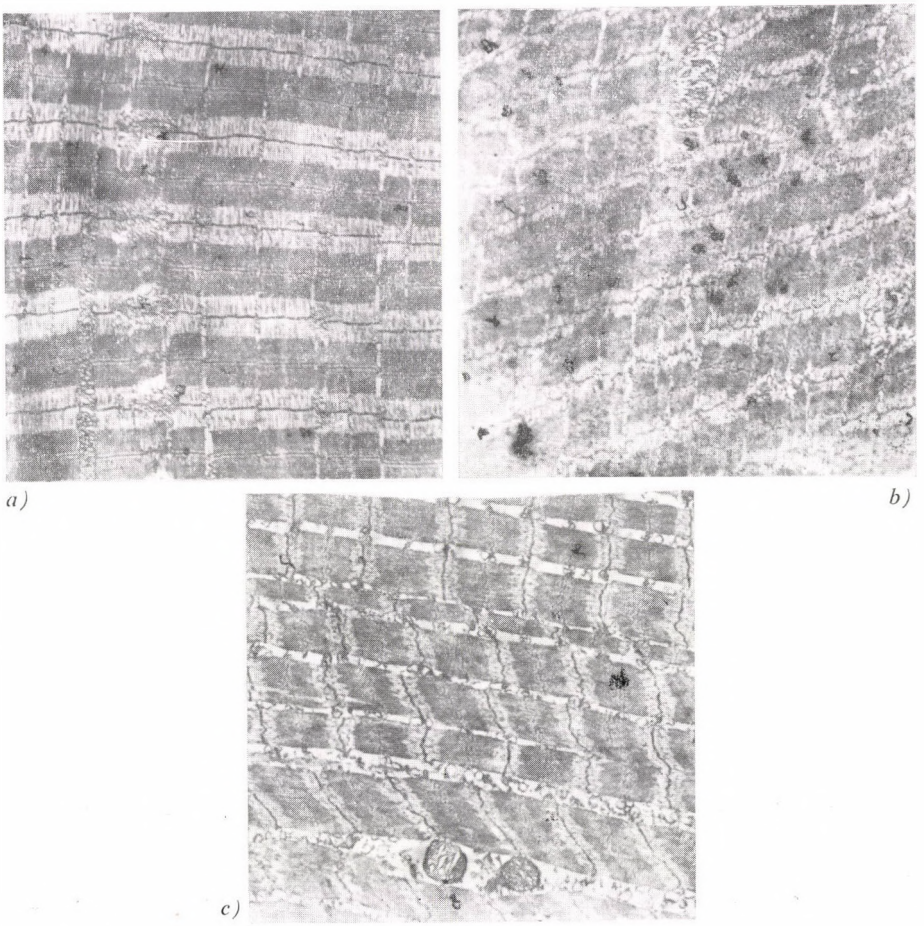


Fig. 4. Electron microscopic autoradiogram of ultrathin section from the sartorius muscle of a frog (Rana esculenta) treated with ^{24}Na, *a)* perfused, *b)* stimulated during perfusion, *c)* inactive control. (M = 6000)

Table 2

Distributions of the density of grains over the muscle fibre in autoradiographic experiments performed with ^{24}Na at electron microscopic level

Treatment of the muscle	Density of grains $\frac{\text{grain}}{100\ \mu^2}$	
	over the A-band	over the I-band
perfused	9.89±1.77	6.75±1.25
perfused and stimulated	12 91±2.29	8.12±1 04
control	2.60±0.42	2.91±0.46

found over the A and I bands is different from the equal distribution even if the level of significance is P = 0.001, 2/3 of the grains being placed in the A band of the muscle fibril, and 1/3 in the I band which included the Z-line too.

The second line of Table 1 shows the average density of grains over a muscle perfused with the same solution and directly stimulated while under perfusion. Here, the density of grains is considerably greater (P = 0.05) than that found over the non-stimulated muscle shown in the first line. But the proportion of grain densities over the A and I bands did not change.

The third line of Table 1 shows the equal distribution of grains originating from the background over the inactive control muscle.

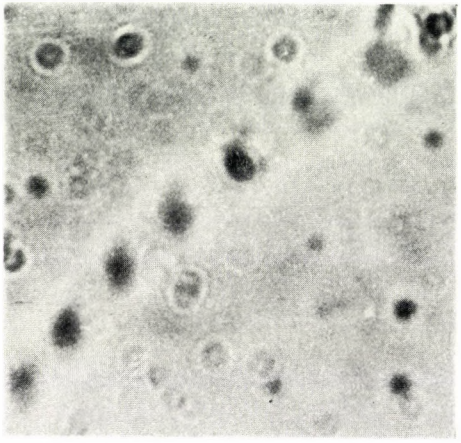


Fig. 5. Autoradiogram of a single muscle fibre obtained from the sartorius muscle of a frog (Rana esculenta) treated with ^{24}Na. (M = 3000)

Table 2 shows the mean values of grain densities found in 12 experiments performed with ^{24}Na. The distribution of grains over the A and I bands is almost entirely the same as that found in the experiments carried out with ^{42}K, 2/3 of the grains being placed over the A band of the muscle fibril, and 1/3 over the I band which included also the Z-line in these experiments too. But it is worthy of attention that though the Na content of the muscle is much less than its K content, the density of grains coming from the ^{24}Na exchanged through perfusion is greater than that found in the experiments performed with ^{42}K (the perfusion was made with the same quantity of solution of the same radioactive concentration).

Direct stimulation caused a remarkable increase in the density of grains also in the case of ^{24}Na (P = 0.05), as it can be seen in the second line of the Table 2. The third line of Table 2 also shows the equal distribution of grains coming from the background over the inactive control muscle, similarly to Table 1.

Our light microscopic investigations also support the assumption that the direct stimulation has an increasing effect on the intrafibrillar ion exchange. We evaluated statistically the numbers obtained by counting under light microscope

the fibrils similar to the single fibrils of Fig. 5 and the grains found above them. Table 3 shows the results of the evaluation of 14 experiments performed with ^{42}K. The density of grains over the stimulated muscle fibrils is, again, considerably greater than that over the non-stimulated ones ($P = 0.05$). After the subtraction of the grain density of the background, the ratio of grain densities over the stimulated and non-stimulated muscles is 1.82 in light microscopic experiments and 1.91 in experiments at electron microscopic level.

Table 3

The density of grains over the muscle fibre in autoradiographic experiments performed with ^{42}K at light microscopic level

Treatment of the muscle	Density of grains $\frac{\text{grain}}{\text{fibre}}$
perfused	0.214 ± 0.033
perfused and stimulated	0.270 ± 0.046
control	0.446 ± 0.030

Discussion

Two beta emitters of relatively short half-life and of great energy, i.e. ^{42}K and ^{24}Na, were applied for autoradiographic purpose with good results in our experiments. The distribution of these two elements between the different sections of the striated muscle fibril was determined with the aid of these beta emitters which are at the same time the radioactive isotopes of the two inorganic elements occurring in a relatively high concentration in the muscle. Our experiments seem to corroborate chiefly the opinion of Ernst (1963) and his colleagues according to which the potassium in the muscle is mostly localized in the muscle fibril, namely in its A band. The relatively great density of grains found over the I band can be partly explained by transradiation from the A band due to the great beta energy, and partly by the residual activity of the muscle soaked with isotopic solution. However, certain inorganic substances contained in the Z-line cannot be neglected either. The solution of this problem needs further investigations.

A distribution of the isotopes equal to, and a density of grains considerably over those in the experiments performed with ^{42}K were observed in the experiments with ^{24}Na. The almost 5 times higher value obtained instead of the expected density of grains can be explained neither by theoretical autoradiographic consideration, nor by the difference in the exchange speed of the two ions. On the basis of the investigations of Heppel (1940) and Ernst (1963) it seems to be an attractive assumption for the explanation of the inhomogeneous distribution of grains, and of their greater density than that found in the experiments with K, that the ^{24}Na content of the perfusing solution is partly exchanged with the intrafibrillar K.

This assumption seems to be supported by the equal distribution of grain density in the two experiments, a finding contrasting with the homogeneous distribution of extracellular Na.

A remarked increase was found in the intrafibrillar ion exchange under the effect of direct stimulation current, in accordance with the results of the pertaining experiments performed on whole muscle. The possible explanation for this increase of ion exchange can be the release during stimulation of the partly-bound K content of the myofibril. But the mode of stimulation can also play a part in this increase of ion exchange; we consider the investigation of this question to be our further task.

The authors thank Prof. E. Ernst for raising the question.

References

Caro, L. G., van Tubergen, R. R. (1962) J. Cell Biol. *15* 173
Bélanger, R. F., Leblond, C. P. (1946) Endocrinology *39* 8
Ernst, E. (1963) Biophysics of the Striated Muscle. Akadémiai Kiadó, Budapest
Ernst, E. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 113
Heppel, L. A. (1940) Am. J. Physiol. *128* 449
Kállay, N., Tigyi-Sebes, A. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 221
Kállay, N., Tigyi-Sebes, A. (1967) Abstr. First Joint Congress of the Hung. Societies of Biochim. Biophys. and Physiol. Pécs. P. 86
Nesterov, V. P., Tigyi-Sebes, A. (1965) Acta Physiol. Acad. Sci. Hung. *27* 97
Tigyi, J. (1960) MTA Biol. Csop. Közl. *4* 71
Tigyi, J. (1968) Symposium on Muscle. Akadémiai Kiadó, Budapest, p. 143
Tigyi-Sebes, A. (1962) Acta Physiol. Acad. Sci. Hung. *22* 234

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (1) pp. 79—87 (1969)

X-ray Induced Alterations in the Mitotic Cycle

L. VARGA, V. VÁRTERÉSZ

"Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest

(Received November 12, 1968)

The authors studied the alterations within the mitotic cycle by the labelled mitosis method using *in vivo* cultured Németh–Kellner mouse lymphoma ascites cells irradiated with 10 to 500 R. They obtained 7 to 8 hours for G_1, 10.6 hours for S, 4.4 hours for G_2 and 22 to 23 hours for the entire mitotic cycle in the non-irradiated control group. Mitotic inhibition developed in the irradiated groups, lasting the longer the higher the dose applied (after 10 R = 5.6; 500 R = 10.4 hours). The ascites cells displayed increased mitotic activity after the release of mitotic inhibition, and in the first postirradiation cycle the reproduction rate of the population was higher than that of the control. Analysing the labelled mitoses curve and relying on the higher value of the reproduction rate, the authors interpret the mitotic overshoot as a result of the shortening of G_1 and S phases and of the accumulation of cells before the G_2 block.

Introduction

The mitosis-inhibitory and mutagenic effects of ionizing radiations are known for a long time and have been first observed by Apolant (1904). Ever since the introduction of the new concept of the mitotic cycle (Howard, Pelc, 1953; Lajtha et al. 1954) the process leading to radiation-induced cell death or to some pathological lesion has been extensively studied.

The first morphological sign of the postirradiation syndrome in mammalian cells is a transitory absence of mitosis figures. Several authors interpret this by a block developing in the G_2 phase (Puck, Steffen, 1963; Kim, Evans, 1964; Watanabe, Okada, 1966). Owing to this block the cells accumulated in the G_2 stage are unable to enter the M phase, though DNA duplication had been accomplished in them and they do contain DNA in amounts sufficient for mitosis. Cells already in division at the time of irradiation pass through the M phase apparently undisturbed and enter G_1.

These early signs of the postirradiation syndrome are generally valid for all mammalian dividing cells studied either *in vivo* or *in vitro*. However, the actual reason of mitotic inhibition is not yet known. More precisely, the alteration in biological functions stopping the mitotic process has not yet been revealed.

It is also known (Watanabe, Okada, 1966) that increased mitotic activity can be observed in the cell population as soon as mitotic inhibition is released

and that the mitotic rate changes periodically, displaying alternate maxima and minima. According to one of the interpretations of these mitotic peaks, as long as mitotic inhibition prevails, cells accumulate before the block. As soon as mitotic inhibition is released, this accumulated cell population enters the M phase and the mitotic index will be increased. According to another hypothesis (Perris, Whitfield, 1967) overshoots would be the consequence of a calcium mobilization after irradiation. However, other factors also seem to play a part in producing mitotic overshoots, since the duration of the overshoots exceeds the mitotic cycle of a given type of cell and their course displays a definite periodicity.

The appearance and role of these factors have been studied on *in vivo* cultured and irradiated NK/lymphoma cells (Németh, Kellner, 1960). The results obtained are reported in the present paper.

Materials and Methods

3×10^7 ascites tumour cells were inoculated intraperitoneally into each of 54 male white mice weighing 20 to 25 g. On the 6th day after inoculation each animal was injected intraperitoneally with 10 μCi of H–3 thymidine (H–3 Tdr) in 0.2 ml of sterile physiological saline (2.5 Ci/mM, TRA 61, The Radiochemical Centre, Amersham, England). After the injection of H–3 Tdr the animals were divided into 6 groups. Animals in 5 groups with 9 mice each were given whole body irradiation with 10, 50, 100, 250 and 500 R. The radiation factors were as follows: 180 kV, 10 mA, 0.5 mm Cu filter, FSD 60 cm, dose rate 26.8 R/min. Thirty to sixty minutes elapsed between Tdr administration and the beginning of irradiation.

At various times after irradiation 0.1 ml ascites fluid was withdrawn from each mouse, by puncture of the abdominal cavity. The cells were spread on cleaned microscopical slides, fixed in a 1 : 3 mixture of acetic acid and methanol, washed and covered with liquid emulsion (Ilford Nuclear Research Emulsion Type G5). After exposure at +4 °C for 5 days, the smears were developed in ORWO A49 developer at 18 °C for 6 minutes, fixed in an acidic fixative and washed in running water. The smears were stained with 5 per cent Giemsa solution for 20 minutes. The staining solution was diluted by 0.05 M phosphate buffer at pH 6.

The autoradiographic slides were examined for the thymidine and mitotic indices and for the ratio of active to inactive cells in mitosis. Mitotic forms ranging from the early metaphase to the late anaphase were considered for the determination of the mitotic index and their frequency was expressed in per cent. A cell was taken for labelled if more than two grains were found over its chromosomes. With the autoradiographic technique used there was little difficulty in distinguishing the labelled and non-labelled as well as the dividing and interphase cells after staining (Fig. 1).

In case of a high S. D. having been found for the mitotic rate values referring to the same point in the graphs, the counting of 1000 cells per smear was continued, or the smears prepared parallel to the above were also exposed to auto-

radiography and evaluated in the same way. The points in the graphs represent the mean value of the two series of smears.

In another experimental series 3.10^7 tumour cells were implanted intraperitoneally into each of 85 male white mice. Forty-five of these animals received a whole body irradiation with 500 R on the 5th day after inoculation. Forty animals served as non-irradiated controls. Following irradiation, 6 to 10 animals were daily killed from both groups and the total tumour cell count in their abdominal cavities was determined. Cell counting was done by means of diluted Türk solution, in a Bürker chamber.

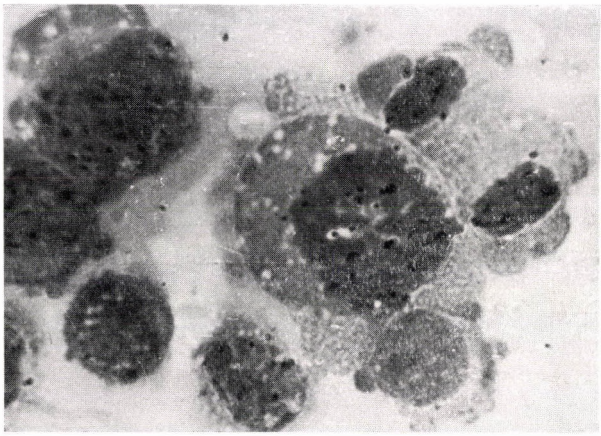


Fig. 1. Autoradiograph of NK/Lymphoma cells. Giemsa staining. Magnification: about 1200×. The smear derives from the group irradiated with 250 R, from the 16th hour of the experiment

Results

The mitotic curves for the non-irradiated group and for the groups irradiated with various doses are shown in Fig. 2. In the non-irradiated group the labelled mitotic forms appeared 4 hours after thymidine administration. By the 5th hour the ratio of labelled cells amounted to 75 per cent, by the 6th hour to the maximum, i.e. to 90 per cent (first ascending part of the curve). This ratio remained unchanged up to the 15th hour. From that time on the labelled mitosis curve showed a slow decline, and reached its minimum between the 25th and 26th hours (descending part of the curve). At 27 to 28 hours the labelled cells entered the M stage again, accordingly, the curve showed an ascending tendency anew.

Owing to the following reasons, the analysis of the curve representing labelled mitoses somewhat differed from the usually recommended methods. Owing to the asymmetry of the ascending part for the irradiated groups, as well as to the lack of the descending part, the $G_2+{}^1/_2M$ stages were determined in the following

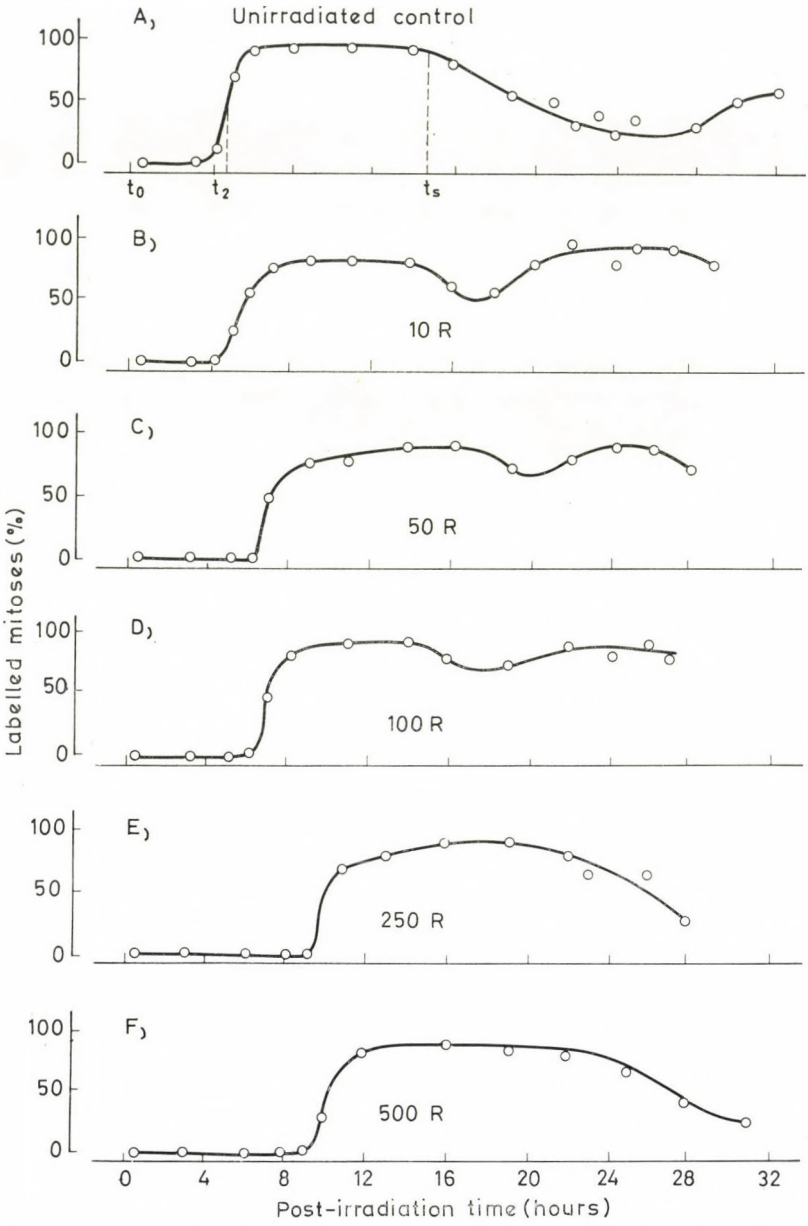


Fig. 2. Percentage of the labelled mitotic figures after the administration of 10 μCi of H–3 thymidine and various X-rays doses. A: non-irradiated control, B to F: curves of the mean values of groups irradiated with 10 R to 500 R 30 minutes after the administration of H–3 Tdr. For detailed analysis of the curves see text

manner. The point where the ascending part reaches 50 per cent of the plateau height was located and connected by a perpendicular with the abscissa (t_2). The time period between t_0 (the administration of H-3 Tdr) and t_2 was taken for $G_2 + 1/2$ M. (The M phase was not determined in these experiments. In the following it should be included with phases G_2 and G_1, respectively). The time between t_2 and the end of the plateau (ts) was taken for the time period of S, and the distance between two identical points of the curve for the mitotic cycle.

According to the above mentioned analysis, 4.4 hours were obtained for G_2, 10.6 for S, 7 to 8 hours for G_1 and 22 to 23 hours for the whole mitotic cycle for the non-irradiated group. Multiple disturbances among the various stages could be observed in the irradiated groups. The duration of G_2 prolonged with increasing dosage (10 R = 5.6; 50 R = 6.8; 100 R = 7; 250 R = 10.1 and 500 R = 10.4 hours). The entering of labelled cells into stage M also displayed essential differences. The ascent of the first ascending part of the curve representing labelled mitoses in the group irradiated with 10 R is milder than in the control group. At higher doses it is the shape of the ascending part that changes and the labelled cells reach the 80 to 90 per cent height of the plateau at a later date. On the other hand, the plateau was shorter for the groups irradiated with 10, 50 and 100 R, resp. (10 R = 9.4; 50 R = 9.2; 100 R = 9 hours).

In case of the same groups, following the first activity wave representing stage S, the curve dropped for a short time (3 to 5 hours) thereafter it rose again. As compared to the control, the site of this rise corresponded to stages G_1 and G_2. As it appears from the Figure, the two active waves approach each other when the dose increases. After irradiation with 250 and 500 R, resp., the two active mitotic waves approached each other and became, indeed, confluent, which resulted in a prolonged plateau.

The mitotic rate values may be correlated with the curve obtained for labelled mitoses (Fig. 3). In the control group mitotic frequency ranged from 1 to 2 per cent during the experimental period. Taking into account the slight fluctuation depending on the part of the day, the 70 per cent of the values obtained was 1.47 ± 0.402 per cent. For the sake of easier comparison this mean control value is indicated by a dotted line also in the irradiated groups.

Thirty minutes after irradiation the mitotic rate dropped to 0.1 to 0.2 per cent. After the completion of mitotic inhibition, the duration of which changed in proportion to the dose applied, no dividing cells were found in the smears. After the release of mitotic inhibition the mitotic rate slowly increased and 2 hours after the restarting of mitoses it exceeded the mean value. Both inhibition and overshoot may be well distinguished even after 10 R, though this phenomenon becomes more marked after higher doses. A certain regularity may be observed in the appearance of mitotic overshoots: 3 mitotic peaks can be seen after 50 and 100 R, resp., with 3 to 5 per cent values at hours 9, 19 and 26; two more marked overshoot waves appeared after 250 and 500 R, with maxima at 16 and 25 hours after irradiation.

The total tumour cell number values obtained in the second experimental series were also appreciably different in the irradiated and the control group. The

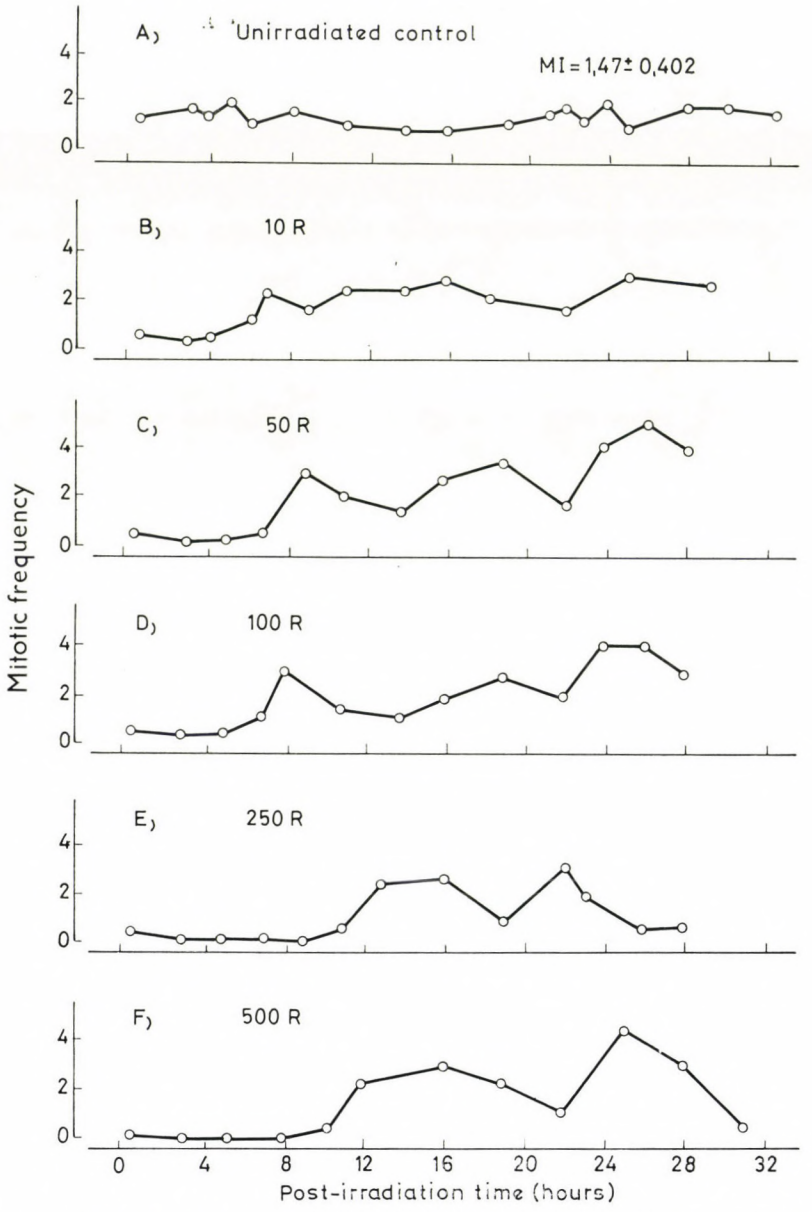


Fig. 3. Mitotic frequency in per cent. A: in the non-irradiated control group; B to F: in the groups irradiated with doses from 10 R to 500 R.

mean tumour cell numbers in the abdominal cavity and the S. D. values relating to the single points in the graphs are shown in Fig. 4. As against the cell number of

$$2.12 \pm 1.18 \times 10^8$$

obtained for the control group on the 5th day after inoculation, the cell population increased to

$$10.9 \pm 1.27 \times 10^8$$

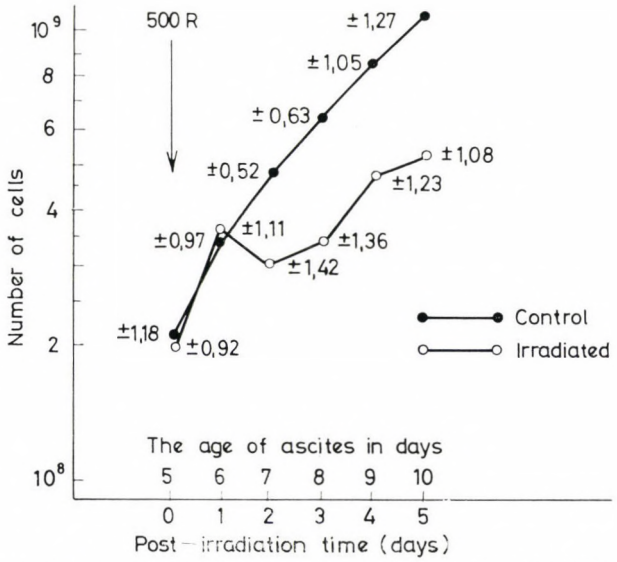


Fig. 4. The change of the total cell count in the ascites fluid of mice with NK/Lymphoma after whole body irradiation with 500 R. Irradiation was done on the 5th day after inoculation. S. D. for the control groups is indicated on the left, and S. D. for the means of the irradiated groups on the right side of the curve

by the 10th day. On the day of irradiation and on the next day the cell number in the irradiated group was identical with that found in the control group. The cell number failed to increase any further on the second and third days. Beginning by the third day, the growth rate of the irradiated ascites tumour cells was almost identical with that of the non-irradiated control cells.

Discussion

According to one of the interpretations of increased mitotic activity cells accumulate during mitotic inhibition in the stage preceding the block. As soon as mitotic inhibition is released and the accumulated cells begin to divide, the mitotic rate will be increased (Watanabe, Okada, 1966). This hypothesis seems to be

acceptable to interpret the first mitotic peak (Fig. 3) observed in our experiments since the maximum of the first overshoot is, indeed, developing a short time after the restart of mitoses. The less steep ascent and asymmetry of the first ascending part of the labelled mitosis curve also confirms that a part of the S stage cells enters stage G_2 already during mitotic inhibition. Accordingly, after the disappearance of the G_2 block a mixed (S and G_2 stage) population of labelled and unlabelled cells enters the M stage. Therefore, the ratio of labelled and unlabelled cells in mitosis will be changed. However, the appearance of the second and third mitotic peaks cannot be explained by the mere accumulation of cells before the block, since the time of their appearance corresponds to stage G_1 of the control curve. It seems plausible to assume a second block, possibly by the end of G_1 or at the beginning of S and interpret its role in a way similar to that of the G_2 block.

Considering the characteristics of the labelled mitosis curve multiple alterations within the mitotic cycle should be assumed to explain the mitotic overshoots. After low doses the first postirradiation mitotic wave of active (S stage) cells is followed by a second in 3 to 5 hours. After moderate doses (250 and 500 R) these two waves are confluent. The fact that between 18 and 20 hours the labelled cells entered the M stage again cannot be explained otherwise than by a shorting of individual phases. In our opinion, as soon as the block is over, the irradiated cell passes through the appreciably shortened G_1, S and the approximately unchanged G_2 stages and enters M phase again, completing mitosis. Owing to the shortening of the phases it is, therefore, possible that cells being at the end of the cycle at the time of irradiation may enter the same stage during the second postirradiation mitotic cycle as the cells in the first cycle. Accordingly, a certain kind of superimposing of cell groups occurs under the effect of irradiation. The potential multiple blocks, shortened mitotic cycle and the superimposing of cell groups may sufficiently account for the slope of the labelled mitosis curve and also for the periodical changes of the mitotic index. In the light of the above arguments, the protracted curve obtained for labelled mitosis in the groups irradiated with 250 and 500 R, resp., is not attributed to the prolongation of the S stage, much rather to the confluence of the double wave due to the shortening of the cell cycle.

The postirradiation change in the total tumour cell number also confirms the correctness of the conclusions we have drawn from the slope of the labelled mitosis curve, namely, that owing to the shortening of the G_1 and S stages, in the first and second postirradiation cycles the cells pass through the cycle more quickly. In spite of the 10.4 hours long mitotic block developing after whole body irradiation with 500 R, the cell count in the irradiated population is identical with that in the control group on the day following irradiation, it is indeed, somewhat higher, though not significantly. Any reduction in the cell count appeared only on the 2nd or 3rd day. This might have been due to increased cell destruction or to the prolongation of the cycle.

When interpreting the result, it arises also the problem whether ionizing radiations affect the incorporation of the labelled precursor. In the experiments reported each animal was given 10 μCi of H–3 Tdr 30 minutes before irradiation,

and this period of time is long enough for incorporation of the amount of Tdr injected in any *in vivo* system (Cleaver, 1967). The labelling index and the average grain count did not change appreciably during the experiment. At the same time this proves that one has not to reckon with an increased cell death immediately after irradiation, at least not in this dose range.

Dewey and Humphrey (1962) irradiated L–P59 mouse fibroblasts cultured *in vitro* with 250 R and Watanabe and Okada (1966) L5178Y mouse leukemia cells with 500 R. Both teams observed a growth rate identical with that of the controls during the first mitotic cycle after the release of the inhibition. According to them, signs suggesting cell damage (abnormal division, chromosomal aberration etc.) and cell death, resp., appeared but from the second mitotic cycle. On the other hand, with the labelled mitosis method Fry et al. (1963) and Lesher et al. (1966) measured a shorter S phase and generation cycle on duodenal crypt cells of mice and rats exposed to chronic irradiation.

In the experiments reported above increased cell reproduction was measured after the disappearance of mitotic inhibition, as a result of the shortening of various phases, hence, of the whole generation cycle. However, the role of the general radiation reaction of the host animal cannot be neglected either, this being such a factor that may appreciably modify the mitotic cycle of the cells. With this in view, our data may be well correlated with the results of the *in vitro* studies

Thanks are due to Miss Elizabeth Szathmáry-Király and Mrs Anna Somossy for their skilful technical assistance.

References

Apolant, H. (1904) Deut. Med. Wschr. *30* 1126
Cleaver, J. E. (1967) Thymidine Metabolism and Cell Kinetics. North Holland Publishing Company, Amsterdam
Dewey, W. C., Humphrey, R. M. (1962) Radiat. Res. *16* 503
Fry, R. J. M., Lesher, S., Sallese, A., Staffeldt, E. (1963) Radiat. Res. *19* 628
Howard, A., Pelc, S. R. (1953) Heredity (suppl.) *6* 261
Kim, J. H., Evans, T. C. (1964) Radiat. Res. *21* 129
Lajtha, L. G., Oliver, R., Ellis, F. (1954) Brit. J. Cancer *8* 367
Lesher, S., Lamerton, L. F., Sacher, G. A., Fry, R. J. M., Gordon, G., Roylance, P. J. (1966) Radiat. Res. *29* 57
Németh, L., Kellner, B. (1960) Naturwissenschaften *47* 544
Perris, A. D., Whitfield, J. F. (1967) Nature *214* 302
Puck, T. T., Steffen, J. (1963) Biophys. J. *3* 379
Varga, L., Várterész, V. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 217
Watanabe, I., Okada, S. (1966) Radiat. Res. *27* 290

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (1) pp. 89—97 (1969)

On the Intrabacterial Phage Development

GY. RONTÓ, G. TUSNÁDY

Biophysical Institute, Medical University and Mathematical Institute, Hungarian Academy of Sciences, Budapest

(Received September 14, 1968)

The burst size distribution of intact T7 coli-phages irradiated with ultraviolet rays for different periods of time has been determined experimentally. For the interpretation of the empirical burst size distribution a stochastic mathematical model was constructed, and the density function parameters of the theoretical distribution required to approximate sufficiently the empirical distribution were examined. The burst size distributions belonging to the irradiated phages were found to consist of several components. The function of compound distribution was separated into several components according to the phage production brought about by intact, once damaged, several times damaged and host cell reactivated phages, respectively. The separation of different components proved to be possible in accordance with our model applied to the interpretation of dose effect curves constructed on the basis of plaque forming ability.

Introduction

The inactivating effect of ultraviolet rays on the plaque forming ability of T7 phages has been studied in our previous investigations (Rontó, Tarján, 1966; Rontó, et al. 1967). According to our experiences the phage production that can be considered intact from the point of view of plaques formation chiefly consists of two parts: intact phages continually decreasing in number with the increase of the time of irradiation, and others damaged by UV light but reactivated on host cells. Considering its mechanism host cell reactivation is an enzyme function which, in the case of UV damage of the phages, includes partly the splitting of pyrimidine-dimers (enzymatic photoreactivation), and partly the excision of the damaged, locally deformed piece of the polynucleotide chain, as well as the synthesis of the piece cut out (dark reactivation) (Szybalski, 1967; Dellweg, 1967). A part of the energy of the host cell is also consumed up in reactivation (Rupert, Harm, 1966).

In the present paper we shall give an account of experiments in which we examined how the phage production brought about by phages reactivated in the host cell after UV irradiation depended on the degree of the damage of the phages. In this connection we want to give an approximative mathematical model of the development of the phages inside the host cell, by the aid of which the phage

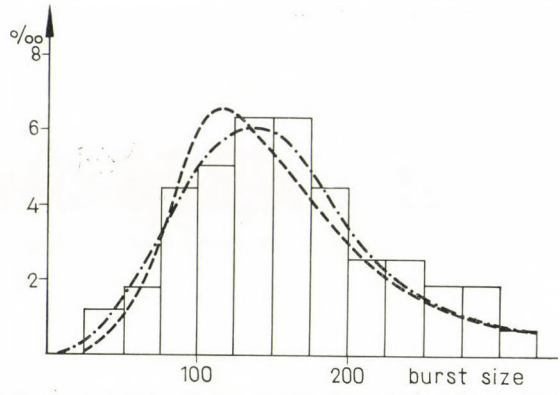


Fig. 1. Comparison of the empirical burst size distribution obtained from E. coli B infected with intact T7 phages with theoretical distributions computed on the basis of theoretical models B1 (— — — —) and B2 (—·—·—)

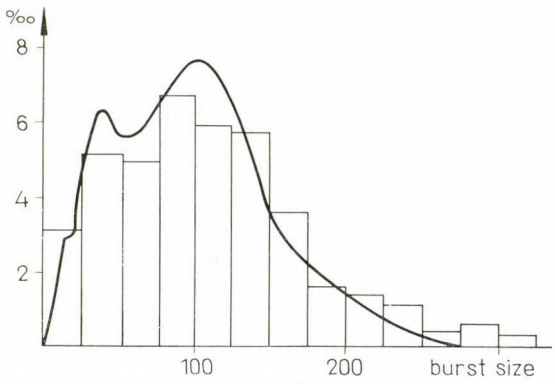


Fig. 2. Comparison of experimental burst size distribution obtained from E. coli B infected with T7 phages irradiated by UV for 2 seconds with the theoretical approximation obtained under the conditions given in paragraph 3.c

production caused by the intact phages, and its radiation-induced changes can also be evaluated in accordance with the experiences.

Methods

The experiments were performed on synchronized host cells Jerusalimsky 1963; Cutler, Evans, 1966). The culture was infected with T7 phages at half-time of the multiplication cycle. In the experiences of Sauerbier (1962) synchronized cultures in the middle of the two division cycles show the same degree of host cell reactivation as do cultures in the logarithmic phase. So our pre-

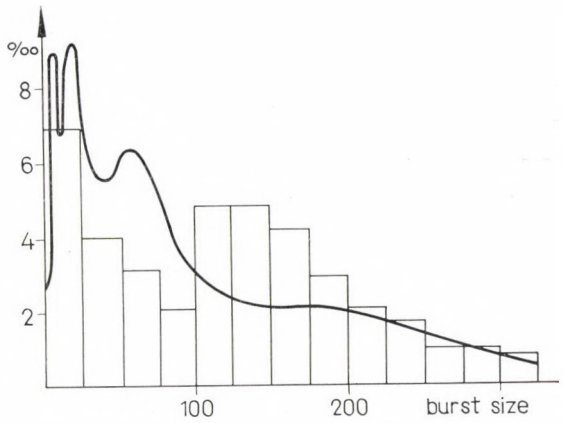


Fig. 3. Distribution of burst size brought about by T7 phages irradiated by UV for 10 seconds, and its theoretical approximation

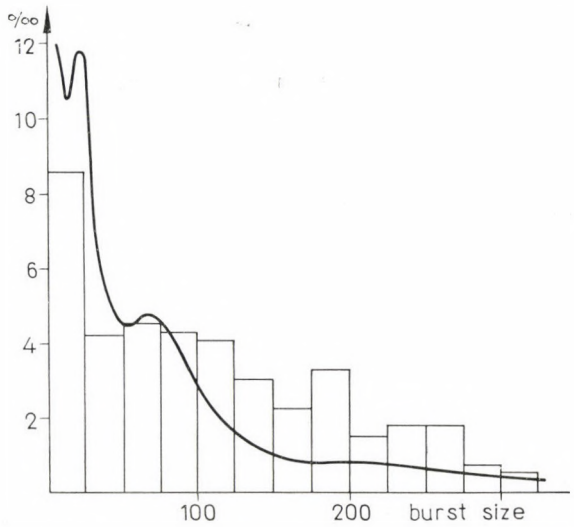


Fig. 4. Distribution of burst size brought about by T7 phages irradiated by UV for 20 seconds and its theoretical approximation

sent measurements can be brought into connection with our former investigations from the aspect of host cell reactivation. The burst size was determined with the method of Adams (Delbrück, 1945; Adams, 1958). The distribution of burst size was determined on host cells infected with *a)* intact phages, *b)* with those irradiated with UV light (germicide lamp) for two seconds, *c)* for 10 seconds, *d)* for 20 seconds (see the frequency diagrams of 1 to 4 Fig.). According to our

Table 1

The empirical density function of burst

Time of irradiation (sec.)	Middle of intervals						
	12.5	37.5	62.5	87.5	112.5	137.5	162.5
0	—	12.5	18.8	43.8	50.0	62.5	62.5
2	31.0	50.8	49.0	67.1	59.4	56.8	36.2
10	69.1	39.7	31.4	20.9	48.2	48.2	41.9
20	85.5	42.7	45.4	42.8	40.3	30.2	22.6

former investigations the value of the survivors in the enumerated cases was: 1; 0.7; 0.27; and 0.08 resp. The multiplicity of infection was 0.1 for all phages, so any possibility of multiplicity reactivation was eliminated.

Results

We should like to remark that the number of those phages which, after irradiation, started phage production at all agreed, within the range of experimental errors with the number of phages proved to be capable of plaque forming in our previous investigations. The results of our experiments are summarized in Table 1, which shows the relative frequency at certain intervals of phage productions in 1/10000-s. Of the results we should like to emphasize that the burst size distribution of phages irradiated by UV light shows two or more maxima.

Presentation of the models

The models constructed in connection with the intrabacterial development of the phages was presented in the literature by Gani (1965). The basic idea of birth-and-death model is that the phages duplicate in the host cell with $\lambda(t)$ density of events and then reach maturity with $\nu(t)$ density of events. The process is interrupted by the lysis occurring independently from the process. In the generation model the phages divide in step k, then they divide with 50 per cent probability in step m, and they mature with 50 per cent probability. The lysis is independent also in this model of the processes taking place in the microbe.

We deviated from the above models in our own ones as follows. The process of phage development was examined from the aspect of the activity of host cell and phage number already produced. We deduced the lysis from the process itself, which led to the fact that we could neglect the time course of the process, and make suitable suppositions in concrete cases about it, not aiming to make them agree with our experimental results.

size distribution expressed in 1/10000-s

Time of irradiation (sec.)	Middle of intervals						
	187.5	212.5	237.5	262.5	287.5	312.5	337.5
0	43.8	25.0	25.0	18.8	18.8	6.2	12.5
2	15.5	12.9	10.3	2.6	5.2	2.6	—
10	29.3	20.8	16.7	10.5	10.5	8.4	4.2
20	32.7	15.1	17.6	17.6	7.5	5.0	—

We constructed two types of models. *Model A* partly characterises the state of the phage-bacterium complex by a quantity of ξ (t) (assumed to be a discrete variable in the model) of *precursor substances* available for the phages (e.g. phage RNA, replicating DNA; Putnam et al., 1952), as well as by the η (t) number of already matured phages. In the system, new precursor substance [$(\xi + 1, \eta)$ transition] appears with $\lambda(\xi, \eta)$ density of events, and the new phage [$(\xi - 1, \eta + 1)$ transition] does it with $\nu(\xi, \eta)$. The two versions of this model were examined. In *model A1* $\lambda(\xi, \eta) = \lambda$ and $\nu(\xi, \eta) = \nu$. (This corresponds, in fact, to the first model of Gani in the case of the process starting from the state $\xi = \alpha$, $\eta = 1$.) The η belonging to $\xi = 0$ gives the amount of burst size. We can calculate the moments of burst size distribution on the basis of the model. In *model A2* $\lambda(\xi, \eta) = \lambda\xi$ and $\nu(\xi, \eta) = \nu\xi\eta$. We think this model gives the best approximation of the real biological process as it includes the experience that precursor substances (eg. phage RNA) quickly increase at the beginning of the development of phages, then the amount of precursors continually decreases with the progress of latency period (Summers, Szybalski, 1968), and the more phage is produced in the host cell, the smaller is their quantity (Herriott, Barlow, 1952). But for the time being we cannot even determine the moments of burst size according to model A2.

Model B emphasizes the circumstance that it is the *substance and energy store resp., of the* phage–host cell *complex*, existing already at the time of infection of the T7 phage (Putnam et al., 1952; Huppert et al., 1967) what is consumed up when new phages (η) are formed. The store decreases with $\lambda(\xi, \eta)$ density of events [$(\xi - 1, \eta)$ transition]. The density of events of phage multiplication [$(\xi, \eta + 1)$ transition] is $\nu(\xi, \eta)$. (We want to stress that the meaning of ξ is different in models A and B.) The multiplying of phages has the consequence in the metabolism of the host cell that the quantity of the substance (energy content) in the host cell (eg. chromosome of microbes, cellular membrane, etc.) gradually decreases to $\xi = 0$. Our model has, again, two variants according to suppositions about λ and ν parameters.

In *model B1* the process starts with the state $\xi = \alpha$ and $\eta = 1$, $-\lambda(\xi, \eta) = \lambda$, as well as $\nu(\xi, \eta) = \nu$. The burst size distribution gives a negative binomial distribution, which can be approximated by gamma-distribution.

Model B2 differs from model B1 in that $\lambda(\xi, \eta) = \lambda\eta$ and $\nu(\xi, \eta) = \nu$. So this model implies as an important feature, also the fact that the more advanced the process of phage production, the greater is the probability of the host cell decomposition. Distribution can be approximated by chi-square distribution. Calculations in the followings were done on the basis of model B1 and B2.

Discussion

1. The expected value of the infected host cells in one Petri-dish was high enough to take into account the probability (calculated according to the appropriate Poisson-distribution) that the number of phages counted in one Petri-dish was originated from more than one infected host cell. So we had to correct the empirical moments in order to obtain a right estimation of the moments of burst size. Table 2 indicates the first ($E\,\eta$), second ($E\,\eta^2$), as well as the reciprocal $\left(E\frac{1}{\eta},\ E\frac{1}{\eta^2}\right)$ moments of the intact,and the 2, 10 second and 20 second burst size distributions resp. The latter two moments show an increasing tendency in the function of irradiation time, which can be observed very well. Beside this, we indicated the Λ parameter of the Poisson-distribution of each series of experiments.

Table 2

The corrected first, second and reciprocal moments of the burst size distributions

	0″	2″	10″	20″
Λ	0.24	0.41	0.48	0.44
$E\eta^2$	23910	9260	12800	11764
$E\eta$	147.2	86.6	97.4	87.8
$E\frac{1}{\eta}$	0.00821	0.01770	0.02173	0.05036
$E\frac{1}{\eta^2}$	0.0000889	0.0005968	0.00105159	0.01037592

2. The parameters of burst size distribution belonging to the intact phages were approximated by distributions corresponding to B1 and B2 models, at the α and μ parameters of Table 3, where α is the value of ξ (t) at the moment: t = 0, and μ shows the probability of $(\xi, \eta) \rightarrow (\xi, \eta + 1)$ transition. In Fig. 1 we indicated the fitting of the density functions of B1 and B2 distributions to the empirical curve, according to the parameters given in Table 3. It can be seen very well from the figure that the empirical distribution function is best approached by model B2.

Table 3

	Model B1	Model B2
α	5.90	1.886
μ	0.0411	0.0000788

3. In connection with the analysis of the burst size distribution of irradiated phages we first examined *a)* whether the second component of the distributions corresponds to the distribution of intact phages, i.e. whether the η_t burst size distribution can be assumed to be the mixture of a η'_t (belonging to a damaged) and a η_0 (belonging to an intact) distribution, the former taking part in the mixture distribution with a probability of p_t, and the latter with $(1-p_t)$. But the splitting on to components of empirical curves on the basis of the above-mentioned conception led to no result.

b) As a second step to the approximation of burst size distributions belonging to treated phages, we supposed that 1. the C_t constant products of distributions belonging to untreated ones give the distribution belonging to the intact fraction of population. (Different burst size distributions come namely from different series of experiments.) 2. Under the conditions given above we tried to split the compound distributions onto two components in such a way that we produced an intact, and a homogeneously damaged but host cell reactivated fraction, according to the ratio of our former model. The latter one was characterized by α and μ parameters identical with those of the intact fraction. The damage suffered by phages irradiated by UV for 2, 10 and 20 seconds, resp., was characterized by a constant factor continually increasing in the function of the time of irradiation. The approximation of empirical burst size distributions appeared to be satisfactory under such conditions with irradiations of 2 and 10 seconds, but not with those of 20 seconds.

c) After this, the condition given under 1) was completed as follows. 2) We assumed that, in the treated population, the intact phages or those damaged once, twice, three times, etc. took part in the compound distribution with such p_{0t}, p_{1t}, p_{2t} $p_{3t}, \ldots$ probabilities as was the ratio of intact, once, twice, three times, etc. damaged phages in our previous investigations on irradiated population still capable of plaque formation.

Table 4

	p_{0t}	p_{1t}	p_{2t}	p_{3t}	p_{4t}	p_{5t}
2″	0.82	0.16	0.02	0.001	—	—
10″	0.40	0.37	0.17	0.05	0.01	0.0020
20″	0.18	0.31	0.26	0.15	0.06	0.02

The p_{tk} probabilities of the participation of phages differently damaged after different times of irradiation are given in Table 4. It should be mentioned that only the ratios of the intact and of the 1 to 5 times damaged ones are indicated in the table as these are the ones practically found in the distributions.

3. In addition, we supposed the phage-bacterium complexes infected with damaged phages to have a substance (energy)- content identical with that found in phages infected with intact phages, i.e. that the α parameter of the theoretical distributions does not change in comparison with the untreated ones. On the other hand, the phages bring about the production of new intact phages in the host cell with a μ_k probability gradually decreasing in dependence on their degree of damage. According to this, the μ_0 parameter of our theoretical distributions was replaced by the expression of

$$\mu_k = \frac{\mu_0}{t_k}$$

where k means the number of damages suffered by one phage. The value of t has been determined empirically in such a way that we tried to find the value of t enabling us to fit best the theoretically obtained distribution to the empirical burst size distributions. Figs 2, 3 and 4 show the empirical distributions of 2, 10 and 20 seconds, as well as the theoretical distribution curves obtained by the above mentioned three conditions in the case of $t = 3$. It can be seen from the figures that the fitting is sufficient. It should be noted in connection with the compound distributions shown by the figures that the maxima of distribution curves of several peaks at continuously decreasing burst size values correspond to phage productions brought about by more and more times damaged and host cell reactivated phages. These results show that the host cell reactivated and differently damaged phages, though appearing equally intact in respect of plaque formation, behave in different ways concerning phage production.

d) Previously we considered the α parameter of our models to be constant in all damaged fraction of phage production, and equal to the α of the intact fraction. The degree of damage suffered by phages is expressed by varying the μ parameter. Also considering our previous knowledge on the mechanism of host cell reactivation, according to which reactivation needs the presence of the energy producing processes of the host cell (Rupert, Harm, 1966), we are of the opinion that the actual biological events are better reflected in our model by such a solution in which the decrease in the energy content of the host cell due to host cell reactivation is characterized by altering the α parameter. Further studies on this topic are going on at present.

We thank Prof. I. Tarján, Director of the Biophysical Institute, Medical University, Budapest, as well as Dr K. Sarkadi, chief scientist of the Mathematical Research Institute of the Hungarian Academy of Sciences, for their help in the course of this work.

References

Adams, H. M. (1959) Bacteriophages. Intersci. Publ. Inc. New York
Cutler, R. G., Evans, J. E. (1966) J. Bact. *91* 469
Delbrück, M. (1945) J. Bact. *50* 131
Dellweg, H. (1968) Strahlentherapie *135* 504
Gani, J. (1965) Appl. Probability *4* 1
Herriott, R. M., Barlow, J. L. (1953) J. gen. Physiol. *36* 17
Huppert, J., Blum-Emerique, L., Breugnon, M. M. (1967) Virology *33* 307
Иерусалимский, Н. D. (1963) Основы физиологии микробов. Акад. Наук СССР, Москва.
Putnam, F. W., Miller, D., Palm, L., Evans, E. A. (1952) J. Biol. Chem. *199* 177
Rontó, Gy., Tarján, I. (1966) Strahlentherapie *132* 143
Rontó, Gy., Sarkadi, K., Tarján, I. (1967) Strahlentherapie *134* 151
Rupert, C. S., Harm, W. (1966) In: Adv. Radiat. Biol. Vol. 2. Acad. Press, New York.
Sauerbier, W. (1962) Virology *17* 164
Summers, W. C., Szybalski, W. (1968) Virology *34* 9
Szybalski, W. (1967) Radiat. Res. Suppl. *7* 147

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (1) pp. 99—108 (1969)

Thermoelectromotive Force in Living Tissues. Frog Muscle

Biological Tissues as Semiconductors. II*

T. LAKATOS, A. KOLLÁR-MÓROCZ

Biophysical Institute, Medical University, Pécs

(Received November 12, 1968)

Three different methods were used to measure the thermoelectromotive force (TEMF) of isolated frog muscle. Its dependence on temperature was investigated between 0 and 35 °C. The TEMF was proportional to the temperature between 0 and 19 °C, and a maximum was found at 23 °C. These findings are interpreted as the result of a possible semiconductor property of biological tissues. Several preliminary results obtained on sciatic nerves are also reported.

Introduction

As it was found almost a century ago (Hermann, 1871) thermoelectric current occurred in frog muscle under the effect of a temperature gradient. Later, others investigated the phenomenon concerning both muscle and nerve, and the sign of the TEMF was disputed (Grützner, 1881; Galeotti and Porcelli, 1910; Pauli and Matula, 1916, 1916a). Theoretical treatment of the phenomenon was attempted by Bernstein (Bernstein, 1902, 1910, 1916). However, the type of the thermoelectric power was not clear enough. One thing was sure: it had a biological nature; when the muscle had been killed, the thermoelectric power disappeared.

On the other hand, the thermoelectricity was found with excitable tissues. The excitation process was suggested by Ernst to be connected with a sort of semiconductor-like behaviour of excitable tissues (Ernst, 1955, 1956) and particularly the biological thermoelectricity was considered as a likely evidence of this hypothesis (Ernst, 1958).

It seemed to be interesting to investigate the details of this phenomenon. Three different methods were used in the course of the experiments. A possible explanation was found for the disputability of the sign of the TEMF, and a remarkable analogy appeared to the thermoelectric effect of semiconductors. The question has been raised whether the thermoelectric power has any connection with excitability.

* The first communication on this topic was: T. Lakatos: Direct Current Conductivity of Dried Frog Muscle. Acta Physiol. Acad. Sci. Hung. *22* 297 (1962).

Methods

1. The first group of experiments was performed in a double plexiglass vessel (Fig. 1). A groove was made in the upper edge of the partition, the muscle or the nerve was laid across this groove onto two non-polarizable electrodes (Ag–AgCl – Ringer solution). The glass tubes filled with Ringer solution were long enough to avoid the cooling or heating of the Ag–AgCl electrodes. Both parts of the vessel were filled with paraffin oil of different temperatures, one of them was nearly 0 °C, the other one was of higher temperature, but not over 20 °C. The temperature was measured by mercury thermometers with an accuracy of 0.2 °C. A Feussner-type

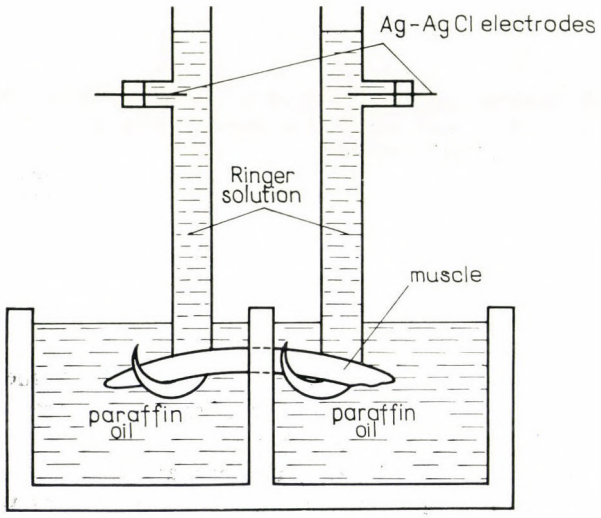


Fig. 1. Vessel for investigating the thermoelectricity of muscle and nerve

potentiometer was used for measuring the TEMF. (The earlier papers had mostly mentioned "Thermostrom" – i.e. thermocurrent – but, in the present experiments, it was always the TEMF what was measured.) The standard cell of the potentiometer was a Weston cadmium cell; the indicator was a galvanometer of a sensitivity of 10^{-9} A/mm. The experiments were carried out as follows:

The excitability of the isolated sartorius muscle or sciatic nerve of Rana esculenta was controlled by electric stimulation, then the preparation was laid onto the connection points of the reference electrodes across the groove of the partition. The two parts of the vessel were simultaneously filled with paraffin oil of different temperature. After the temperature of both sides had been showed by the thermometers to be stabilized, the thermoelectromotive force was measured. The procedure was repeated after changing the position of the cold and warm oil, and once again after killing the muscle or nerve by immersing them in warm (45 °C) Ringer solution, chloroform, or ether. This method allowed a quick change of

temperature, the thermal equilibrium set in speedily, but a fine regulation of temperature was difficult to achieve.

2. In order to make possible a finer regulation of temperature another apparatus was developed (Fig. 2). Two brass rods, 30 mm in diameter, were connected by a 2 mm thick brass plate covered by Epokitt (a synthetic epoxy resin) to isolate it electrically. One of the brass rods was warmed by an electric heater of a

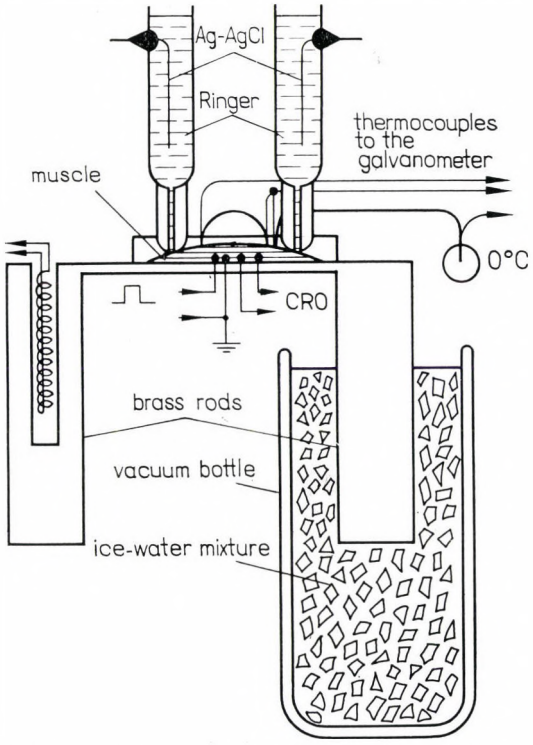


Fig. 2. Apparatus to bring about temperature gradient in a muscle. For a more detailed explanation see text

power 5 W, the other one was cooled by an ice-water mixture. A uniform temperature gradient developed along the brass plate in a steady state. The muscle was laid on the brass plate and covered by a plexiglass box. There were four borings in the top wall of this box for the two reference electrodes and for the thermocouples by which the temperature difference and the temperature of the cold end of the muscle was measured. In addition, four platinum electrodes were mounted in the box in order to control the stimulus threshold of the muscle. The stimulus was supplied by a transistorized square wave generator, the duration of stimulus was 0.1 ms and its voltage was adjusted with an accuracy of 0.01 Volt. A double beam cathod ray oscilloscope (CRO) was used as an indicator of activity. The thermoelectro-

motive force was measured by the CRO the sensitivity of which was adjustable between 0.5 mV/cm and 100 mV/cm. The TEMF was connected with the input only for a short time (< 0.5 sec) with the aid of a switch. Thus the TEMF was not loaded (the input resistance of the differential amplifier of the CRO was 4 Mohm) and it could be measured with a good reproducibility. The drawback of this method was that the temperature of the "cold point" was influenced by that ot the "warm

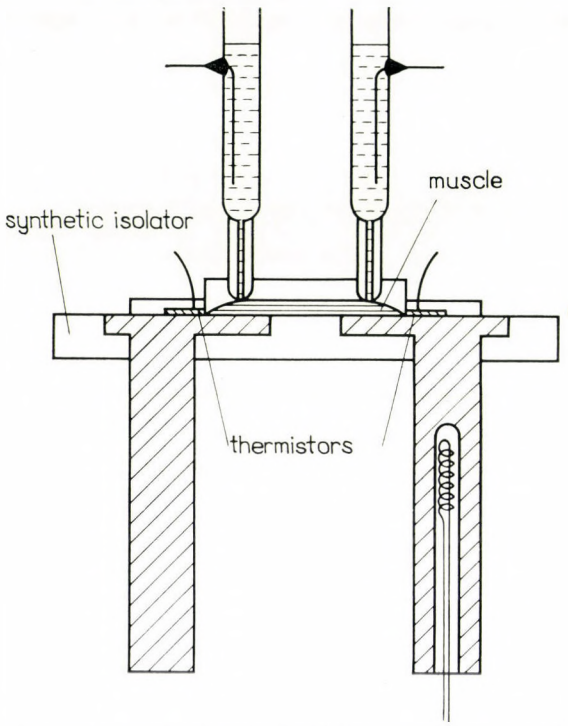


Fig. 3. Scheme of the apparatus of the 3rd method. The arrangement was similar to that shown by Fig. 2, but its brass plate was replaced by a synthetic insulator

point" and the evaluation of results became difficult because of the continuous shift of the temperature of cold point. On the other hand, in these experiments the external temperature gradient (which occurred in the brass plate) produced a gradient in the muscle. Most experiments were carried out with this method.

3. The above-mentioned imperfections were eliminated by a third method. An apparatus similar to that described in the previous method was used but the muscle was laid on an insulator made of plexiglass and Epokitt (Fig. 3), and only its two ends touched the brass plates of the heater and the cooler. The temperature was measured by two thermistors with an accuracy of 0.2 °C. The same sort of Ag–AgCl electrodes were used as in the previous method. The TEMF was measured by a DC amplifier, the input resistance of which was higher than 50

Mohm, the limiting frequency was 15 Hz, and a CRO as indicator was used. The sensitivity of the systems was adjustable between 0.04 mV/cm and 4 mV/cm (this system was a unit of an ELEMA–SCHÖNANDER Mingograf-81 connected with the cathod ray oscilloscope). The stimulus threshold of muscles was determined before and after the measuring of TEMF.

Results

1. Both sartorius muscle and sciatic nerve were investigated with the first method. Results of these experiments are summarized as follows:

a) The cooled place was always negative in comparison with the heated one.

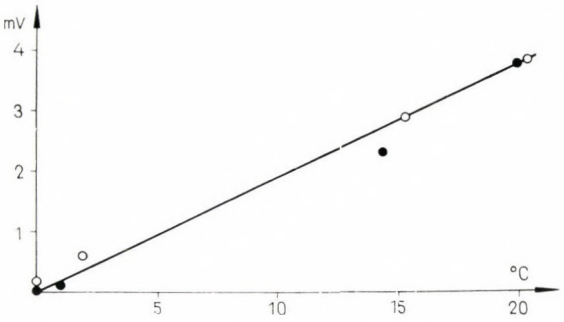


Fig. 4. Tipical result of an experiment carried out with the first method. The TEMF is plotted against temperature ●; after the cold and warm ends had been changed ○. The warm part of muscle was always positive in respect of the cold one

b) The differential thermoelectric power (i.e. the TEMF per 1 °C; or more exactly the slope of TEMF as a function of the temperature) of the muscle was 230 ± 40 μV on the average of 30 experiments. A typical example is shown by Fig. 4. After the muscle had been killed by immersion in chloroform no TEMF was found.

c) The sign of the TEMF of nerve was the same as that of the muscle.

d) The differential thermoelectric power of nerves was found to be 45 ± 15 μV/°C on the average of 27 experiments. There was no TEMF after the nerve had been killed.

e) Further 32 nerves were excised and kept in Ringer solution at a temperature of +1 °C. Four of them were investigated on the first day, another four on the next day, etc., through ten days with no measuring on the 5th and 7th days. Each day the TEMF was measured, the average of the differential thermoelectric power of each four nerves was calculated and plotted against time (Fig. 5). The thermoelectric power diminished from day to day as the nerves became less and less excitable. After nine days the nerves were dead, i.e. perfectly inexcitable, and they did not show any thermoelectric effect.

2. The other two methods were used to study the details of the effect on muscle. While the highest temperature difference in the experiments carried out with the first method was 20 °C, the temperature range of investigations was expanded to 35 °C in the experiments performed with the 2nd and 3rd method. The temperature range of measuring had to be cut in two parts, in light of the results. Under 19 °C the differential thermoelectric power was independent of the temperature, with an average value of 200 μV/°C (the error of the mean was ± 24 μV/°C, the number of experiments was 70). In the temperature range from 19 °C to 35 °C the differential thermoelectric power was influenced by the temperature with its sign changing at 23 °C (Fig. 6).

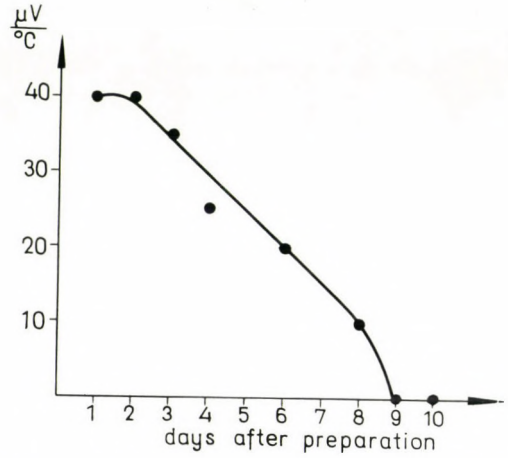


Fig. 5. The differential thermoelectric power of nerve plotted against time. Each point represents the mean of four experimental values

It appears from plot *b* of Fig. 6 that the TEMF is in direct proportion to the temperature under 19 °C, it begins to diminish above 23 °C, and its sign changes at 30 °C. Such a change in the sign of the TEMF was found but a few times, and most experiments gave the result shown by plot *a* of Fig. 6, i.e. a proportional part below 19 °C, and a maximum at 23 °C. The killed controls did not produce similar results, though low voltages could be measured also here, their value being one tenth of that of a living muscle, these values hardly depended on the temperature (plot *c* of Fig. 6) and their sign was either positive or negative at random.

The results of all three methods are in good agreement. The differential thermoelectric power found in the first series of experiments (230 μV/°C) was somewhat higher than that given by the other two methods (200 μV/°C); but the difference between them was not significant at all, as it was proved by a *t*-test of two samples ($t = 1.07$; $n_1 + n_2 - 2 = 98$; the significance level at $p = 0.05$ is 1.98, the computed value of t is by far less).

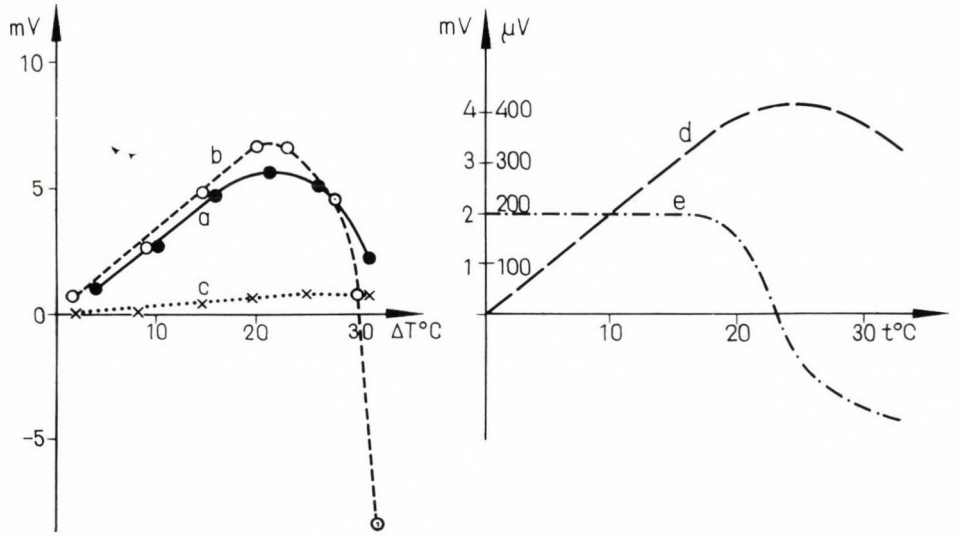


Fig. 6. TEMF of muscle plotted against temperature. *a* and *b:* TEMF of two different, excitable muscles; *c:* inexcitable muscle. *d:* average plot of 70 experiments; *e:* differential thermoelectric power of muscle as the slope of plot *d*

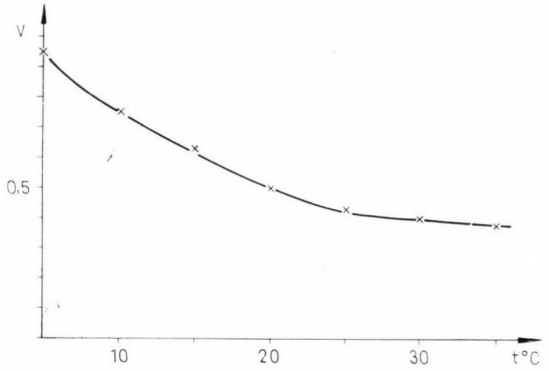


Fig. 7. Stimulus threshold of sartorius muscle plotted against temperature. Each point represents the mean of 10 measurements

The physiological state of the muscle was checked by measuring its stimulus threshold at temperatures between 5 and 35 °C. A monotonous decrease in stimulus threshold (i.e. an increase of the excitability) was found with increasing temperature (Fig. 7). The measuring of the stimulus threshold was carried out under conditions in which the temperature both increased and decreased at the same muscle. The change of stimulus threshold was found to be reversible over the investigated temperature range.

Discussion

As it was mentioned above a controversy existed as to the sign of the TEMF which was found by various authors to be different between 20 and 35 °C (Galeotti and Porcelli, 1910; Verzár, 1912; Pauli and Matula 1916; 1916a). In Verzár's opinion this non-uniformity was due to the damage of tissue caused by the high temperature. But the reversible change of the stimulus threshold (Fig. 7) between 5 to 35 °C suggests that the state of the system generating excitation did not suffer any damage. Consequently, the result that above 23 °C the TEMF decreased (in

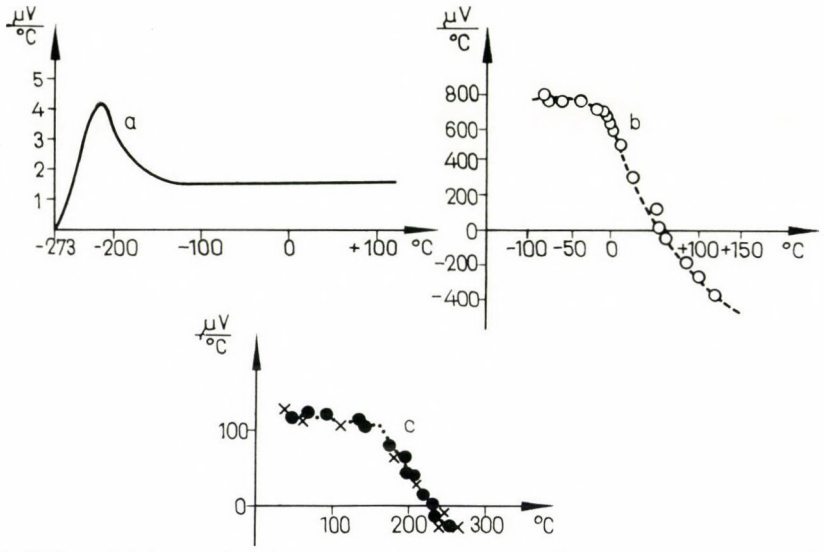


Fig. 8. Differential thermoelectric power of different materials. *a:* Zn-crystal — Cu thermocouple (Laski, 1928); *b:* p-type germanium sample with platinum contact electrodes (Middleton and Scanlon, 1953); *c:* paracyanogen (Toptchyew, 1966)

other words: the sign of the differential thermoelectric power turned for negative) is not to be considered as an "uncertainty", but as a characteristic behaviour on the excitable structure of muscle, for unexcitable muscles do not show any TEMF. The findings that the TEMF was "uncertain" (i.e. it had a low voltage the sign of which was sometimes positive, sometimes negative) above 20 °C could be interpreted by our results: when the TEMF was investigated by the above-mentioned authors between 20 and 35 °C, the colder end of preparation was kept at 20 °C. As it is shown by Fig. 6, when the cold point of muscle is kept at 20 °C, the TEMF between the cold and warm points can be a small positive, zero, or a small negative value as an obvious consequence of the temperature dependence of the thermoelectromotive force.

The decrease of the TEMF shown by Fig. 5 is a proof of the TEMF being connected with the excitability (though this decrease appeared with nerves, very

likely there is no dissimilarity between the thermoelectricity of nerve and muscle). However, more experiments are necessary to elucidate the quantitative relationship between excitability and thermoelectromotive force.

In our experiments on muscles below 19 °C a positive sign of differential thermoelectric power was found, a fact indicating that the TEMF was produced by positive carriers, while the change in the sign of the differential thermoelectric power at 23 °C (and its negativity above this temperature) indicated that either negative carriers were dominating over this temperature range or the mobility of the negative carriers was greater than that of the positive carriers. Though further detailed investigations are required to gain more exact informations as to the nature of the carriers, we call attention to a remarkable analogy offered by solid state physics.

The differential thermoelectric power of different metals is a few times 10 μV/°C, but not higher than 110 μV/°C (Sb – Bi thermocouple). It hardly depends on temperature over a wide temperature range, but it could be a complicated function of temperature at extreme low or high temperatures (Fig. 8*a*). However, the behaviour of a doped semiconductor is unlike to this over a relatively small temperature range (Fig. 8*b*). The approximately constant positive value of the differential thermoelectric power shown by this plot over the temperature range (−80 °C, −20 °C) is produced by mixed intrinsic and impurity conduction; the positive sign of the differential thermoelectric power indicates that the majority of carriers are holes. The differential thermoelectric power decreases quickly, and its sign turns for negative, as the temperature rises and the impurity conduction is overwhelmed by the intrinsic one, the number of electrons and holes are nearly the same, but the mobility of electrons is higher. Similar phenomenon was found also with organic semiconductors (Fig. 8*c*). There is an evident analogy between these data and our results.

To sum up, these new data suggest that there exists a mechanism dissimilar to that postulated by the membrane theory, which mechanism is connected with the excitation generating system and is capable of producing the thermoelectricity in living tissues. This mechanism probably represents a semiconductor property of the excitable tissue of muscle or nerve.

The authors are indebted to Prof. E. Ernst for raising the problem and for his encouragement during the work.

References

Bernstein, J. (1902) Arch. ges. Physiol. *92* 521
Bernstein, J. (1910) Arch. ges. Physiol. *131* 589
Bernstein, J. (1916) Arch. ges. Physiol. *164* 102
Ernst, E. (1955) Wiss. Z. Humboldt-Univ. Berlin, Math.-Naturwiss. Reihe *5* 167

Ernst, E. (1956) Biofizika *1* 297 (Russian)
Ernst, E. (1958) Die Muskeltätigkeit. Verlag d. Ungarischen Akademie d. Wissenschaften, Budapest
Galeotti, G., Porcelli, F. (1910) Z. f. Algem. Physiol. *11* 317
Grützner, P. (1881) Arch. ges. Physiol. *25* 255
Hermann, L. (1871) Arch. ges. Physiol. *4* 149
Laski, G. (1928) Thermoelektrizität. in Handbuch der Physik *13* 183 Springer
Middleton, A. E., Scanlon, W. W. (1953) Phys. Rev. *92* 219
Pauli, W., Matula, J. (1916) Arch. ges. Physiol. *163* 355
Pauli, W., Matula, J. (1916a) Arch. ges. Physiol. *165* 157
Toptchyew, A. W. (editor) (1966) Organische Halbleiter. Akademie Verlag, Berlin
Verzár, F. (1912) Arch. ges. Physiol. *143* 252

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (1) pp. 109—110 (1969)

Remark on the Question of the Ion- and Water-Binding of Biological Substances

(Short Communication)

G. MASSZI

Biophysical Institute, Medical University, Pécs

(Received October 12, 1968)

The high-frequency and microwave investigations reported earlier from this Institute gave data to the study of the ion- and water-binding in biological substances (Ernst, 1935, 1963; Masszi, Tigyi-Sebes, 1962; Masszi, Örkényi, 1967). According to high frequency measurements published recently (Pauly, Schwan, 1966) the conductivity of the erythrocyte content is about four times smaller than that which we could expect on the basis of the inorganic substance content presuming complete dissociation. The above authors considered the role of both the ion-binding and hydration of the Hb molecule as unimportant factors in the explanation of the decrease of measured conductivity. They came to this conclusion on the basis of the fact that, if the difference is explained *only* by ion-binding or *only* by hydration, they obtain irreal result.

In our opinion this two phenomena play, in the reality, their parts together and we must take this fact into consideration while calculating. In this case the Fricke equation (Fricke, 1924) can be written as follows:

$$\kappa_c = \alpha\kappa_2 \frac{0.84}{1 + \frac{p}{x}}$$

where $\kappa_c = 5.2$ mmho/cm is the measured conductivity of the erythrocyte content, $\kappa_2 = 17.3$ mmho/cm is the conductivity of the whole water content of the erythrocyte in the case of complete dissociation, α is the degree of dissociation characteristic of the ion-binding, p is the volume rate of hydrated Hb, and x is a constant with a value between 1 and 2. On the basis of the above equation, at $p = 0.6$ the value of α lies, for instance, between 0.5 and 0.6, which is already acceptible (e.g. for an 0.1 mole/liter of KCl solution the value of α is 0.86). The connection of α and p is shown by Fig. 1. Here, $p = 0.6$ corresponds to the water binding of 1 g of water/g Hb. This value agrees well with our estimation made on the basis of the microwave conductivity of gelatine solutions (Masszi, Örkényi, 1967).

It was assumed in connection with this calculation that the quantity of the bound water is well-defined, and it does not take part in the dissolving of ions. However, we cannot probably draw a sharp border-line between "free" and "bound" water, and bound water also takes part in the dissolving of ions. Considering this fact, an even greater α belongs to a given p value. All these show that the diminution of conductivity can be well explained if we take the ion and water binding into consideration.

According to Pauly and Schwan the main cause of the diminution of conductivity can be explained by a hydrodynamical model. The hydrated ions 1–2 Å in radius flow among Hb molecules of 27 Å radius in a gap of about 23 Å filled

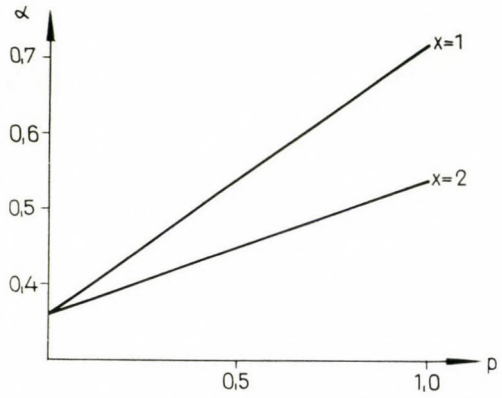


Fig. 1. The connection of the degree of dissociation (α) with the volume rate of the hydrated Hb (p) if $x = 1$ and $x = 2$

with water. This arrangement could be the cause of the increase of friction of ions. But from the point of view of hydrodynamics the dislocation of ions at 10^8 cps would be even less than 10^{-3} Å which would mean a movement much inside atomic proportions. High frequency field can be assumed to influence the heat-motion of ions, and so the main question in understanding the motion of ions is, what is the structure of water like in the gap of 23 Å which corresponds about to a layer of 8 water molecules and which is in close interaction beside protein with the ions as well.

References

Ernst, E. (1935) The Sechanov Journal of Physiology of the USSR. Progr. XV. Int. Physiol. Cong 21

Ernst, E. (1963) Biophysics of the Striated Muscle. Publ. House of Acad. Sci. Budapest

Fricke, H. (1924) Phys. Rev. *24* 575

Masszi, G., Tigyi-Sebes, A. (1962) Acta Physiol. Acad. Sci. Hung. *22* 27

Masszi, G., Örkényi, J. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 131

Pauly, H., Schwan, H. P. (1966) Biophys. Journal *6* 621

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (1) pp. 111—112 (1969)

Excitation Induced by Ultraviolet Rays in Stopped Frog Heart

Short Communication

T. LAKATOS, A. KOLLÁR-MÓROCZ

Biophysical Institute, Medical University, Pécs

(Received November 27, 1968)

The effect of visible light on stopped frog hearts, and the effect of visible light and ultraviolet rays on the stimulus threshold of frog muscle were reported in previous papers (Lakatos, Kollár-Mórocz, 1966, 1967; Lakatos, 1967). In those experiments Na-eosin was used as sensitizer (and partly also methylene blue); the stopped and sensitized frog hearts restarted beating under the effect of illumination by visible light; a decrease was found in stimulus threshold of sensitized frog sartorius muscles stimulated by short electric pulses, when they were irradiated by strong visible light or ultraviolet rays. The opinion was expressed that the sensitizer absorbed the light quanta which excited electrons in the dye molecule, and the excitation energy might have migrated over the network of π-electrons to the site of formation of biological excitation.

Proteins have an optical absorption maximum in the ultraviolet range of the spectrum, exactly at 280 mμ due to tyrosine and tryptophan (e.g. Bücher and Kaspers, 1947; Weber, 1961). It was presumable that stopped frog hearts would start beating again when illuminated by ultraviolet rays having a spectral component of wavelength of 280 mμ, even if the hearts were not stained with any sensitizer.

A series of experiment was carried out to check this assumption. The same method was used as in the previous experiments (Lakatos, Kollár-Mórocz, 1966), except one condition: the potassium-free solution, by which the hearts were stopped, did not contain sensitizer. The stopped but excitable frog hearts were irradiated by the light of an HBO 50 mercury lamp (Zeiss, Jena) having a peak in its spectrum at 280.4 mμ and thus capable of exciting the corresponding energy state of proteins in the heart muscle. Control experiments were performed, in which hearts were illuminated by the same light source, but the ultraviolet range of its spectrum was eliminated by a CG 11/2 glassfilter of Jena.

The temperature of hearts was increased during illumination by both the total radiation of the lamp and the visible light alone. However, the increase in temperature was approximately the same in both cases, with a maximum of 2.5 °C. Contractions elicited by irradiation occurred from the end of the first minute of irradiation, they were repeated in the next several minutes, and ceased after the

ultraviolet rays had been switched off (usually five minutes from the outset of illumination). The results can be summarized as follows:

35 per cent of the stopped frog hearts (24 out of 68 experiments) started beating again during irradiation by ultraviolet rays, without any sensitizer present. However, a smaller number of the hearts (6 out of 60 experiments) illuminated by visible light restarted as well. Therefore a χ^2-test was applied to determine the probability that the rate of restarting was greater only by chance in the presence of ultraviolet rays than that in the presence of visible light. It was found that $\chi^2 = 11.2$, that means $P < 0.001$ (being $\chi^2 = 10.2$ at $P = 0.001$), thus indicating that the exciting effect of ultraviolet rays very likely exists.

It has to be accentuated that there is an essential difference between the results of our experiments and those of similar experiments performed by others (Viale, 1917 1931; Agnioli, De Barbieri, 1929; Santamaria et al., 1959; Arvanitaki, Chalazonitis, 1947); because these earlier experiments were carried out with rhythmically beating hearts, their amplitude and the frequency of beating having been increased by illumination. In the case of our recent and present experiments it was clear that quite new excitation process was formed under the effect of light or ultraviolet quanta.

The authors are indebted to Prof. Ernst for raising the problem and for his encouragement during the work.

References

Agnioli, R., De Barbieri, A. (1929) Arch. exp. Path. *143* 46
Arvanitaki, A., Chalazonitis, N. (1947) Arch. des Sciences Physiologiques *1* 385
Rücher, Th., Kaspers, J. (1947) Biochim. Biophys. Acta *1* 21
Lakatos, T., Kollár-Mórocz, A. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 413
Lakatos, T., Kollár-Mórocz, A. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 191
Lakatos, T. (1967) Studia Biophysica (Berlin, GDR) *3* 263
Santamaria, L., Prino, G., Pace, G. P. (1959) Atti. Soc. It. Patol. *6* cited by Santamaria, L. (1960) Recent Contributions to Cancer Research in Italy *1* 167
Viale, G. (1917) Giorn. Regia Acc. Med. Torino, *3–4–5* 1
Viale, G. (1931) Arch. Scien. Biol. *2* 231
Weber, G. (1961) Excited States of Proteins. in "A Symposium on Light and Life." W. D. McElroy and B. Glass (editors) The John Hopkins Press, Baltimore

Instructions to Authors

The following instructions should be met in order to keep terms in publishing the Acta Biochimica et Biophysica Academiae Scientiarum Hungaricae. Manuscripts which do not meet these requirements will be returned to the author(s). Only the data of receipt of the properly completed manuscript will be indicated in the publication. All responsibility for delays in publication is returned to the author(s).

The aim of Acta Biochimica et Biophysica Academiae Scientiarum Hungaricae is to publish experimental or theoretical results from the field of biochemistry, biophysics and functional microstructure. Articles of normal length and also short and preliminary communications are published in the journal in English, Russian, German or French.

Submission of a manuscript implies that the same work has not been and will not be published elsewhere.

Manuscripts on biochemistry should be sent to the Institute of Biochemistry of the Hungarian Academy of Sciences (Budapest XI., Karolina út 29, Hungary), while manuscripts on biophysics to the Institute of Biophysics, University of Pécs (Pécs, Rákóczi út 80, Hungary) in *two* copies and should be recommended by the director of the institute where the work has been done. The whole text including the legends to figures, footnotes and references should be typewritten in double spacing.

Manuscripts should be written in a concise and plain form. Generally accepted technical terms should be used as far as possible. Do not use laboratory slang. The length of the manuscript should not exceed 10 typed pages.

Manuscripts will be accepted in Hungarian as well as in foreign languages (preferably in English). It is advised to enclose a copy in Hungarian to papers sent in from Hungary in a foreign language in order to facilitate the editorial work. In case a commonly unknown special term is used it is suggested to the author(s) to propose the equivalent expression in the language of the publication.

Structure of manuscripts of normal length

1. Title. If needed, explanation of the title can be given in a sub-title.
2. Name of author(s)
3. Name of the laboratory where the work was done.
4. Summary. Length of the summary should not exceed 20 lines. The summary should be intelligible in itself by presenting the methods applied and the new conclusions reached. Do not use abbreviations.
5. Text
   a) Introduction. The most important preliminaries of the work and the aims of the investigations should be described. Lengthy reviews of literature should be avoided.
   b) Experimental procedures. This section should contain a description of the used materials, methods and instruments, in sufficient details. With respect to the form in which dimensions should be given it is advised to consult international recommendations (Chemical Abstracts *56* IB—98N (1962) International Enzyme Committee).

c) Results. This section should contain the documentation and description of the most important observations as well as the conclusions to which they lead.

d) Discussion. Comparison of the observations of the author(s) to the literary data.

e) Other notes (e.g. acknowledgements) should follow the Discussion.

f) References. References in the text should be given by the name of the author and the year of publication (Boyer, 1960), (Lambert, Jones, 1966). In the case of more than two authors only the name of the first author is to be given (Kaplan et al., 1963). When more than one paper of the same author is cited from the same year, these should be distinguished: (Scheraga, 1964, 1964a). References are to be listed alphabetically at the end of the text (without the title of the cited paper). Personal communications (e.g. Hill, personal communication) and unpublished data (e.g. Colm, unpublished data) should only be given in the text. Form of references:

Paper from a journal:

Pauling, L., Corey, R. B. (1953b) Nature *171* 59.

Paper under publication:

Kovács, G., Nagy, Z., Kis, I. (1965) Acta Biochim. Biophys. Hung. in press

Book:

Szent-Györgyi, A. (1960) Introduction to a Submolecular Biology. Academic Press, New York, p. 112

Collective volume:

Doty, P. (1961) in Biophysical Science, Oncley, J. C. (editor), Wiley et Sons, Co., New York. p. 142

g) Address of author(s). It is suggested to give the postal address of the author to whom correspondence is to be sent from the Editorial Office.

6. Tables

Tables should be numbered with Arabic numerals. They should be typed with their title and legend on separate pages.

7. Legends to Figures and Tables

Legends to figures should be typed on a separate page, consecutively. Legends to both figures and tables should contain the experimental conditions in order to make the documentation intelligible in itself.

8. Figures

Two copies of each figure are required. The first copy should be drawn on a graph paper of 18×12 cm, the second one may be a photocopy. Authors are requested to indicate the required reduction in size of the figures. Do not write lengthy explanations on the figures. if possible, only use symbols on the graphs. Photographs of good quality are required, printed on bright white photographic paper. The name of the author(s), the brief title of the paper and the number of the figure should be written in ink on the back of each figure. Figures are to be numbered with consecutive Arabic numerals.

Short papers

Maximal length is four typewritten pages with 1 table and 1 figure.

a) Short communication

Concise representation of a completed investigation in a limited field (e.g. an analytical or methodical problem). Data published in a short communication are not to be presented later. Short communications should give a complete list of references.

b) Preliminary communication

Results from current investigations can be published as a preliminary communication. Since, presumably a publication of normal length will follow, the most important references should only be cited.

ANNOUNCEMENT OF AN IAEA SYMPOSIUM

Title: *In vitro* Procedures with Radioisotopes in Clinical Medicine and Research

Date: 8–12 September, 1969

Location: Vienna

Organizers: International Atomic Energy Agency
Kärtnerring 11–13, 1010 Vienna, Austria

Scientific Secretaries: Dr. E. H. Belcher and Dr. T. Nagai
Section of Nuclear Medicine

This symposium will be concerned with all procedures with radioisotopes in clinical medicine and research in which the radioactive material is added to the sample *in vitro* but is not administered to the subject. (Topics to be covered include saturation analysis, dilution analysis, derivative analysis, radioimmunoassay, studies of enzyme activity, immunological studies and cytological studies on isolated tissues). The symposium will deal both with the procedures themselves and with their applications, and will give emphasis to procedures which have direct applications in clinical medicine and research. Neutron activation analysis will be excluded from the programme.

Further information and forms to accompany abstracts of papers intended for presentation at the Symposium may be obtained from national authorities for atomic energy matters. Abstracts must be submitted through these authorities so as to reach the International Atomic Energy Agency before 1 May, 1969.

Hoppe-Seyler's Zeitschrift für Physiologische Chemie

Editors in Chief
A. Butenandt · K. Thomas · G. Weitzel

Subscription Rates
For one volume (12 parts) DM 360,—

Vol. 349, No. 11 November 1968

Contents

Walter de Gruyter & Co · Berlin 30

Hoppe-Seyler's Zeitschrift für Physiologische Chemie

Editors in Chief

A. BUTENANDT · K. THOMAS · G. WEITZEL

Subscription Rates

For one volume (12 parts) DM 360,—

Vol. 349, No. 12 **Contents** December 1968

Walter de Gruyter & Co · Berlin 30

T. Dévényi, P. Elődi, T. Keleti und G. Szabolcsi

Strukturelle Grundlagen der biologischen Funktion der Proteine

In deutscher Sprache . Etwa 710 Seiten . 120 Abbildungen . 80 Tabellen
Ganzleinen

Der Band gibt eine zusammenfassende Darstellung einiger wichtigsten Forschungsergebnisse auf dem Gebiet der strukturellen Grundlagen des Fragenkomplexes der Proteinchemie in erster Reihe der Enzyme, u.zw. in Zusammenhang mit ihren funktionellen Eigenheiten. In der Arbeit werden die Ergebnisse und Probleme der Sequenz-Untersuchungen von Aminosäuren, der Bemühen nach einer Beschreibung der Raumstruktur der Reaktionskinetik der Enzyme, der Evolution der Proteine und der Funktion der Enzyme analysiert.

AKADÉMIAI KIADÓ

Verlag der Ungarischen Akademie der Wissenschaften

Budapest

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója Műszaki szerkesztő: Farkas Sándor

A kézirat nyomdába érkezett: 1968. XII. 20. — Terjedelem 10,50 (A/5) ív, 73 ábra

66823/1969 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

Reviews of the Hungarian Academy of Sciences are obtainable at the following addresses:

ALBANIA
Ndermarja Shtetnore e Botimeve
Tirana

AUSTRALIA
A. Keesing
Box 4886, GPO
Sydney

AUSTRIA
Globus Buchvertrieb
Salzgries 16
Wien I

BELGIUM
Office International de Librairie
30, Avenue Marnix
Bruxelles 5
Du Monde Entier
5, Place St. Jean
Bruxelles

BULGARIA
Raznoiznos
1, Tzar Assen
Sofia

CANADA
Pannonia Books
2, Spadina Road
Toronto 4, Ont.

CHINA
Waiwen Shudian
Peking
P. O. B. 88

CZECHOSLOVAKIA
Artia
Ve Směčkách 30
Praha 2
Poštovná Novinová Služba
Dovoz tisku
Vinohradská 46
Praha 2
Mad'arská Kultura
Václavské nám. 2
Praha I
Poštová Novinová Služba
Dovoz tlače
Leningradská 14
Bratislava

DENMARK
Ejnar Munksgaard
Nörregade 6
Copenhagen

FINLAND
Akateeminen Kirjakauppa
Keskuskatu 2
Helsinki

FRANCE
Office International de Documentation et Librairie
48, rue Gay Lussac
Paris 5

GERMAN DEMOCRATIC REPUBLIC
Deutscher Buch-Export und Import
Leninstraße 16
Leipzig 701
Zeitungsvertriebsamt
Fruchtstrasse 3—4
1004 Berlin

GERMAN FEDERAL REPUBLIC
Kunst und Wissen
Erich Bieber
Postfach 46
7 Stuttgart S.

GREAT BRITAIN
Collet's Holdings Ltd.
Dennington Estate
London Rd.
Wellingborough. Northants.
Robert Maxwell and Co. Ltd.
Waynflete Bldg. The Plain
Oxford

HOLLAND
Swetz and Zeitlinger
Keizersgracht 471—487
Amsterdam C.
Martinus Nijhof
Lange Voorhout 9
The Hague

INDIA
Current Technical Literature
Co. Private Ltd.
India House OPP
GPO Post Box 1374
Bombay I

ITALY
Santo Vanasia
Via M. Macchi 71
Milano
Libreria Commissionaria Sansoni
Via La Marmora 45
Firenze

JAPAN
Nauka Ltd
92, Ikebukur O-Higashi 1-chome
Toshima-ku
Tokyo
Maruzen and Co. Ltd.
P. O. Box 605
Tokyo-Central
Far Eastern Booksellers
Kanda P. O. Box 72
Tokyo

KOREA
Chulpanmul
Phenjan

NORWAY
Johan Grundt Tanum
Karl Johansgatan 43
Oslo

POLAND
RUCH
ul. Wronia 23
Warszawa

ROUMANIA
Cartimex
Str. Aristide Briand 14—18
Bucureşti

SOVIET UNION
Mezhdunarodnaya Kniga
Moscow G—200

SWEDEN
Almquist and Wiksell
Gamla Brogatan 26
Stockholm

USA
Stechert Hafner Inc.
31, East 10th Street
New York, N. Y. 10003
Walter J. Johnson
111, Fifth Avenue
New York, N. Y. 10003

VIETNAM
Xunhasaba
19, Tran Quoc Toan
Hanoi

YUGOSLAVIA
Forum
Vojvode Mišića broj 1
Novi Sad
Jugoslovenska Knjiga
Terazije 27
Beograd

48, — Ft

Index

30. IV. 1969

Index: 26.030

VOLUME 4
NUMBER 2
1969

Acta biochimica et biophysica

ACADEMIAE SCIENTIARUM HUNGARICAE

AKADÉMIAI KIADÓ, BUDAPEST

Acta Biochimica et Biophysica

Academiae Scientiarum Hungaricae

Szerkeszti:

STRAUB F. BRUNÓ és ERNST JENŐ

Technikai szerkesztők:

SAJGÓ MIHÁLY és NIEDETZKY ANTAL

Szerkesztőség címe: Budapest XI., Karolina út 29. (biokémia);
Pécs, Rákóczi út 80. (biofizika)

Az *Acta Biochimica et Biophysica*, a Magyar Tudományos Akadémia idegen nyelvű folyóirata, amely angol nyelven (esetleg német, francia vagy orosz nyelven is) eredeti tanulmányokat közöl a biokémia és a biofizika — fehérjék (struktúra és szintézis), enzimek, nukleinsavak, szabályozó és transzport-folyamatok, bioenergetika, izom-összehúzódás, radiobiológia, biokibernetika, funkcionális és ultrastruktúra stb. — tárgyköréből.

A folyóirat negyedévenként jelenik meg, a négy füzet évente egy kb. 400 oldalas kötetet alkot. Kiadja az Akadémiai Kiadó.

Előfizetési díja belföldre 120,— Ft, külföldre 165,— Ft kötetenként. Megrendelhető az Akadémiai Kiadónál (Bp. V., Alkotmány u. 21), a külföld részére pedig a Kultúra Könyv és Hírlap Külkereskedelmi Vállalatnál (Budapest I., Fő u. 32).

The *Acta Biochimica et Biophysica*, a periodical of the Hungarian Academy of Sciences, publishes original papers, in English, on biochemistry and biophysics. Its main topics are: proteins (structure and synthesis), enzymes, nucleic acids, regulatory and transport processes, bioenergetics, excitation, muscular contraction, radiobiology, biocybernetics, functional structure and ultrastructure.

The Acta Biochimica et Biophysica is a quarterly, the four issues make up a volume of some 400 pp, per annum. Manuscripts and correspondence with the editors and publishers should be sent to

Akadémiai Kiadó Budapest 24 P.O.B. 502.

The subscription rate is 165,— Ft per volume. Orders may be placed with *Kultúra* Trading Co. for Books and Newspapers (Budapest 62, P.O.B. 149) or with its representatives abroad, listed on p. 3 of the cover.

Acta Biochimica et Biophysica — журнал Академии Наук Венгрии, публикующий на английском языке (возможно и на немецком, французском и русском языках) оригинальные статьи по проблемам биохимии и биофизики — белков (структура и синтез), энзимов, нуклеиновых кислот, процессов регуляции и транспорта, биоэнергетики, мышечного сокращения, радиобиологии, биокибернетики, функциональной структуры и ультраструктуры и т. д.

Журнал выходит ежеквартально, выпуски каждого года составляют том объемом около 400 страниц. Журнал выпускает Издательство Академии Наук Венгрии.

Рукописи и корреспонденцию просим направлять по следующему адресу:

Akadémiai Kiadó Budapest 24 P.O.B. 502.

Подписная цена — 165 Фт за том. Заказы принимает:

Предприятие по внешней торговле книгами и газетами «Kultúra» (Budapest 62. P. O. B. 149) или его заграничные агентства.

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (2) pp. 112–129 (1969)

Comparative Studies on the Composition and Properties of EGTA-sensitizing Factors*

W. DRABIKOWSKI, RENATA DABROWSKA, EWA NOWAK

Department of Biochemistry, Nencki Institute of Experimental Biology, Warsaw, Poland

(Received October 14, 1968)

Three kinds of protein preparations were compared with respect to their composition and to their ability to sensitize reconstituted actomyosin to calcium chelating agents such as EGTA.**

Preparation A was essentially the native tropomyosin of Ebashi, preparation B, crude actin extracted at room temperature and preparation C, crude Bailey's tropomyosin. All of them contain tropomyosin, troponin and a nucleoprotein fraction and differ only in the relative proportions of these components.

The quantity of troponin is the highest in preparation A, whereas preparation C contains the largest amount of contaminating nucleoproteins. The prepations were characterized by their E_{278}/E_{260} ratio, and by their content of ribose and sulfhydryl groups, both directly and after fractionation at pH 4.6 and by using Sephadex G-200 and DEAE-cellulose chromatography.

All preparations revealed EGTA sensitizing activity, the highest activity being in preparation A. The activity seems to be equal when calculated for the amount of troponin present in the preparations.

Perry and Grey (1956) were the first to notice that the Mg^{++} activated ATPase of reconstituted actomyosin was not inhibited by the removal of calcium, contrary to that of "natural" actomyosin (myosin B). Subsequently, Weber and Winicur (1961) observed that some of the preparations of reconstituted actomyosin were sensitive to calcium-chelating agents, such as EGTA, and that this property was connected with the actin component of actomyosin.

These observations have suggested that the requirement of actomyosin for minute amounts of calcium is not characteristic of highly purified actomyosin preparations.

A new light on this problem was shed by the discovery of Ebashi and Ebashi (1964), who have shown that the calcium sensitivity of actomyosin was dependent on the presence of a specific protein factor obtained by prolonged extrac-

* A brief account of this work was given at the Symposium on Functional and Structural Aspects of the Myofibrillar Proteins, Balatonboglár, Hungary, 1968.

** Abbreviations: EGTA — 1,2-bis-(2-dicarboxylaminoethoxy)-ethane; DTE — dithioerythritol; HEDD — β-hydroxyethyl-2,4-dinitrophenyl disulphide; DAM — desensitized actomyosin; RNA — ribonucleic acid; IEP — isoelectric precipitate fraction; IES — isoelectric supernatant fraction.

tion with low ionic strength solution of the muscle residue devoid of myosin. This protein factor caused the inhibition by EGTA of superprecipitation and of ATP-ase activity of reconstituted actomyosin. The protein was called by Ebashi "native" tropomyosin in view of many similarities to the classical Bailey's tropomyosin. The latter, however, failed to sensitize actomyosin to the action of EGTA.

Shortly after Ebashi's discovery other authors have reported about the isolation of protein preparations sharing with the "native" tropomyosin the property of sensitizing actomyosin to the removal of calcium. Thus, Azuma and Watanabe (1965) have shown that a so-called minor component of metin (Szent-Györgyi, Kaminer, 1963) was able to sensitize actomyosin to EGTA, whereas Katz (1966) isolated an active preparation from purified actin extracted at room temperature, previously shown to contain tropomyosin contamination (Drabikowski, Gergely, 1962).

Subsequently, Ebashi and Kodama (1965) have pointed out that "native" tropomyosin consisted in fact of two proteins – one, classical tropomyosin – and the other, called troponin (Ebashi, Kodama, 1966) and that both components are necessary for the activity (Ebashi, Nonomura, 1967). Contrary to that, Mueller (1966) has reported that tropomyosin itself, prepared in the presence of dithiothreitol for protection of its sulfhydryl groups, also revealed EGTA-sensitizing activity.

Watanabe and Staprans (1966), on the one hand, have confirmed the heterogeneity of "native" tropomyosin by gel filtration but, on the other hand, have suggested that only one protein component, different from tropomyosin and troponin, was responsible for EGTA-sensitizing activity.

The present studies have been undertaken in order to compare the protein composition of different kinds of EGTA-sensitizing preparations and to elucidate which component is responsible for their activity. Fractionation with ammonium sulphate, isoelectric precipitation, and subsequent chromatography on Sephadex G–200 and DEAE cellulose were employed.

Part of this work has already been published (Dabrowska et al., 1967; Nowak et al., 1968).

Materials and Methods

Rabbit back and leg muscle was used for all preparations. Three kinds of crude protein extracts served as a starting material for fractionation.

Extract A

In this case the procedure essentially followed that of Ebashi for the isolation of the "active component without acetone treatment" (Ebashi, Ebashi, 1964). Minced muscle was extracted twice with 3 vols of Guba–Straub solution for half an hour at 0°C. Subsequently, the residue was washed twice with 0.02 M KCl + + 0.3 mM $NaHCO_3$, then once with H_2O (for each washing 100 ml per 100 g of

initial fresh muscle were used) and suspended in 0.3 mM $NaHCO_3$ solution. The pH was adjusted to 7.5 and the suspension was kept at 2°C with occasional stirring. Every 24 hours the suspension was filtered through cheese-cloth and the residue was extracted with fresh $NaHCO_3$ solution. Changing the $NaHCO_3$ solution was found to shorten the time required for extraction of active material, as indicated by an increase in viscosity.

Extract B

Acetone dried muscle powder used for the preparation of actin (Feuer et al., 1948) was extracted with deionized water for two hours at 25°C (Drabikowski, Gergely, 1962). The extraction was repeated and all extracts were combined. Sometimes, for comparison, the extraction was performed at 0°C.

Extract C

Alcohol-ether dried muscle powder, prepared according to Bailey (1948) was repeatedly extracted for several hours with 1 M KCl at room temperature and the extracts were combined. Also the residue left after a previous extraction of actin at 0°C from the aceton dried powder was used as a starting material for this preparation.

To extracts A, B and C 22.5 g ammonium sulfate per 100 ml (about 40 per cent saturation) was added, following the original procedure of Ebashi and Ebashi (1964). The precipitates removed by centrifugation were discarded and 15 g $(NH_4)_2SO_4$ was added per 100 ml of supernatant, corresponding to about 60 per cent saturation. The precipitates collected by centrifugation were dialyzed against 0.3 mM $NaHCO_3$. The insoluble material remaining at the end of dialysis was removed by centrifugation. The supernatant solutions are denoted in the text as A 40–60, B 40–60 and C 40–60.

Preparations 40–60 were further fractionated at pH 4.6 in 1 M KCl (Ebashi, Kodama, 1966). The precipitates formed under these conditions were suspended in 1 mM $NaHCO_3$ (if necessary the pH was adjusted to 7.5) and dialyzed against 0.3 mM $NaHCO_3$. The obtained solution contained crude tropomyosin and is denoted in the text as IEP. It served for the preparation of purified tropomyosin by repeated fractionation with ammonium sulphate and precipitation at pH 4.6 in 1 M KCl according to Bailey (1948).

The pH of the supernatant obtained at pH 4.6 was adjusted to 7.5 and dialyzed against 1 mM $NaHCO_3$; the precipitate formed during dialysis was centrifuged off and discarded. The obtained solution corresponded to crude troponin (Ebashi, Kodama, 1966) and is denoted in the text as IES. When the viscosity of the solution at this stage was high, indicating the presence of some tropomyosin, the fractionation at pH 4.6 in 1 M KCl was repeated. The final IES solutions were collected, and, when necessary, concentrated either by lyophilization or by the use of Sephadex G 25 or Ficoll.

Sephadex G–200 chromatography was carried out essentially according to Watanabe and Staprans (1966) on a column bed with a diameter of 2.5 cm and a

height of 90–100 cm, and containing approximately 20 g (dry wt) of Sephadex G–200. The elution medium was 0.3 M KCl containing 20 mM Tris-HCl, pH 7.5. The outflow time was 10–30 ml per hour, the temperature about 4°C. Usually 7 ml samples were collected and absorbances at 278 mμ and 260 mμ were measured.

DEAE cellulose (Sigma, Medium) chromatography was carried out under the conditions described by Perry and Żydowo (1959) on a column 24 cm high and 2 cm in diameter. DEAE cellulose was equilibrated with 20 mM Tris-HCl, pH 7.5. For the elution, first 20 mM Tris-HCl, pH 7.5, then increasing KCl concentrations (from 0.1 to 2 M) and finally 1 per cent NaOH were used.

Adenosinetriphosphatase activity was measured at 25°C in a system containing 50 mM KCl, 20 mM Tris-maleate buffer, pH 7.3, 1 mM $MgCl_2$, 1 mM EGTA, actomyosin desensitized according to Schaub et al. (1967a) and varying amounts of proteins investigated for their EGTA sensitizing activity. The reaction was started by the addition of 1 mM ATP. The samples were usually removed after 3 or 5 min., and the reaction was stopped by the addition of an equal volume of 10 per cent TCA. After removal of the precipitated protein by centrifugation, the amount of inorganic phosphate was measured by the method of Fiske and Subbarow (1925).

Protein was determined with the biuret method (Gornall et al., 1949) or, occasionally, according to Lowry et al. (1951).

Ribose was determined with the orcinol method according to Mejbaum (1939).

Sulfhydryl groups were determined with HEDD, as previously described (Drabikowski, Nowak, 1965).

Viscosity was measured in an Ostwald type viscometer having an outflow time of 50 sec. for water, at 21°C.

All the reagents used were of analytical reagent grade. ATP disodium salt was purchased from Boehringer and Soehne GmbH, Mannheim, Germany. DTE was supplied by Cyclochemical Corp.

Results

Crude extracts

Extract A. As a result of prolonged extraction with 0.3–1 mM $NaHCO_3$ at 2°C of muscle residue devoid of myosin about 25 mg protein per 1 g of fresh muscle were obtained. As it was stated in Methods, every 24 hours the residue was extracted with fresh $NaHCO_3$ solution. The composition of the successive extracts changed with the time of extraction. The first extracts had low viscosity. Usually the extract turned viscous on the third day, when the extraction was performed at pH 7.5 and already on the second day, when it was done at pH above 8.0. Below pH 7.0 no viscous material was extracted. Usually after 6–7 days no more protein was extracted.

The viscous extracts (usually about 16 mg per 1 g fresh muscle) yielded about 5 mg of 40–60 fraction per 1 g fresh muscle.

The residue left after extraction still contained some tropomyosin. When it was dehydrated with alcohol, about 1 mg of protein per 1 g of initial fresh muscle could be extracted with 1 M KCl from the obtained powder.

Extract B. As a result of repeating 4–5 one hour extractions at 20–22°C about 60–80 mg protein per 1 g powder was obtained. The amount of the material precipitated between 40 and 60 per cent of ammonium sulfate saturation was only about 7 mg protein per 1 g of muscle powder, or about 0.7 mg per 1 g of fresh muscle.

When the extraction of aceton dried muscle powder was performed at 0°C the amount of 40–60 fraction was somewhat lower than that in case of extraction at room temperature.

Extract C. Repeating extraction of alcohol-ether dried muscle powder yielded about 100–120 mg protein per 1 g powder. About 25 mg protein precipitated between 40 and 60 per cent of ammonium sulfate saturation, which corresponded to about 6 mg protein when calculated for 1 g fresh muscle.

To sum up, the yield of A 40–60 and C 40–60 preparations was about the same, whereas that of B 40–60 was much lower.

40–60 preparations

Some properties of the 40–60 preparations are presented in Table 1. One can see that all of them are viscous, with the highest values for A 40–60, and the lowest for B 40–60. In all cases the viscosity dropped considerably after addition of salt. The viscosity of the control B 40–60 preparations, obtained from crude 0°C extract, was musch less (η_{red} of 1 mg/ml was usually 0.2–0.5).

Preparation C contained the greatest amount of ribose. The assumption that the ribose content is due to RNA agrees well with the finding that preparations C had the lowest E_{278}/E_{260} ratio and the highest E_{278} per mg protein.

Table 1 also shows the sulfhydryl content of the 40–60 fractions. The number of SH groups reacting with HEDD varied according to the conditions, presumably due to changes in the protein structure. In the absence of salt and in 1.8 M KCl about 2.5 and 3.4 SH equivalents per 100 000 *g* were found, respectively, in the preparations A 40–60 and B 40–60. In the preparations C 40–60 these values were slightly lower. When 6 M urea was present about 6 SH equivalents per 100 000 *g* were detected in A 40–60, 5 in B 40–60 and even less than 4 SH equivalents in C 40–60. After preincubation with 0.5 mM DTE and subsequent dialysis the number of SH equivalents increased only slightly.

Fractionation at pH 4.6

Ebashi and Kodama (1966) have shown that native tropomyosin can be separated at pH 4.6 in the presence of salt into tropomyosin, which precipitates under these conditions, and troponin which remains in the supernatant. One can see from Table 1 that the amount of protein precipitating in 1 M KCl at pH 4.6

Table 1

Properties of the 40—60 preparations

Preparation	E_{278}^{1cm}[1]	E_{278} to E_{260} ratio	Reduced viscosity[1]		IEP to[2] IES ratio	Ribose, moles per 10^5 g protein	Reactive SH groups per 10^5 g protein		
			in H_2O	in 0.1 M KCl			in H_2O	in 1.8 KCl	in 6 M urea
A	0.395	1.60	20.0	2.45	3.2	0.18	2.81	3.50	7.09
	0.419	1.57	18.0	2.60	2.3	0.44	1.90	2.64	5.04
	0.378	1.50	16.8	2.45	3.5		2.58	3.78	6.10
	0.525	1.64	20.0	2.45	3.2		2.73	3.53	5.60
	0.520	1.50		2.45	2.8				
av.	0.450	1.56	18.7	2.48	3.0	0.31	2.50	3.40	5.95
B	0.490	1.56	2.3	0.75	5.6	0.11	2.56	2.84	5.04
	0.445	1.61	3.2	0.70	5.6	0.35	2.48	2.77	4.24
	0.430	1.43	2.5	1.00	5.1		2.94	3.81	4.90
	0.430	1.44	3.8	0.60	5.0		2.61	3.55	5.60
	0.446	1.47	4.2	1.15					
	0.474	1.76	4.9	0.90					
		1.64	2.2	0.89					
av.	0.450	1.56	3.3	0.85	5.3	0.23	2.65	3.32	4.94
C	0.675	1.27	8.1	0.8		2.4	1.94	2.10	3.73
	0.905	1.21	8.4	1.6	5.4	1.6	2.18	2.83	3.44
	0.968	1.21	9.5	1.2	5.1	1.8			
		0.87	6.2	2.4		2.9			
		0.91							
av.	0.850	1.05	8.0	1.5	5.2	2.1	2.06	2.47	3.59

[1] for a solution of 1 mg per ml

[2] weight ratio of the amount of protein precipitated in 1 M KCl at pH 4.6 to that remaining in solution

(IEP) was about three times as much as that remaining in the solution (IES) in preparations A, and about five times as much as in the two other preparations. In the former case the above ratio represents the value obtained by fractionation of the total 40—60 material collected from all viscous crude extracts. During the extraction of muscle residues this ratio changed so that the 40—60 preparations from the first viscous extracts contained less IES than the last one, in which the ratio of IEP to IES was almost 1 : 1.

In the protein fraction remaining at pH 4.6 in the supernatant and containing crude troponin (IES) almost all ribose present in the original material (Table 2) was found. The E_{278}/E_{260} ratio of IES was lower than that of the original 40—60 fractions, the lowest in case of the fraction derived from preparation C. Con-

Table 2

Properties of supernatants (IES) and precipitates (IEP) obtained from the 40—60 preparations at pH 4.6 in 1 M KCl

Preparation	IES				IEP			
	E_{278} to E_{260} ratio	Reduced viscosity[1]		Ribose, moles per 10^5 g protein	E_{278} to E_{260} ratio	Reduced viscosity[1]		Ribose, moles per 10^5 g protein
		in H_2O	in 0.1 M KCl			in H_2O	in 0.1 M KCl	
A	1.20	0.61	0.57	1.49	1.62	15.0	2.08	0.08
	1.13	0.77	0.65	1.13	1.80	12.5	1.80	
	1.18	0.78	0.72	1.57	1.83	12.5	1.60	
	1.12	0.80		1.67	1.69	12.5	1.60	
	1.12	0.75		1.96		14.0	1.98	
	1.10			1.12				
av.	1.14	0.75	0.65	1.49	1.73	13.5	1.81	0.08
B	1.08	0.65	0.55	2.31	1.69	4.0	0.76	0.10
	1.18	0.46	0.19		1.84	9.4	1.12	0.15
	1.29				1.65	4.0	0.50	
	1.01				1.71	6.2	1.02	
	1.21				1.64			
	1.04							
av.	1.14	0.55	0.34	2.31	1.70	5.9	0.85	0.12
C	1.00	0.30	0.10	4.46				
	0.88	0.30	0.20	3.40				
	0.69	0.25	0.10	4.85	1.66			0.60
av.	0.86	0.28	0.13	4.23	1.66			0.60

[1] for a solution of 1 mg per ml

sequently, the E_{278}^{1cm} of the 1 mg/ml solution of IES from C 40—60 was the highest, on the average above 1.1.

The protein present in IES had a low viscosity which practically did not change after addition of 0.1 M KCl.

The protein fraction precipitated at pH 4.6 in 1 M KCl, corresponding to crude tropomyosin (IEP), contained only traces of ribose and had a higher E_{278}/E_{260} ratio than original 40—60 preparations. Its viscosity was high in the absence of salt, but lower than that of the 40—60 fractions.

EGTA-sensitizing activity

All 40—60 preparations were tested for their ability to sensitize DAM to EGTA. It can be seen from Fig. 1A that A 40—60, corresponding to Ebashi's "native tropomyosin", was the most active. It seems that the difference in the

activity among preparations A, B and C is mainly due to the different ratio of troponin to tropomyosin, since the average percentage of inhibition of ATP-ase by various preparations was almost identical when it was calculated for one μg of troponin (Fig. 1B).

After addition of an excess of troponin (IES from A 40–60) to all 40–60 preparations at a final ratio of tropomyosin to troponin of about 0.65 : 1, some increase in EGTA-sensitizing activity was observed (smaller in preparation A and

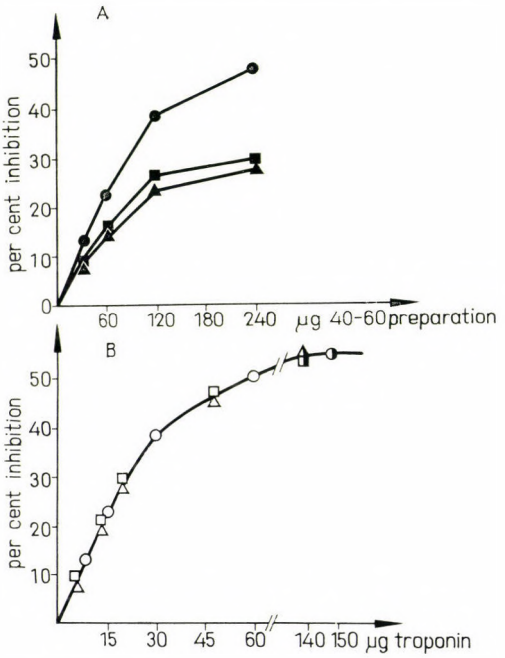


Fig. 1. Effect of 40—60 preparations on Mg^{++} activated ATP-ase of desensitized actomyosin (DAM). The medium contained 20 mM Tris-maleate buffer, pH 7.3, 0.05 M KCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP, 0.6 mg DAM and the amount of investigated preparations as indicated on the abscissa. Total volume 2 ml. A) Per cent inhibition of ATP-ase activity expressed with respect to total protein content in 40—60 preparations (●) A 40—60; (■) B 40—60; (▲) C 40—60. B) Per cent inhibition expressed with respect to troponin content. The amount of the latter was calculated on the basis of the assumption that the average ratio of tropomyosin to troponin in A 40—60 was 3.0 to 1.0, and in B 40—60 and C 40—60 it was 5.0 to1.0, i.e. the same as the ratio of IEP to IES in these preparations (see Table 1). (○) A 40—60; (□) B 40—60; (△) C 40—60 ◑, ◨, ◭ — 120 μg troponin (IES from A 40—60) added to 120 μg of all corresponding 40—60 preparations

higher in preparations B and C) and the same precentage of inhibition of ATP-ase activity was achieved in all kinds of preparation (Fig. 1B).

It seems therefore that the ratio of troponin to tropomyosin in all preparations, especially in preparations B and C, was below optimum, i. e. they contained an excess of tropomyosin over troponin. It is to be mentioned that control B 40–

60 preparations, obtained from 0°C crude extract, did not reveal any EGTA-sensiziting activity.

Sephadex G–200 chromatography

All 40–60 preparations as well as the IEP and IES fractions were chromatographed on Sephadex G–200 column with 0.3 M KCl as eluent. With all 40–60 preparations three peaks were obtained (Fig. 2). They differed, however, in their relative proportions depending on the kind of preparation. Peak I of 40–60 preparations was non-retarded. With IEP almost all protein present was eluted as

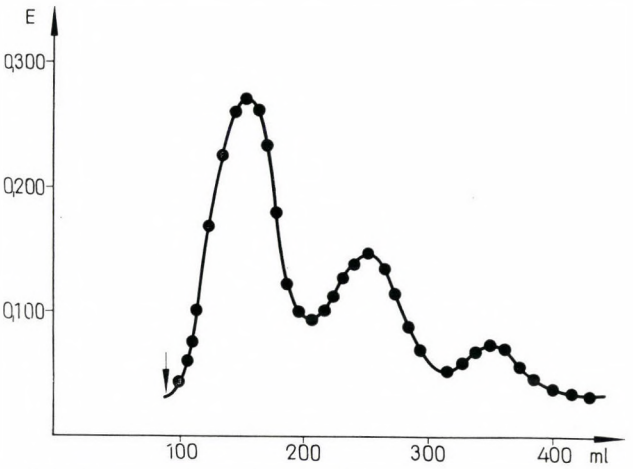


Fig. 2. Sephadex G–200 column chromatogram of A 40—60 preparation. The applied sample contained 15 mg protein. For details see Methods. Arrow indicates void volume

peak I (Fig. 3). A similar elution pattern was obtained for pure tropomyosin. On the other hand, the elution pattern of IES consisted of the two other peaks (II and III) of the original 40–60 preparations (Fig. 3). The relative ratios of peaks II and III also depended on the kind of the original preparation. In the case of preparations A and B peak III was rather small, whereas in preparation C it was even greater than peak II.

The resolution of the 40–60 preparations seemed optimal with 0.3 M KCl. At a lower KCl concentration the solutions were very viscous, whereas at a much higher KCl concentration peak I became somewhat retarded and rather poorly separated from peak II.

Table 3 shows some properties of the proteins present in the peaks obtained from original 40–60 preparations. Since peak III was rather small, in order to get more material for its characterization the IES fraction was chromatographed on Sephadex G–200, instead of the whole 40–60 preparation.

Viscometric properties of peak I corresponded to those of tropomyosin. Its E_{278}/E_{260} ratio, however, was only about 1.5–1.6, markedly lower than that of

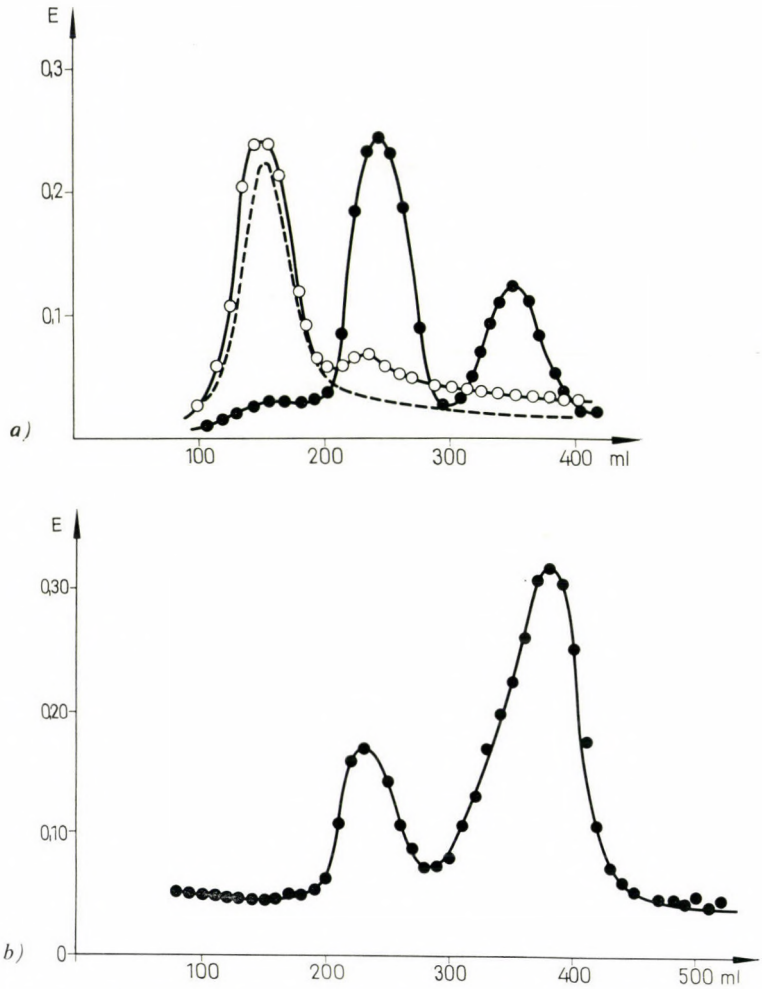


Fig. 3. Sephadex G-200 column chromatograms of IES and IEP fractions. *a)* ○ — IEP from the A 40—60 preparation (15 mg protein) ● — IES from the A 40—60 preparation (15 mg protein) — — — Bailey's tropomyosin. *b)* IES from the C 40—60 preparation (15 mg protein)

pure tropomyosin (in our case 2.0 – 2.2). The viscosity of the second peak was very low. Its E_{278}/E_{260} ratio, irrespective of its origin, was about 1.1 – 1.2, slightly higher when IES instead of 40 – 60 preparations was chromatographed. Both peaks I and II contained small and rather variable amounts of ribose, the highest in the case of preparations C.

Peak III contained in all cases large amounts of ribose, the most when it was obtained from preparation C. The E_{278}^{1cm} of a 1 mg per ml solution of this peak was very high, in preparations A about 1.0 and in preparations C around 5.0, indicating a large nucleic acid content, as also shown by a very low E_{278}/E_{260} ratio,

Table 3

Properties of the peaks separated from the 40—60 preparations on Sephadex G-200

Original extract	Starting material	PEAK I				PEAK II				PEAK III	
		E_{278} to E_{260} ratio	Reduced viscosity[1]		Ribose, moles per 10^5 g protein	E_{278} to E_{260} ratio	Reduced viscosity[1]		Ribose, moles per 10^5 g protein	E_{278} to E_{260} ratio	Ribose, moles per 10^5 g protein
			in H_2O	in 0.1 M KCl			in H_2O	in 0.1 M KCl			
A	40—60 prep.	1.58				1.15	0.25	0.25	0.65		
		1.48				1.05					
		1.60				1.05					
		1.69	7.4	2.4	0.65						
		1.68	7.9	2.1	0.11						
	IES					1.19				0.69	4.28
						1.30	0.36		0.13	0.72	3.20
						1.25	0.24	0.24	0.22	0.72	5.95
						1.35			0.13	0.89	5.80
						1.20			0.72	0.77	4.75
B	40—60 prep.	1.34	8.8	1.3	0.31	1.09	0.28	0.15	0.28	0.72	
		1.48			0.79	1.19	0.40	0.10	0.63	0.68	
		1.42				1.12					
		1.54									
	IES					1.24					
						1.19					
						1.12					
						1.24					
C	40—60 prep.	1.47				0.97			1.70		
		1.77				0.93			1.87		
		1.62	7.2	1.9	1.09	1.02					
		1.54				1.05					
		1.69				0.96			1.76		
	IES					1.30				0.50	43.0
						1.22	0.30	0.15	1.34	0.60	21.0
						1.33			1.87	0.58	60.7
						1.19			1.60		24.0
						1.28			1.22		43.0
Average		1.56	7.8	1.8	—	1.16	0.30	0.18	—	0.69	—

[1] for a solution of 1 mg per ml

which was in all cases below one. Hence, although the areas of peak III as measured by E_{278} were in preparations C relatively large, the protein content in this peak was very small.

Fig. 4 shows the absorption spectra of all three peaks and that of pure tropomyosin for comparison. One can see that peak III possesses a spectrum characteristic of nucleoproteins.

Some EGTA-sensitizing activity appeared in peak I, although smaller than that present in the unfractionated 40–60 preparations. On the other hand, the protein present in peak II did not inhibit *per se* the ATP-ase activity in the presence of EGTA, but it showed a remarkable inhibiting effect when Bailey's tropomyosin

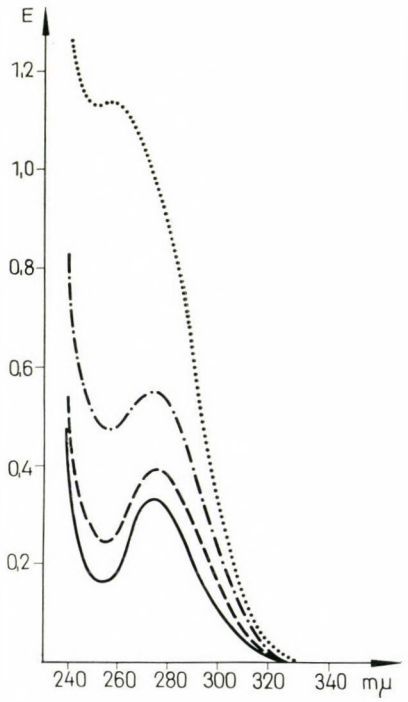


Fig. 4. Absorption spectra of the Sephadex G–200 peaks from A 40—60 preparation. The solutions contained 1 mg protein per ml. — — —, peak I; — · —, peak II; · · ·, peak III; ———, Bailey's tropomyosin

was added. Peak III alone or in combination with other fractions showed no effect on the actomyosin ATP-ase in the presence of EGTA.

On the basis of all observations (see also below) it seems that on Sephadex G–200 under our conditions only partial separation of tropomyosin-troponin complex into its components was achieved. Peak I contained a complex of tropomyosin with troponin rather than pure tropomyosin. Both pure tropomyosin and that in form of the complex with troponin were non-retarded. Hence, one could not distinguish between them only on the basis of elution pattern. Peak II corresponded to troponin, whereas the third peak contained some nucleoproteins.

DEAE-cellulose chromatography

The 40–60 preparations as well as fractions IEP and IES were also chromatographed on DEAE cellulose columns with KCl solutions of various concentrations as eluent. The elution pattern of IES, i.e. crude troponin, showed a main peak eluted at 0.2 M KCl with the ratio E_{278}/E_{260} of about 1.2 (Fig. 5B). This peak did not show any inhibiting activity on actomyosin ATP-ase in the presence of EGTA, unless tropomyosin was added. It is evident that the peak eluted with 0.2 M KCl corresponds to the troponin-containing peak II of the Sephadex–G–200 chromatograms.

The elution pattern of crude tropomyosin (IEP) showed a main peak at 0.3 M KCl, with a E_{278}/E_{260} ratio of about 2.1–2.2, corresponding to that of pure tropomyosin (Fig. 5C). On the other hand, the 40–60 preparations directly chromatographed on DEAE cellulose revealed at 0.2 and 0.3 M KCl only very small peaks, whereas a main peak appeared at 0.1 M KCl with a E_{278}/E_{260} ratio of about 1.6 and with remarkable EGTA-sensitizing activity (Fig. 5A).

The observations suggested the lack of separation on DEAE cellulose of tropomyosin and troponin from the complex. Indeed, when the mixture of purified tropomyosin and crude troponin (IES) was chromatographed on DEAE cellulose column, instead of two peaks at 0.2 and 0.3 M KCl, which should correspond to troponin and tropomyosin, respectively, a new main peak appeared, eluted at 0.1 M KCl, with an E_{278}/E_{260} ratio of about 1.6. The non-retarded peak I from Sephadex G–200 column on rechromatography on DEAE cellulose also showed the main peak eluted at 0.1 M KCl, lending additional support to the view that peak I obtained on Sephadex G–200 during separation of 40–60 fraction was in fact not pure tropomyosin but the complex of tropomyosin and troponin.

During chromatography of 40–60 preparations and especially of IES on DEAE cellulose two other small peaks were obtained, the first eluted at 0.6 M KCl and the second still bound at 2.0 M KCl and released from the column only with 1 per cent NaOH. On the basis of the presence of large amount of ribose and of an E_{278}/E_{260} ratio less than one it seems assured that these peaks contain nucleoproteins.

Discussion

Three kinds of protein preparations were compared in this work with respect to their composition and activity as the factor sensitizing actomyosin to calcium ions. All of them were found to exhibit EGTA-sensitizing activity and to consist of tropomyosin, troponin and a fraction containing nucleoprotein, and to differ only in the relative proportions of these fractions. One of the protein preparations studied, "native" tropomyosin of Ebashi and Ebashi (1964), the best characterized so far, reveals the highest EGTA-sensitizing activity. The second, similar fraction obtained from crude actin extracted at room temperature, possesses only weak EGTA-sensitizing activity (Ebashi, Ebashi, 1964) and its composition has not been studied so far.

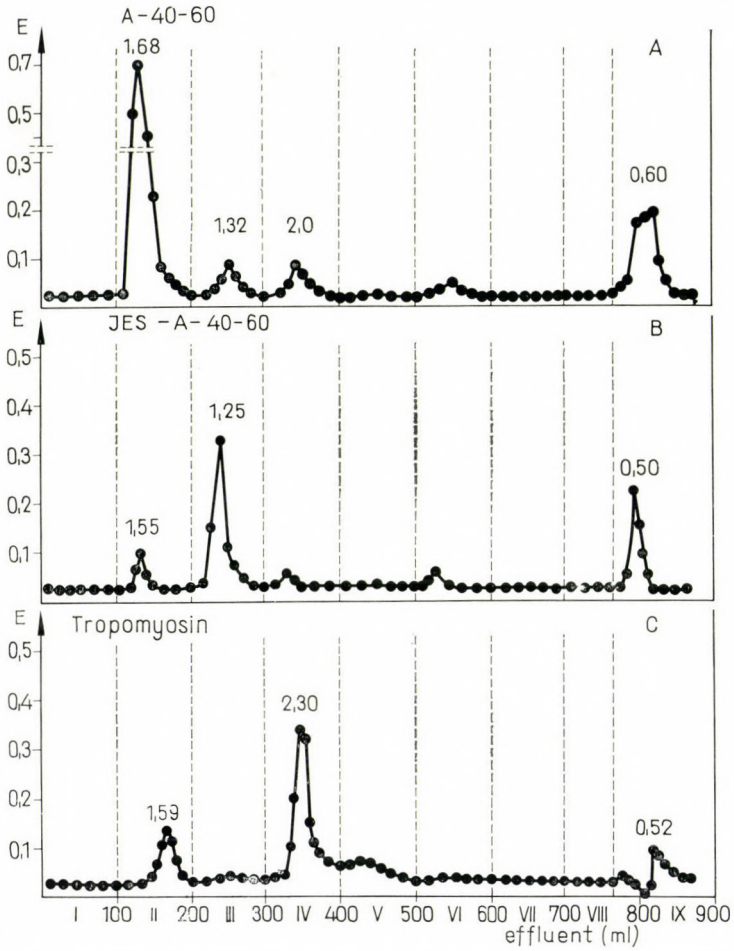


Fig. 5. DEAE cellulose column chromatograms of some of the investigated fractions. *A)* preparation A 40—60 (50 mg protein). *B)* IES preparation A 40—60 (50 mg protein). *C)* Bailey's tropomyosin (20 mg protein). Eluent: I — 10 mM Tris, pH 7.5; II — 0.1 M KCl; III — 0.2 M KCl; IV — 0.3 M KCl; V — 0.4 M KCl; VI — 0.6 M KCl; VII — 0.8 M KCl; VIII — 2.0 M KCl; IX — 1 per cent NaOH. The numbers placed above the peaks indicate E_{273} to E_{260} ratios

An analogous ammonium sulfate fraction obtained from the so-called γ-fraction (Martonosi, 1962) of purified "room temperature" actin was studied by Katz (1966) who also observed that it contained some other unknown protein besides tropomyosin. The results of this work directly demonstrate the presence of troponin in the EGTA-sensitizing preparations obtained from "room temperature" actin.

Crude actin, extracted at 0°C and used in this work as a control preparation for B 40–60 also contains some material salting out between 40 and 60

per cent ammonium sulfate saturations (Drabikowski, 1968). All present results indicate, however, that these preparations do not contain tropomyosin-troponin complex. The same unknown protein material is most probably present also in B 40–60 preparations, as indicated by their relatively low viscosity and weak EGTA-sensitizing acitivity. Our previous studies (Drabikowski, 1968) have shown that this contaminating protein material remains in the supernatant fluid during centrifugation of crude F-actin, according to the purification procedure of Mommaerts (1952), contrary to the tropomyosin-troponin complex sedimenting together with actin. Hence, the EGTA-sensitizing preparations obtained by Katz (1966) from purified room-temperature actin were much more active.

The third kind of active preparation studied in this work was crude tropomyosin, prepared according to Bailey (1948), i. e. a 1 M KCl extract from alcohol-ether muscle powder. This kind of preparation was not used so far as EGTA-sensitizing factor, although Yasui et al. (1968) have recently used the supernatant from isoelectric precipitation of crude Bailey's tropomyosin as a source of troponin.

All the active preparations have different troponin to tropomyosin ratios, the highest in "native" tropomyosin. Even in the latter case the ratio depends on which successive extract of muscle residue is used. Essentially in all of them, however, this ratio seems to be below optimum, so that the addition of troponin still increases their activity to some extent. The complex of tropomyosin and troponin seems to be saturated with troponin with respect to the EGTA-sensitizing activity at a ratio of about 1.5 to 1.0 by weight of troponin to tropomyosin, similar to that found recently by Hartshorne and Mueller (1967) and by Yasui et al. (1968). On the other hand, viscosity of the solution continuosly increases when troponin is added to tropomyosin up to a ratio of 3.0 to 1.0 or even more (Dabrowska, Drabikowski, 1968).

One could expect that the lack of EGTA-sensitizing activity of tropomyosin prepared according to Bailey was due to the action of organic solvents before extraction. This possibility is excluded by the results of the present work. Crude Bailey's tropomyosin was fully active as an EGTA-sensitizing factor.

The preparations lost their activity only during purification by precipitation at pH 4.6 in the presence of salt, due to removal of troponin remaining in the supernatant under these conditions. Troponin prepared from the latter was perfectly active in combination with tropomyosin.

Another view was represented by Mueller (1966) who suggested that Bailey's tropomyosin with free SH groups was active as EGTA-sensitizing factor. If one assumes, however, that his preparations of tropomyosin contained troponin, as judged by the fact that the E_{278}/E_{260} ratio remained low after repeated isoelectric precipitations, one could explain their EGTA-sensitizing activity. In fact Yasui et al. (1968) have recently shown by acrylamide disc electrophoresis the presence of troponin in Mueller's "SH tropomyosin" preparations. Also the recent work of Hartshorne and Mueller (1967) clearly indicates that the EGTA-sensitizing factor requires both tropomyosin and troponin.

The high content of SH groups, found previously by Mueller in "SH tropomyosin" preparations, could also be due, at least partially, to the presence of troponin. According to our determinations, troponin contains up to 10 SH

equivalents per 10^5 g protein in the presence of urea, i.e. much more than tropomyosin (Drabikowski et al. in preparation). Similarly, the higher number of SH groups found previously by us (Drabikowski, Nowak, 1965) in unpurified tropomyosin was probably not only due to the smaller oxidation of SH groups, but also partially to troponin contamination.

All EGTA-sensitizing preparations contain a fraction of nucleoprotein origin. The highest amount of this fraction has been found in crude tropomyosin prepared according to Bailey (1948), and the lowest in Ebashi's "native" tropomyosin. Probably the dehydration of muscle favours subsequent extraction of nucleoproteins. All these results suggest that the nucleoprotein fraction does not play any role as far as the specific property of tropomyosin-troponin complex with respect to actomyosin is concerned and that it is present in the investigated preparations as a contamination.

In this work the removal of a nucleoprotein fraction from troponin was achieved by chromatography on Sephadex G–200 or DEAE cellulose. In their recent paper Ebashi et al. (1968) introduced salting out of troponin with $(NH_4)_2SO_4$ to 70 per cent saturation as an additional step during preparation of this protein. Our recent results show that under these conditions all ribose containing material remains in the supernatant, and, hence, troponin prepared in this way is practically no more contaminated with nucleoproteins.

The complex of tropomyosin and troponin seems to dissociate at a pH around 4.6 only if high salt concentration is present. In the absence of salts the separation is not complete. At a pH around neutral the conditions of the dissociation of the complex are less known. The results of this work show that chromatography on DEAE cellulose columns with KCl solution does not lead practically to the dissociation of the complex.

The elution pattern from DEAE cellulose obtained in this work was essentially similar to that obtained recently by Schaub et al. (1967b) during chromatography of another type of EGTA-sensitizing preparation, a low ionic strength extract from natural actomyosin. The data obtained by these authors suggest, however, that at least a partial dissociation of the tropomyosin-troponin complex occurred on the DEAE cellulose and that a peak corresponding to tropomyosin was eluted.

On Sephadex G–200 in 0.3 M KCl only partial separation of troponin from its complex with tropomyosin takes place. This phenomenon is at present difficult to explain since in the preparations applied to the column tropomyosin does not seem to be "saturated" with troponin. Watanabe and Strapans (1966) obtained four peaks during chromatography of Ebashi's native tropomyosin on Sephadex G–200 under similar conditions. Detailed examination of their data, however, shows no essential discrepancy between their results and ours. Similarly to us, upon chromatography of the unfractionated native tropomyosin, Watanabe and Strapans obtained one main non-retarded peak exhibiting EGTA-sensitizing activity. The authors thought that that peak (called α) contained a "relaxing" protein different from tropomyosin and troponin. It is rather evident from our data that peak α contained the complex of tropomyosin and troponin. The only difference is that in our case pure tropomyosin was also eluted as a non-retarded peak, where-

as in the experiment of Watanabe and Strapans the peak corresponding to tropomyosin (peak β) was eluted as the second one, after the non-retarded peak α. These authors, however, obtained peak β as a relatively large one only in some ammonium sulfate subfractions of "native" tropomyosin, which were rich in tropomyosin and devoid of troponin.

In conclusion, the results of this work are in full accordance with the hypothesis of Ebashi (cf. Ebashi, Nonomura, 1967) that for the EGTA-sensitizing activity a complex of tropomyosin and troponin is necessary.

The authors wish to thank Mrss U. Koperska and J. Klonowska for skillful technical assistance. This work was partly supported by Foreign Research Agreement No. 6×7715 of N. I. H. under P. L. 480.

References

Azuma, N., Watanabe, S. (1965) J. Biol. Chem. *240* 3852
Bailey, K. (1948) Biochem. J. *43* 271
Dabrowska, R., Nowak, E., Drabikowski, W. (1967) Abstr. 4th FEBS Meeting Oslo, p. 83
Dabrowska, R., Drabikowski, W. (1968) Symposium of Functional and Structural Aspects of the Myofibrillar Proteins, Balatonboglár
Drabikowski, W., Gergely, J. (1962) J. Biol. Chem. *237* 3412
Drabikowski, W., Nowak, E. (1965) Acta Biochim. Polon. *12* 61
Drabikowski, W. (1968) in E. Ernst and F. B. Straub (edrs) Symposium on muscle, Budapest September 1966, Akadémiai Kiadó, Budapest, p. 103
Ebashi, S., Ebashi, F. (1964) J. Biochem. (Tokyo) *55* 604
Ebashi, S., Kodama, A. (1965) J. Biochem. (Tokyo) *58* 107
Ebashi, S., Kodama, A. (1966) J. Biochem. (Tokyo) *60* 733
Ebashi, S., Nonomura, Y. (1967) Abstr. VIIth International Congress of Biochemistry, Vol. II, p. 329, Tokyo
Ebashi, S., Kodama, A., Ebashi, F. (1968) J. Biochem. in press.
Feuer, G., Molnar, F., Pettko, E., Straub, F. B. (1948) Acta Physiol. Hung. *1* 150
Fiske, C. H., Subbarow, Y. (1925) J. Biol. Chem. *66* 375
Gornall, A. G., Bardawill, C. J., David, M. M. (1949) J. Biol. Chem. *177* 751
Hartshorne, D. J., Mueller, H. (1967) J. Biol. Chem. *242* 3089
Katz, A. M. (1966) J. Biol. Chem. *241* 1522
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) J. Biol. Chem. *193* 265
Martonosi, A. (1962) J. Biol. Chem. *237* 2795
Mejbaum, W. (1939) Z. Physiol. Chemie *258* 117
Mommaerts, W. F. H. M. (1952) J. Biol. Chem. *198* 469
Mueller, H. (1966) Biochem. Z. *345* 300
Nowak, E., Dabrowska, R., Drabikowski, W. (1968) Symposium on Functional and Structural Aspects of the Myofibrillar Proteins, Balatonboglár
Perry, S. V., Grey, T. C. (1956) Biochem. J. *64* 5p
Perry, S. V., Żydowo, M. (1959) Biochem. J. *71* 220
Schaub, M. C., Hartshorne, D. J., Perry, S. V. (1967a) Biochem. J. *104* 263
Schaub, M. C., Perry, S. V., Hartshorne, D. J. (1967b) Biochem. J. *105* 1235
Szent-Györgyi, A., Kaminer, B. (1963) Proc. Natl. Acad. Sci. US *50* 1033
Watanabe, S., Staprans, I. (1966) Proc. Natl. Acad. Sci. U. S. *56* 572
Weber, A., Winicur, S. (1961) J. Biol. Chem. *236* 3198
Yasui, B., Fuchs, F., Briggs, N. (1968) J. Biol. Chem. *243* 735

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (2), pp. 131–139 (1969)

($Mg^{++}-K^{+}-Na^{+}$)-activated ATPase Activity and its Properties in Scenedesmus Obtusiusculus Alga Cells

Gabriella MESZES, L. ERDEI

Institute of Plant Physiology, L. Eötvös University, Budapest

(Received October 4, 1968)

(Mg^{++})-activated ATPase activity was detected in Scenedesmus obtusiusculus CHOD unicellular alga. Simultaneous addition of K^{+} and Na^{+} ions enhanced the activity and ouabain proved to be a specific inhibitor.

This (Mg^{++}–K^{+}–Na^{+})-activated ATPase activity increased in the light-period of synchronous cells, whereas it decreased in the dark-period and became nearly insensitive to ouabain.

As a result of illumination the (Mg^{++}–K^{+}–Na^{+})-activated ATPase activity exhibited a further increase according to a light-saturation curve. It is suggested that this ATPase might be connected with the active K^{+}–Na^{+}-transport system in this organism and both ATPase activity and active K^{+}–Na^{+}-transport are enhanced on illumination.

Introduction

The great number of papers dealing with the problems of transport in animal cells, microorganisms, and higher plants provide evidence, in general, for the existence of active transport processes proceeding against concentration or electrochemical gradients.

In the case of algae, species of different organizational forms have been used for such studies. Bioelectric measurements on coenocyte species also proved the existence of active ion transport (Blount, Levedahl, 1960; MacRobbie, 1962, 1964, 1965; Fujita, 1962; Dainty, 1962), whereas the transport processes in unicellular algae are still obscure.

The fact that transport can proceed against concentration and electrochemical gradients raises the question: where does the energy that maintains the process come from? The answers given differ with different authors.

Literary data concerning mainly animal cells, but also other objects, have shown that in active biological transport processes, especially in the active transport of K^{+} and Na^{+}, the energy conserved in ATP becomes directly or indirectly utilized and the role of transport-ATPases seems to be well founded (Jeschke, Simonis, 1967; Weigl, 1964; Skou, 1964; Bonting et al., 1961; Hafkenscheid, Bonting, 1968). A (Mg^{++}–K^{+}–Na^{+})-activated ATPase, sensitive to inhibition by ouabain, has been found, which is closely related to, or identical with, the K^{+}–Na^{+}-transport system. The question arises, are such ATPases present in unicellular algae, and if so, what is their role in ion transport. In fact, investigations

with higher plants, algae, and microorganisms indicate that K^+- and Cl^--transport is connected with the electron transport rather than directly with ATP (Atkinson et al., 1966; Barber, 1968; McRobbie, 1965, 1966; Raven, 1967a, b, 1968).

We have undertaken a comparative study on the alkali and haloid transport of the green alga Scenedesmus obtusiusculus (Meszes et al., 1967) parallel with measuring the ATP-pool and ATPase activity in the cells. Unicellular algae seem to be particularly suitable for such studies since they generate ATP partly by respiration and partly by certain steps of photosynthesis. Thus the ATP-pool is presumably affected by light–dark or dark–light transitions. Another outstanding feature of the chosen organism is the fact that it is fairly easily grown in synchronous cultures. Such cultures, in turn, provide excellent possibilities to study the changes that take place in the energy-pool of the cell.

The aim of the present experiments was to examine, whether such ATPase activity, activated by (Mg^{++}–K^+–Na^+) and inhibited by ouabain, can be found in Scenedesmus obtusiusculus cells, and if so, what are its general properties.

Materials and Methods

The experiments were carried out with the unicellular alga Scenedesmus obtusiusculus CHOD obtained from the alga collection of the Research Institute for Biology, Hungarian Academy of Sciences. Samples were taken from synchronous and non-synchronous flask cultures, respectively, at various times during the light and dark period. Synchronization was achieved by the method of Meszes and Sipos (1968). Non-synchronous cultures were grown in the same media and the cells were also grown in a 13/11 hour light/dark period by bubbling air containing 5 per cent CO_2 through the culture, at 15 000 lux illumination.

For ATPase assay the cells were centrifuged, washed with distilled water and broken up with ethylacetate. After adding Tris buffer, pH 7.2, ATPase activity was determined from the suspension using the following systems:

1. ATP + Tris buffer
2. Alga extract + Tris buffer
3. Alga extract + Tris buffer + ATP
4. Alga extract + Tris buffer + ATP + Mg^{++}
5. Alga extract + Tris buffer + ATP + Mg^{++}–K^+–Na^+
6. Alga extract + Tris buffer + ATP + Mg^{++}–K^+–Na^+ + inhibitor

The final volume of the reaction mixture was 3 ml. ATP concentration was 3 mM throughout, while Mg^{++}, K^+, and Na^+ were used in a concentration range of 5–200 mM. As inhibitor 0.001 or 1 mM ouabain was used.

The reaction mixtures were incubated in a 37 °C water bath for 30 minutes in diffuse light. At the end of incubation the enzyme reaction was stopped by the addition of 3 ml of 10 per cent TCA. The precipitate was centrifuged and the amount of phosphate split by ATPase was determined from a fraction of the supernatant according to Fiske and Subbarow (1925). After subtracting the blank (ATP + buffer) from the absorbancy values, the enzymic activity was expressed in terms of mg phosphorus per 10^{10} cells. Calibration curves were made to each

experiment with KH_2PO_4. In each case two parallels were run and the figures and tables give the average values.

When the effect of light was studied, the incubation mixtures were illuminated for various times in a 37 °C water bath and incubation mixtures kept in the usual diffuse light served as control. The illumination was 42 000 lux. When the dark-effect was studied, the incubation chamber was put in the dark.

Results

As mentioned above, there is no direct evidence for the existence in microorganisms of such an ATPase, which, similarly to that in animal cells, can be activated by (Mg^{++}–K^{+}-Na^{+}) and inhibited by ouabain. In case of algae, the only attempt to demonstrate the classical transport ATPase, known from animal cells, was made with Ulva lactuca (Bonting, Caravaggio, 1966). In this instance, however, K^{+}–Na^{+} and ouabain had no activating and inhibitory effect, respectively.

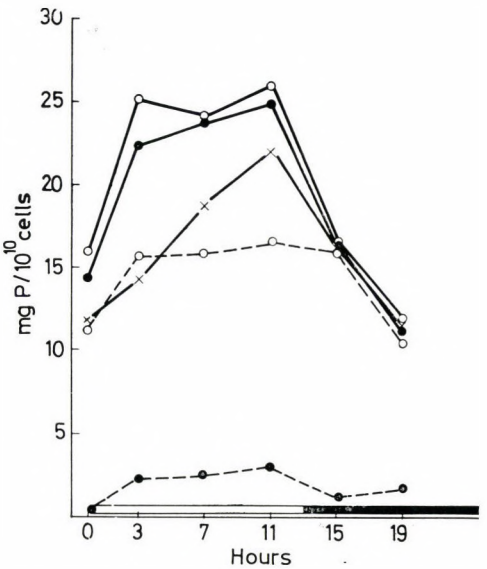


Fig. 1. Changes in ATPase activity during a synchronous cycle of Scenedesmus obtusiusculus. (The hatched part on the abscissa denotes the dark period of the cycle.) ●– – –● alga extract; ○- - -○ alga extract + ATP; ●——● alga extract + ATP + Mg^{++}; ○——○ alga extract + ATP + Mg^{++} + K^{+} + Na^{+}; × ——× alga extract + ATP + Mg^{++} + K^{+} + Na^{+} + ouabain

First we had to examine whether the species under investigation contained such ATPase activity at all. We could prove the existence of this enzyme with alga cells grown in simple flask cultures. The ATPase activity of 5-day-old Scenedesmus cultures varied between 8–50 mg P per 10^{10} cells. The activity was increased by 20 mM Mg^{++} and further enhanced by 10 mM K^{+} and 20 mM Na^{+} ions. In

Table 1

Inhibition by ouabain of the ATPase activity of Scenedesmus obtusiusculus during a synchronous cycle

Time of sampling	Inhibition %	
	0.001 mM ouabain	1 mM ouabain
Light period 0 hour	10.16	26.73
Light period 3rd hour	25.03	42.56
Light period 7th hour	15.12	22.16
Light period 11th hour	8.21	15.46
Dark period 3rd hour	0.98	3.96
Dark period 7th hour	0.81	3.52

agreement with literary data K^{+} and Na^{+} increased the activity only in the presence of Mg^{++}. The inhibitory effect of ouabain could also be demonstrated with this organism, but inhibition occurred only at an unusually high ouabain concentration: 1 mM ouabain caused a 25 per cent decrease in the activity of (Mg^{++}–K^{+}–Na^{+})-activated ATPase.

We studied the changes of this ATPase activity during a synchronous life cycle. For this purpose samples were taken from synchronous cell cultures at various times during the light and dark periods. Four samples were taken during the 13-hour light period, and two samples during the 11-hour dark period. The first sample was withdrawn immediately before starting the light period.

As it can be seen in Fig. 1, the mg P per 10^{10} cells values for (Mg^{++}–K^{+}–Na^{+})-activated ATPase change according to the same curve both in the light and dark periods. In the light period Mg^{++} markedly increased the activity as compared to the control shown in Fig. 1. The control contained only alga-extract and ATP. The activity was further enhanced by the addition of K^{+} and Na^{+} and was maintained at a maximal level from the 3rd to the 11th hour of the light

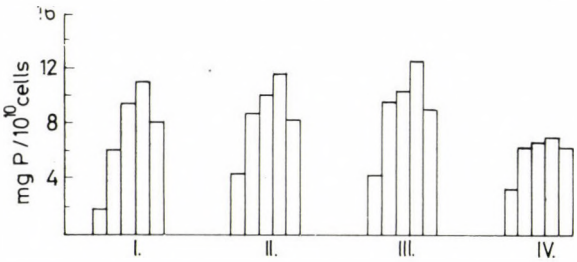


Fig. 2. Effect of light and darkness on the ATPase activity of non-synchronous Scenedesmus obtusiusculus cells. I: cells taken during the dark period. II: ATPase activity after half an hour of light period. III: ATPase activity after 8 hours of light period. IV: ATPase activity after half an hour of darkness following an 8-hour light period. Columns within clusters I–IV: Column 1: alga extract. Column 2: alga extract + ATP. Column 3: alga extract + ATP + Mg^{++}. Column 4: alga extract + ATP + Mg^{++} + K^{+} + Na^{+}. Column 5: alga extract + ATP + Mg^{++} + K^{+} + Na^{+} + 1 mM ouabain

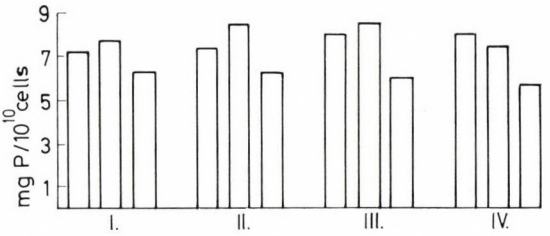


Fig. 3. Effect of the duration of illumination on the (Mg^{++}–K^{+}–Na^{+})-activated ATPase activity of Scenedesmus obtusiusculus. Cluster I: Column 1: activity after incubation for 30 minutes at 37 °C in diffuse light. Column 2: Activity after 5 minutes of illumination. Column 3: Activity after 5 minutes of illumination in the presence of 1 mM ouabain. Cluster II: Column 1: the same as Column 1 in Cluster I. Column 2: Activity after 10 minutes of illumination. Column 3: Activity after 10 minutes of illumination in the presence of 1 mM ouabain. Cluster III: the same as cluster I, but instead of 5 minutes, 30 minutes of illumination was applied. Cluster IV: Column 1: Activity after incubation for 30 minutes in diffuse light. Column 2: Activity after 30 minutes in the dark. Column 3: Activity after 30 minutes in the dark in the presence of ouabain

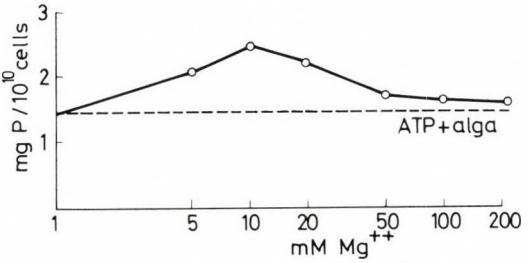


Fig. 4. Effect of Mg^{++} concentration on the ATPase activity of Scenedesmus obtusiusculus. ---- ATP + alga extract; o——o activity in the presence of various Mg^{++} concentration (Activity expressed in mg P per 10^{10} cells.)

period. On the other hand, inhibition by ouabain showed a different pattern and maximal inhibition was attained in the 3rd hour of the light period. In the dark period both activation and inhibition decreased considerably.

The percentage inhibition by ouabain is shown in Table 1. In the 3rd hour of the light period the inhibition was 42.56 per cent with 1 mM ouabain. Less pronounced inhibition was observed in the dark period with both concentrations applied, but it remained nearly constant between the 3–7 hours of the dark period.

To ascertain that light has an activating effect, the ATPase activities of non-synchronous alga cultures were determined with cells taken during the dark period, 0.5 and 8 hours after illumination, and with cells kept in the dark for 0.5 hours following an 8-hour light period (Fig. 2).

Fig. 2 shows that in addition to the activating effect of the ions light also increased ATPase activity in non-synchronous alga cultures. As to the activating effect there is no appreciable difference between a 0.5 or several-hour-long illumination. If the (Mg^{++}–K^{+}–Na^{+})-activated ATPase activity of cells taken during

the dark period is regarded as control, then 0.5 hours of illumination caused a 6.5 per cent whereas 8 hours of illumination a 10 per cent increase in activity. However, if kept in the dark for 0.5 hours the activating effect of ions as well as the inhibitory effect of ouabain decreased in the same manner as in the dark period of synchronous cultures. Quantitatively, when kept in the dark for half an hour a 42 per cent decrease in activity occurred as compared to the value obtained after 8 hours of illumination. Compared to the same control, 1 mM ouabain caused a 30 per cent inhibition of the ATPase activity during the light period. On the other hand, if the inhibitory effect of ouabain is compared to the ATPase values obtained in the dark period, then the inhibition amounted only to 11 per cent. Thus the extent of inhibition decreased as compared to synchronous cell cultures.

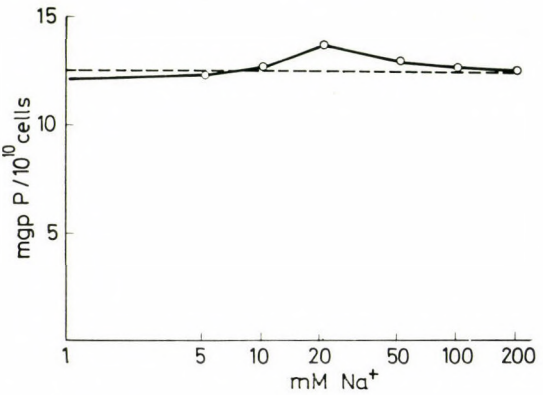


Fig. 5. Effect of Na^{+} concentration on the ATPase activity of Scenedesmus obtusiusculus. ------ activity in the presence of ATP + 10 mM Mg^{++} + 10 mM K^{+} + alga extract without (Na^{+}). o———o activity in the presence of various Na^{+} concentrations

To decide whether light had any stimulating effect on the activity of the enzyme, the cell suspension was illuminated for various times (5, 10 and 30 minutes); during incubation mixtures kept in diffuse light for 30 minutes served as control. The results are shown in Fig. 3.

As seen in Fig. 3, light stimulated the (Mg^{++}–K^{+}–Na^{+})-activated ATPase, but the activity did not disappear in the dark as described in the case of Ca^{++}-activated ATPase (Bennun, Avron, 1964). Five minutes of illumination resulted in a 5 per cent, and 10 minutes of illumination in a 14 per cent increase in activity, compared to the control that was kept in diffuse light. By illuminating for 30 minutes only a 6 per cent increase could be achieved. Complete darkness elicited only partial inhibition (7 per cent). In the presence of 1 mM ouabain the activity of the enzyme fell to the same level in all mixtures.

Since the concentrations of Mg^{++}, K^{+}, and Na^{+} used by us appeared to be somewhat high as compared to those reported in the literature, we studied the effect of these ions on ATPase activity in a wide concentration range. Thus incubation mixtures were prepared with 5, 10, 20, 50, 100 and 200 mM concentrations of the three ions. Fig. 4 shows the effect of various Mg^{++} concentrations.

The highest activity was obtained with 10 mM Mg^{++}, where the increase in activity amounted to 72 per cent. Higher concentrations (50–200 mM) were much less effective (11–20 per cent activation).

Figs 5 and 6 show the effect of Na^{+} and K^{+} concentrations, respectively. Mixtures containing 10 mM Mg^{++} served as control. Maximal activating effect (22 per cent) was observed with 20 mM Na^{+} and 10 mM K^{+}, i.e. the concentra-

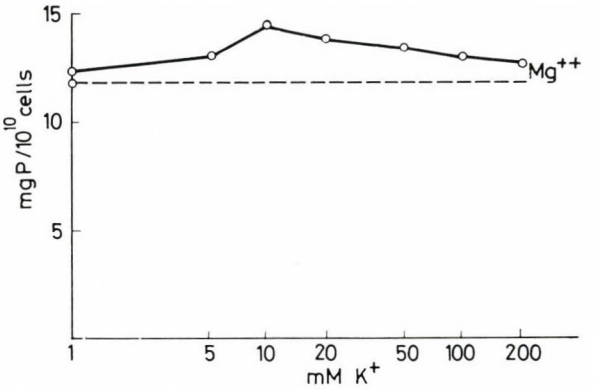


Fig. 6. Effect of K^{+} concentration on the ATPase activity of Scenedesmus obtusiusculus. ---- activity in the presence of ATP + 10 mM Mg^{++} + 20 mM Na^{+} + alga extract (without K^{+}). o——o activity in the presence of various K^{+} concentrations

tions used in the first series of our experiments. It should be noted that, in contrast to Mg^{++} and Na^{+} ions, K^{+} exhibited considerable activating effect at higher concentrations. Thus 20 mM K^{+} caused 14 per cent, 50 mM K^{+} 17 per cent activation.

Discussion

There are widely different opinions in the literature concerning the origin of energy needed for the maintenance of transport processes. Authors who relate active transport to the utilization of ATP, suggest the existence of transport ATP-ase(s) (Dunham, Glynn, 1961; Post et al., 1960; Bonting et al., 1961; Jeschke, Simonis, 1967). In the case of bacteria we may say that, in general, the activator of ATPase is Mg^{++}. Ca^{++} has no effect, whereas monovalent cations may enhance the effect of Mg^{++}. The inhibitory effect of ouabain could not be demonstrated. In the chloroplasts of Euglena gracilis Mg^{++}- and Ca^{++}-activated ATP-ases were found (Carell, Kahn, 1967), which could be separated on the basis of pH optima and their localization in the structure. Among higher plants a soluble ATPase was prepared from bean roots (Gruener, Neumann, 1966). This enzyme was activated by Mg^{++} in the presence of K^{+} and Na^{+}. Ouabain had no inhibitory effect.

In our present experiments we have demonstrated the existence of (Mg^{++}–K^{+}–Na^{+})-activated ATPase in Scenedesmus obtusiusculus cells. Ouabain,

known to be a specific inhibitor of active transport without affecting respiration or glycolysis, inhibited this activity.

In synchronous Scenedesmus obtusiusculus cells ATPase activity changed during the life cycle. In the light period Mg^{++} had a strong activating effect and Na^{+} and K^{+} increased the activation. Ouabain had an inhibitory effect. In the dark period both activation by the ions and inhibition by ouabain were less pronounced.

Similar results were obtained with non-synchronous alga cultures. In these experiments samples were taken four times at the beginning and towards the end of the light period. Light increased the ATPase activity but the effect of ions and the inhibition by ouabain were significantly reduced after half an hour in the dark following a light period.

It has been known for some time that there are two enzymes that split ATP in the chloroplasts of plants (Bennun, Avron, 1964). One of them requires Ca^{++} and light for its action, whereas the other is active in the dark provided that a short illumination preceded the darkness. Carmeli and Avron (1967) studied the so-called "light-triggered" ATP-phosphate exchange reactions in chloroplasts. An ATPase activated by Ca^{++} and Mg^{++} could also be demonstrated in Euglena gracilis cells (Chang, Kahn, 1966).

In our experiments light enhanced the activity of (Mg^{++}–K^{+}–Na^{+})-activated ATPase in Scenedesmus obtusiusculus. The enzyme activity, in terms of mg P per 10^{10} cells, reached a maximum on 10 minutes of illumination and did not change appreciably when illumination was extended to 30 minutes. These results are in agreement with our so far unpublished data concerning the uptake of Na^{+} and K^{+}, where light had a similar effect on the ^{42}K-uptake of Scenedesmus obtusiusculus cells. That the inhibition of ATPase activity by ouabain was of about the same extent throughout (Fig. 3, the 3rd column in each cluster), cannot yet be precisely explained. We may suggest the following explanation. As it is known, ATPase activity, activated by Mg^{++}, K^{+}, and Na^{+} and inhibited by ouabain, is in close connection with the active K^{+} and Na^{+} transport through the membrane in other organisms (Dunham, Glynn, 1961; Post et al., 1960; Bonting et al., 1961; Hafkenscheid, Bonting, 1968). If a part of the ATPase activity in Scenedesmus obtusiusculus is located in the membrane and ouabain inhibits it to the same extent irrespective of illumination conditions, we might say that as a result of increased ATPase activity in the membrane, due in turn to the light, the concentrations of K^{+} in the cell should increase and that of Na^{+} decrease. However, for a conclusion to be drawn we have to examine the ATPase activity and ATP-pool in the various cell compartments as well as their role in ion transport.

Finally, it is to be noted that concentrations of the three ions, Mg^{++}, K^{+}, and Na^{+}, which have the maximal activating effect, differ from those observed with other organisms. In Scenedesmus obtusiusculus ATPase activity showed a maximum with 10 mM Mg^{++} and K^{+}, and 20 mM Na^{+} concentration.

References

Atkinson, M. R., Eckermann, G., Grant, M., Robertson, R. N. (1966) Proc. Natl. Acad. Sci. *55* 560
Barber, J. (1968) Nature *217* 877
Bennun, A., Avron, M. (1964) Biochim. Biophys. Acta *79* 640
Blount, R. W., Levedahl, B. H., (1960) Acta Physiol. Scand. *49* 1
Bonting, S. L., Caravaggio, L. L. (1966) Biochim. Biophys. Acta *112* 519
Bonting, S. L., Simon, K. A., Hawkins, N. M. (1961) Arch. Biochem. Biophys. *95* 416
Carell, E. F., Kahn, J. S. (1967) Biochim. Biophys. Acta *131* 571
Carmeli, C., Avron, M. (1967) Europ. J. Biochem. *2* 318
Chang, I. C., Kahn, J. S. (1966) Arch. Biochem. Biophys. *117* 282
Dainty, J. (1962) Ann. Rev. Plant Physiol. *13* 379
Dunham, E. T., Glynn, I. M. (1961) J. Physiol. *156* 247
Fiske, C. H., Subbarow, Y. (1925) J. Biol. Chem. *66* 375
Fujita, M. (1962) Plant Cell Physiol. *3* 229
Gruener, N., Neumann, J. (1966) Physiol. Plant. *19* 678
Hafkenscheid, J. C. M., Bonting, S. L. (1968) Biochim. Biophys. Acta *151* 204
Jeschke, W. D., Simonis, W. (1967) Z. Naturforsch. *226* 873
McRobbie, E. A. C. (1962) J. Gen. Physiol. *45* 861
McRobbie, E. A. C. (1964) J. Gen. Physiol. *47* 859
McRobbie, E. A. C. (1965) Biochim. Biophys. Acta *94* 64
McRobbie, E. A. C. (1966) Aust. J. Biol. Sci. *19* 363
Meszes, G., Kralovánszky, J., Cseh, E., Böszörményi, Z. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 239
Meszes, G., Sipos, D. (1968) Bot. Közl. *55* 93
Post, R. L., Merritt, C. R., Kinsolving, C. R., Albright, C. D. (1960) J. Biol. Chem. *235* 1796
Raven, J. A. (1967a) J. Gen. Physiol. *50* 1607
Raven, J. A. (1967b) J. Gen. Physiol. *50* 1627
Raven, J. A. (1968) J. Exp. Botany *19* 233
Skou, J. C. (1960) Biochim. Biophys. Acta *42* 6
Skou, J. C. (1964) Progress Biophys. *14* 131
Weigl, J. (1964) Z. Naturforsch. *19b* 845

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (2), pp. 141—149 (1969)

Enzymic, ATP-dependent Cleavage of β-Methyl-δ-keto-$\Delta^{\alpha\beta}$-hexenoic Acid

I. β-Methyl-δ-keto-$\Delta^{\alpha\beta}$-hexenoic Acid as Acetyl Donor

I. ALKONYI, A. SÁNDOR, D. SZABÓ, F. TÁRNOK

Institute of Biochemistry, University Medical School, Pécs

(Received November 18, 1968)

1) β-methyl-δ-keto-$\Delta^{\alpha\beta}$-hexenoic acid is able to acetylate sulfanylamide in the presence of pigeon liver extract, ATP*, and CoA.

2) Acetylation is brought about *via* acetyl-CoA, by splitting off the terminal acetyl group of the ketoacid.

3) The lactone of the ketoacid as well as its decarboxylated derivative, mesityl oxide, show similar behaviour. Examinations of several compounds of related structure indicate that we are dealing with a specific enzyme reaction rather than with a general cleavage of ketones. The fraction between 0.4 and 0.7 ammonium sulphate saturation exhibited higher activity than the crude extract.

Several enzymological studies have provided evidence for the biotin-dependent carboxylation of β-methyl-crotonyl-CoA (Tustanoff, Stern, 1966; Rilling, Coon, 1960), which takes place exclusively on the terminal methyl group. It is probably the tautomerism of the unsaturated acid that causes the shift of the nucleophylic center from the α to the γ-carbon atom (Lane et al., 1960). Besides carboxylation, we do not know of any other biochemical reaction which involves electrophylic substitution on the γ-carbon atom of β-methyl-crotonic acid.

Organic chemical experience shows, however, that ordinary esters of β-methyl-crotonic acid can be readily acylated on the γ-carbon atom by various cationoid electrophylic agents (Alkonyi, 1965; Lohaus et al., 1967). To elucidate the possible biochemical relation of this reaction, the first question to be answered was: can the acetylated derivative of β-methyl-crotonic acid serve as acetyl-donor in enzymic reactions?

In the present paper we attempt to answer this question.

Materials and Methods

Chemicals

The following chemicals were used: sulfanylamide, adenosine-5'-triphosphate, sodium acetate, L-cysteine-HCl, ethanol, ammonium sulfamate, N-(1-naphthyl)-ethylene-diamine (REANAL, Budapest); coenzyme A, 95 per cent pure (BDH);

* *Abbreviations:* ATP = adenosine-5'-triphosphate; CoA = coenzyme A; ketoacid = β-methyl-δ-keto-$\Delta^{\alpha\beta}$-hexenoic acid; lactone = 4,6-dimethyl-α-pyrone (lactone of ketoacid); TCA = trichloroacetic acid.

glutathione (Serva); β-methyl-crotonic acid (Theodor Schuchardt, Munich). The inorganic chemicals and the rest of the organic chemicals were commercial preparations of reagent grade.

Synthetic preparations

β-methyl-δ-keto-$\Delta^{\alpha\beta}$-hexenoic acid-ethylester and lactone were prepared as described earlier (Alkonyi, 1965).

Ketoacid was prepared as follows: 124.1 mg of crystalline lactone and 40 mg of sodium hydroxide were dissolved in 6 ml of water and allowed to stand at room temperature for 50 hours. The complete cleavage of the lactone was controlled by the optical test: the lactone has an absorption maximum at 294 mμ, whereas the sodium salt of ketoacid at 244 mμ; the opening of the lactone ring can thus be followed by measuring the absorbancy at 294 mμ. The pH of the solution thus obtained was between 7 and 8, and this solution was used in experiments where ketoacid served as substrate.

δ-keto-geranic acid was prepared as described in a previous paper (Alkonyi, Sándor, 1969).

Pigeon liver extract

The crude extract designated in the Tables by E was prepared from the acetone powder of pigeon liver according to the procedure of Kaplan and Lipmann (1948), with the omission of the ageing process. The protein concentration of this solution was 30.7 mg/ml. Fraction E 40–70 was prepared from the crude extract as follows:

32 ml of extract E was centrifuged at 22 000 *g* (20 000 r.p.m.) for 25 minutes at 0–5 °C. The supernatant was then fractionated with ammonium sulphate and the fraction between 40 and 70 per cent saturation was dissolved in 10 ml of 0.02 M $NaHCO_3$ at 0 °C. The solution was dialyzed against 0.02 M $NaHCO_3$ at 2 °C for 1 hour with continuous stirring. The final volume was 15.2 ml, the protein concentration was 32.7 mg/ml.

The protein content of fractions E and E 40–70 was determined by the biuret reaction (Ditterbrandt, 1948).

Sulfanylamide-test

The composition of incubation mixtures is given in the Tables. The final volume was 1.1 ml unless otherwise stated. The incubations were made at 37 °C for 2 hours, then 4 ml of 5 per cent TCA was added to the mixtures. After filtration 2 ml of the filtrate was diluted to 10 ml with 15 per cent TCA solution. Sulfanylamide was determined in this solution according to the method of Bratton and Marshall (1939).

Paper chromatography

For paper chromatography 6 cm wide strips of Schleicher–Schüll 2043 b paper were used. Chromatography was carried out by the ascending technique

with the following solvent system:

n-butanol	50 per cent v/v
96 per cent acetic acid	10 per cent v/v
H_2O	40 per cent v/v

The duration of a run varied from 16 to 24 hours depending on the temperature. The spots were located by the aid of a UVIFOT UG-5 lamp and filter (253.7 mμ). As a rule, 100 μl of protein-free incubation mixture was applied to the start line.

Results and Discussion

On the bottom of Table 1 are given the absorbancy values that are proportional to the amount of sulfanylamide. It can be seen that tubes which do not contain substrate (tubes 2 and 4) also show a lower absorbancy than the control (tubes 5 and 6), since both the crude and purified enzymes contain substances in a certain amount which can acetylate sulfanylamide to a small extent. Therefore we express the acylating ability of the examined ketoacid in absorbancy units, using the sulfanylamide content of the incubated but substrate-free mixture as reference. As shown in Table 1 the purified enzyme (E 40–70) exhibits higher activity than the crude one.

Table 1

Acylating ability of ketoacid measured with sulfanylamide

To tubes 5 and 6 the enzyme was added after incubation and was immediately precipitated with TCA. For experimental details see Methods

Components	Tubes					
	1	2	3	4	5	6
Sulfanylamide μmoles	0.4	0.4	0.4	0.4	0.4	0.4
Ketoacid μmoles	20.0	—	20.0	—	20.0	20.0
ATP μmoles	3.9	3.9	3.9	3.9	3.9	3.9
CoA nanomoles	39.6	39.6	39.6	39.6	39.6	39.6
Glutathione μmoles	9.0	9.0	9.0	9.0	9.0	9.0
$NaHCO_3$ μmoles	80.0	80.0	80.0	80.0	80.0	80.0
$MgCl_2$ μmoles	3.9	3.9	3.9	3.9	3.9	3.9
E ml	0.3	0.3	—	—	0.3	—
E 40-70 ml	—	—	0.3	0.3	—	0.3
Absorbancy (545 mμ)	0.328	0.515	0.210	0.510	0.570	0.570
Acylating ability	0.187		0.300			

We have also examined what coenzymes are needed for this reaction. As demonstrated in Table 2, acylation by the ketoacid takes place only in the presence of both CoA and ATP. If ATP and CoA are omitted (tube 4), the sulfanylamide content of the mixture agrees with that of tube 5, i.e. a mixture incubated without ketoacid. If only ATP is omitted, but CoA is added (tube 3), again there is practically no acylation. Very slight acylation occurs if only CoA is omitted but ATP added (tube 2). This effect can be explained by the CoA content of the enzyme

preparation. At any rate, the significantly lower sulfanylamide content of tube 1 proves unequivocally that CoA participates in the mechanism of acylation.

Since evidence has been provided that acylation occurs and its coenzyme requirement is known, the next question to be answered is, what acyl-group takes part in the acylation of sulfanylamide. Whether the carboxylic group of the keto-acid acylates the sulfanylamide, or acetylation involves only the terminal acetyl-group, as we assumed in our working hypothesis. We sought the answer to this question by paper chromatography.

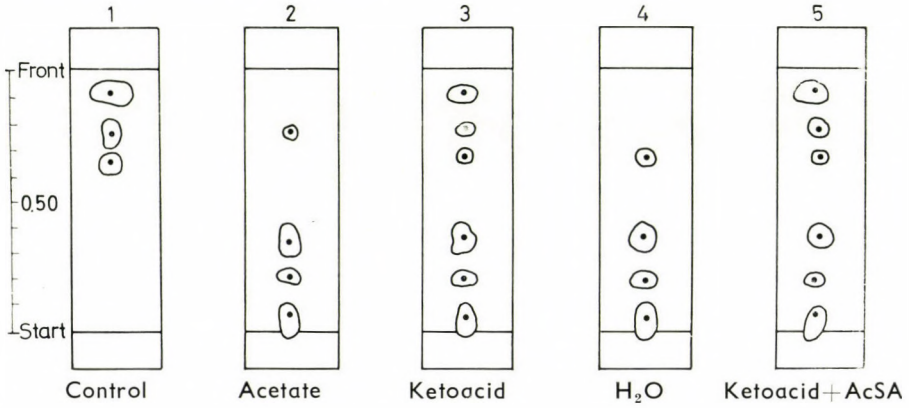


Fig. 1. Detection of acetylsulfanylamide. The following samples were applied to the start line of the chromatograms: Strip 1: 0.1 μmole of sulfanylamide + 0.1 μmole of acetylsulfanylamide + 4.1 μmoles of ketoacid, dissolved in 75 μl of water. Strip 2: The composition of the incubation mixture corresponded to tube 3 in Table 1, but instead of 20 μmoles of ketoacid 20 μmoles of Na-citrate + 24 μmoles of Na-acetate were used. After incubation 0.5 ml of 15 per cent TCA was added to the mixture and after filtration 100 μl of the filtrate was applied to the paper. Strip 3: The mixture corresponded to tube 1 in Table 1 and was treated as described with Strip 2. Strip 4: The mixture corresponded to tube 4 in Table 1 and was treated as described with Strip 2. Strip 5: The same mixture as in Strip 3 + 0.06 μmoles of acetylsulfanylamide dissolved in 15 μl of water

Fig. 1 shows the chromatographic patterns schematically. Chromatogram No. 1 was obtained by resolving an aqueous mixture of sulfanylamide, acetyl-sulfanylamide, and ketoacid. The Rf-values of the components were determined separately and found to be the following: sulfanylamide (Rf = 0.63), acetyl-sulfanylamide (Rf = 0.75), ketoacid (Rf = 0.88).

Chromatogram No. 2 shows the resolution of a protein-free solution obtained from a mixture that was incubated with acetic acid instead of ketoacid. The spots corresponding to ketoacid and sulfanylamide are missing here, as there was a complete acetylation in the mixture. The three lower spots with Rf-values 0.04, 0.20, and 0.38, respectively, were present in all mixtures incubated with the native enzyme. Since they were not identical with any of the substances examined, we disregarded them in the following.

Chromatogram No. 3 shows the resolution of a protein-free solution obtained from a mixture incubated with ketoacid (tube 1 in Table 1). The spot on the top

corresponds to ketoacid applied in excess, the lower ones are acetyl-sulfanylamide and a small amount of unchanged sulfanylamide. Chromatogram No. 4 shows the components of a mixture incubated without substrate: there is no acetyl-sulfanylamide, only sulfanylamide. Though according to Table 1 a slight acylation takes place even in the absence of substrate, this small quantity cannot be detected on the chromatogram.

Chromatogram No. 5, like No. 3, shows the analysis of a mixture incubated with ketoacid, but in this case some acetyl-sulfanylamide was added to the protein-free solution and consequently that corresponding to Rf = 0.75 became more pronounced. These spots (Rf = 0.75) were then cut out from chromatograms No. 3 and 5, eluted with water, and the ultraviolet absorption spectrum of the eluate was taken. In this way we could also identify acetyl-sulfanylamide and the differences between chromatograms No. 3 and 5 could be quantitatively determined on the basis of absorbancy values. Finally, the two chromatograms were submitted

Table 2

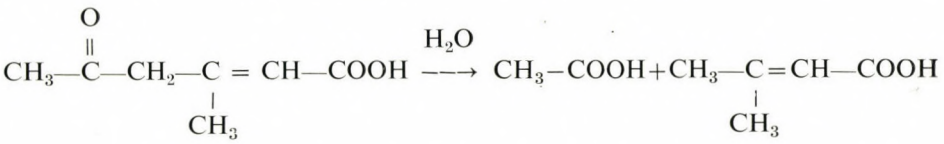
Coenzyme requirement of acylation by ketoacid

All tubes were incubated with native enzyme. The determination of sulfanylamide was carried out as described in Methods

Components	Tubes				
	1	2	3	4	5
Sulfanylamide μmoles	0.4	0.4	0.4	0.4	0.4
Ketoacid μmoles	20.0	20.0	20.0	20.0	—
ATP μmoles	3.9	3.9	—	—	3.9
CoA nanomoles	39.6	—	39.6	—	—
Glutathione μmoles	9.0	9.0	9.0	9.0	9.0
$NaHCO_3$ μmoles	80.0	80.0	80.0	80.0	80.0
$MgCl_2$ μmoles	3.9	3.9	3.9	3.9	3.9
E 40 – 70 ml	0.3	0.3	0.3	0.3	0.3
Absorbancy (545 mμ)	0.210	0.420	0.460	0.470	0.470

to ketone-staining and developed with salicylaldehyde (Straub, 1936). In both cases oly the uppermost band was stained. This showed that the uppermost band corresponded to the ketoacid. Sulfanylamide acylated with the whole ketoacid was not encountered in the system.

Paper chromatography has thus shown that the ketoacid acetylates through splitting off its acetyl-group and the carboxylic group of the ketoacid is not involved in the reaction. In the further investigation of the mechanism of acetylation we had to exclude a not very probable but still feasible mechanism. It might be conceived that the ketoacid is decomposed by a hydrolytic enzyme to yield acetic acid and β-methylcrotonic acid:



The acetic acid formed could be coupled with CoA by a thiokinase reaction and the acetyl-CoA would acetylate sulfanylamide. To test this possibility, ketoacid was incubated with enzyme without coenzymes and with enzyme in the presence of ATP, respectively. The study of hydrolytic cleavage was made possible by the fortunate circumstance that the ketoacid, unlike most acids, forms spontaneously hydroxamic acid with hydroxylamine and this, similarly to acetyl-hydroxamic acid (Lipmann, Tuttle, 1945), can be determined photometrically. Acetic acid and β-methyl-crotonic acid, the products of the hypothetical hydrolysis, do not form hydroxamic acid spontaneously and therefore the method is suitable for the detection of the cleavage.

As shown in Table 3, in tube 2 the enzyme was incubated with acetic acid and hydroxylamine. This served only as a control of the activity of the enzyme. The absorbancy values in tubes 1, 3, and 4 indicate that the ketoacid, when incubated with native enzyme either in the presence (tube 3) or absence (tube 4) of ATP, does not undergo any kind of cleavage. In our opinion the decomposition of ketoacid takes place according to the following scheme:

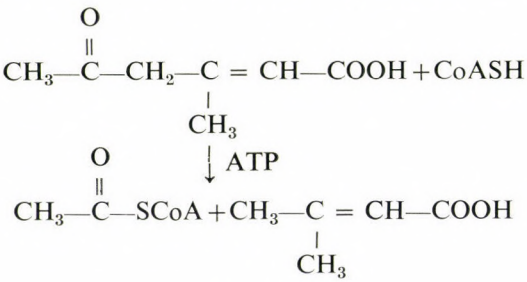


Table 3

Hydrolytic cleavage of ketoacid

To tube 2 hydroxylamine-HCl (200 μmoles dissolved in 1 ml of water) was added before incubation. All tubes were incubated for 2 hours at 37 °C, then 1 ml of 15 per cent TCA solution was added. After filtration 1 ml of hydroxylamine-HCl was added also to tubes 1, 3, and 4, and then all tubes were incubated for 1 hour at 37 °C. At the end of incubation 1 ml of reagent (0.5 ml of 3 N HCl + 0.5 ml of 5 per cent $FeCl_3$) was added to all tubes and the absorbancies were read at 540 mμ. To tube 1 the enzyme was added after incubation and immediately precipitated

Components		Tubes			
		1	2	3	4
Hydroxylamine-HCl	μmoles	—	200.0	—	—
Na-acetate	μmoles	—	24.6	—	—
Na-citrate	μmoles	—	19.7	—	—
Ketoacid	μmoles	20.0	—	20.0	20.0
ATP	μmoles	3.9	3.9	3.9	—
CoA	nanomoles	19.8	19.8	—	—
Glutathione	μmoles	9.0	9.0	9.0	9.0
$NaHCO_3$	μmoles	80.0	80.0	80.0	80.0
$MgCl_2$	μmoles	3.9	3.9	3.9	3.9
E 40—70	ml	0.3	0.3	0.3	0,3
Absorbancy	(540 mμ)	0.143	0.270	0.145	0.143

It is a striking observation that lactone, similarly to ketoacid, can also acetylate sulfanylamide in the presence of ATP and CoA. It is of special interest from the point of view of the mechanism that this cleavage is not preceded by the hydrolytic splitting of the lactone, that is the formation of ketoacid. Apparently, the opening of the lactone ring and the splitting off of the acetyl-group are simultaneous processes.

Table 4 shows the acetylating ability of lactone compared to that of ketoacid. The lactone exhibits considerable acetylating ability, though less than the ketoacid. The data in Table 5 provide evidence that there is no hydrolytic activity in the pigeon liver enzyme which would result in the opening of the lactone ring, since there is no change in absorbancy at the absorption maximum of the lactone.

It remains to be clarified how specific the enzyme is that brings about this peculiar cleavage of ketoacid. Are we facing a general cleavage of ketones and

Table 4

Comparison of acylating abilities of ketoacid and lactone

To tube 4 the enzyme was added after incubation and immediately precipitated. For experimental details see Methods

Components		Tubes			
		1	2	3	4
Sulfanylamide	μmoles	0.4	0.4	0.4	0.4
Ketoacid	μmoles	—	20.0	—	—
Lactone	μmoles	20.0	—	—	20.0
ATP	μmoles	3.9	3.9	3.9	3.9
CoA	nanomoles	39.6	39.6	39.6	39.6
Glutathione	μmoles	9.0	9.0	9.0	9.0
$NaHCO_3$	μmoles	80.0	80.0	80.0	80.0
$MgCl_2$	μmoles	3.9	3.9	3.9	3.9
E 40—70	ml	0.3	0.3	0.3	0.3
Absorbancy	(545 mμ)	0.195	0.100	0.500	0.570

Table 5

Hydrolytic cleavage of lactone

Final volume of mixtures 3.48 ml. In tube 2 the enzyme was precipitated with 0.5 g of ammonium sulphate immediately after mixing. Tubes 1 and 2 were then incubated for 2 hours at 37 °C. After incubation 0.5 g of ammonium sulphate was also added to tube 1, both mixtures were filtered and 0.1 ml of the filtrate was diluted to 100 ml with water. The absorbancy of this solution was measured at 294 mμ in cells of 1 cm light path

Components		Tubes	
		1	2
Lactone	μmoles	20.0	20.0
$NaHCO_3$	μmoles	80.0	80.0
E 40—70	ml	0.3	0.3
Absorbancy	(294 mμ)	0.340	0.342

the observed phenomenon is only one special case of it, or are we dealing with a highly specific enzyme? To approach this question we examined the acylating abilities, with sulfanylamide, of the following ketones: acetone, acetonyl-acetone, acetyl-acetone, methyl-ethyl-ketone, acetophenone, methyl-propyl-ketone, and mesityl oxide. The first six ketones did not exhibit any detectable acylating ability, whereas mesityl oxide, the decarboxylated derivative of our ketoacid, displayed extremely vigorous acetylation.

It can be seen in Table 6 that mesityl oxide is an acetylating agent stronger even than ketoacid. This phenomenon is noteworthy from two aspects. On the one hand, it proves by itself the occurrence of acetylation, proved already by paper chromatography: mesityl oxide is not a carboxylic acid, it has no CoA-ester, thus the acetylation of sulfanylamide can be explained only by the cleavage of the acetyl-group. On the other hand, it is interesting that a relatively small change in the structure of ketoacid, e.g. formation of ethylester, leads to the complete abolition of acylation.

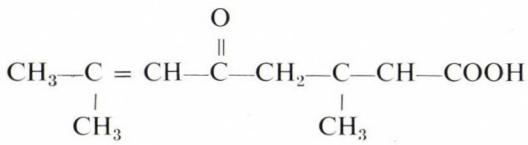
Table 6

Acylating ability of mesityl oxide

To tube 4 the enzyme was added after incubation and precipitated immediately. For experimental details see Methods

Components		Tubes			
		1	2	3	4
Sulfanylamide	μmoles	0.4	0.4	0.4	0.4
Mesityl oxide	μmoles	20.0	—	—	20.0
Ketoacid	μmoles	—	20.0	—	—
ATP	μmoles	3.9	3.9	3.9	3.9
CoA	nanomoles	39.6	39.6	39.6	39.6
Glutathione	μmoles	9.0	9.0	9.0	9.0
$NaHCO_3$	μmoles	80.0	80.0	80.0	80.0
$MgCl_2$	μmoles	3.9	3.9	3.9	3.9
E 40—70	ml	0.3	0.3	0.3	0.3
Absorbancy	(545 mμ)	0.150	0.328	0.515	0.570

We may add that δ-oxo-geranic acid



does not acylate under conditions given in Table 1.

Consequently, the observed phenomenon is not a general reaction of ketones and is not even a result of the structural properties of the terminally acylated derivatives of β-methyl-crotonic acid. Only three compounds can serve as substrates: ketoacid, its lactone, and mesityl oxide, the decarboxylation product of the latter. On this basis we may claim that we have found a rather specific enzyme.

There are numerous examples for an artificial substrate, not encountered in the metabolism of living cells, to react with an enzyme. This is common chiefly with hydrolases. The specific character of the described ketone-cleavage, however, prompts us to investigate the biochemical background of the phenomenon. This question will be dealt with in our next paper.

References

Alkonyi, I. (1965) Chem. Ber. *98* 3099
Alkonyi, I., Sándor, A. (1969) Acta Chim. Acad. Sci. Hung. *59*, 397
Bratton, A. C., Marshall, E. K. (1939) J. Biol. Chem. *128* 545
Ditterbrandt, M. (1948) Am. J. Clin. Path. *18* 439
Kaplan, N. D., Lipmann, F. (1948) J. Biol. Chem. *174* 37
Lane, M. D., Halenz, D. L., Kosow, D. P., Hegre, C. S. (1960) J. Biol. Chem. *235* 3082
Lipmann, F., Tuttle, C. (1945) J. Biol. Chem. *159* 21
Lohaus, G., Friedrich, W., Jeschke, J. P. (1967) Chem. Ber. *100* 658
Rilling, H. C., Coon, M. J. (1960) J. Biol. Chem. *235* 3087
Straub, F. B. (1936) Z. Physiol. Chem. *236* 42
Tustanoff, E. R., Stern, J. R. (1966) Canad. J. Biochem. *44* 861

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (2), pp. 151—159 (1969)

The Reaction of Sulfhydryl Groups of Dehydrogenases with Iodine

Susan MÓRA, Piroska HÜVÖS, Susan LIBOR, P. ELŐDI

Institute of Biochemistry, Hungarian Academy of Sciences, Budapest

(Received December 10, 1968)

1. Iodine reacts at neutral pH very rapidly and specifically with sulfhydryl groups of lactic dehydrogenase and D-glyceraldehyde-3-phosphate dehydrogenase. It appears to react as an oxidizing agent converting the sulfhydryl groups into disulfides. Substitution reaction does not occur as shown by the lack of binding of radioactive ^{131}I.

2. The reaction of eight sulfhydryl groups per mole protein causes complete inhibition of the enzyme activity of both dehydrogenases. The inhibition can be partially reversed by the addition of thiol compounds.

3. In D-glyceraldehyde-3-phosphate dehydrogenase only intra-chain disulfides are formed while in lactic dehydrogenase the formation of inter-chain disulfides was also detected. From these data inferences might be drawn concerning the steric distribution of sulfhydryl groups within the polypeptide chain.

In an earlier paper (Libor et al., 1965) the reaction of iodine with the tyrosyl sidechains of GAPD* and LDH at slightly alkaline pH was described. It was found that iodine reacted both with sulfhydryl and tyrosyl groups of these enzymes. At neutral pH, however, the reaction with tyrosine is very slow and an almost specific reaction with sulfhydryl groups can be achieved.

GAPD and LDH contain 16 and 20 sulfhydryl groups per 140 000 molecular weight, i.e. 4 and 5 sulfhydryls per subunit, respectively (Harris, 1964; Beney, Elődi, 1965). At neutral pH iodine acts on the dehydrogenases as an oxidizing agent and disulfide bonds are formed. In the present paper the effect of iodine oxidation of sulfhydryl groups on some molecular properties of dehydrogenases is presented. Since disulfide formation depends, among others, on certain steric requirements, the localization of sulfhydryl groups in these dehydrogenases is also discussed.

Experimental

Materials and Methods

Swine muscle GAPD and LDH were isolated and recrystallized as described earlier (Elődi, Szörényi, 1956; Jécsai, 1961). Protein solutions were dialyzed overnight against 0.1 M phosphate buffer, pH 6.5, at 0 °C. Protein concentration was determined spectrophotometrically in 0.1 M NaOH at 280 mμ, on the basis

* Abbreviations: GAPD, glyceraldehyde-3-phosphate dehydrogenase [D-glyceraldehyde 3-phosphate:NAD oxidoreductase (phosphorylating) (EC.1.2.1.12)]; LDH, lactic dehydrogenase [L-lactate:NAD oxidoreductase (EC.1.1.1.27)

of an extinction coefficient of $E_{1\,cm}^{0.1\%} = 1.00$ and 1.29 for GAPD and LDH, respectively. A molecular weight of 140 000 was accepted for both proteins.

NAD and NADH coenzymes were purchased from Reanal (Budapest) and solutions were always freshly prepared. Glyceraldehyde-3-phosphate was prepared from fructose diphosphate according to Szewczuk et al. (1961).

Iodine solution was prepared from resublimated iodine by dissolving it in 0.15 M KI. This stock solution was diluted with 0.15 M KI to the desired concentrations. The iodine content of the solution was checked by titration with arsenite. Guanidine hydrochloride was a product of Fluka. All other compounds were of reagent grade.

Iodine reaction was carried out in 0.1 M phosphate buffer, pH 6.5, at 0 °C. The concentration of KI in the protein solution was kept at 0.15 M.

$Na^{131}I$ was supplied by the Isotope Institute of the Hungarian Academy of Sciences. 0.1 M stock solutions were prepared by diluting with cold KI_3 solution up to about 6 to 10×10^4 cpm per μmole of I_2. Radioactivity was measured with a Friesecke-Hoeppfner (Germany) counter.

Sulfhydryl content was determined with 5,5′-bisdithio-2-nitro-benzoic acid (Ellman, 1959). The reaction was carried out in the presence of 7 M urea, which was found to be necessary for the reaction of all sulfhydryl groups.

Enzyme activity was measured spectrophotometrically as described earlier (Előd i, Szörényi, 1956; Jécsai, Előd i, 1963). Iodine treated samples were incubated for 5 to 10 minutes, then diluted to the desired concentration with phosphate buffer and enzyme activity was tested immediately after dilution.

Viscosity measurements were made in Ostwald viscometers with an outflow time of 100–120 sec for 2 ml of water. A dilution-type viscometer was also used with an outflow time of 200 sec for 3 ml of water. Measurements were made at 25 ± 0.05 °C, in a protein concentration range of 0.2 to 1 per cent.

Molecular weight of subunits was determined by viscometry in the presence of 6 M guanidine hydrochloride as introduced by Tanford et al. (1967) and was calculated from the equation

$$[\eta] = 0.716\ n^{0.66}$$

where $[\eta]$ is the intrinsic viscosity given in cc/g and n is the number of residues per polypeptide chain. The molecular weight of subunits is obtained by multiplying n with the mean residue weight. These values were taken as 109 and 112 for GAPD and LDH, respectively, calculated from the amino acid compositions (Harris, 1964; Beney, Előd i, 1965).

Ultracentrifugation experiments were carried out in a MOM G 120 ultracentrifuge equipped with schlieren optics. Absorption measurements were performed in a Spectromom 201 spectrophotometer.

Results and Discussion

The reaction of iodine with sulfhydryl groups may occur essentially in two ways. Substitution, i.e.

$$P{-}SH + I_2 = P{-}SI + HI \qquad (1$$

was demonstrated to occur in ovalbumin and β-lactoglobulin by Cunningham and Nuenke (1959, 1960) and Cunningham (1964), and in tobacco mosaic virus by Fraenkel-Conrat (1959).

The oxidation reaction:

$$2\ P-SH+I_2 = P-S-S-P+2\ HI \qquad (2)$$

involves oxidation of free sulfhydryl groups to a disulfid. The molar ratio between sulfhydryl groups and iodine is 1 : 1 in the substitution (1) and 2 : 1 in the oxidation (2) reaction.

Study on the binding of iodine to GAPD and LDH

To decide between the two alternatives delineated above, we examined first whether any radioactivity appeared in proteins after treatment with $^{131}I_2$. To samples containing 5×10^{-5} M protein a 5- to 15-fold molar excess of $K^{131}I_3$ (3 to 10×10^4 cpm per ml solution) was added in 0.1 M phosphate buffer, pH 6.5, at 0 °C and the samples were incubated for five minutes. The iodine treated samples were gel-filtered on 1.5×25 cm Sephadex G-50 columns and 1 ml fractions were

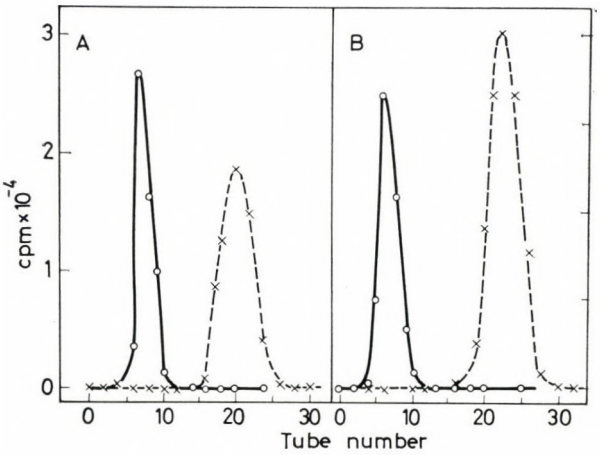


Fig. 1. Separation of protein and radioactive iodine on Sephadex G-50 column. Elution was carried out with 0.1 M phosphate buffer, pH 7. A — LDH; B — GAPD; solid lines — protein absorption at 280 mμ; dotted lines — radioactivity, counts per minute per fraction

collected. Both protein content and radioactivity of the fractions were measured. As it is seen in Fig. 1, protein and the radioactivity appeared in completely separate fractions. These experiments exclude the formation of a stable substitution compound of iodine with the dehydrogenases under the experimental conditions applied.

In agreement with the above results no indication of mono- or diiodotyrosine formation was found on the basis of absorption measurements at 305 and 312 mμ.

Oxidation of sulfhydryl groups with iodine

The lack of formation of a stable P–SI compound may suggest that iodine acts as an oxidizing agent on the sulfhydryl groups. The product of the reaction can be identified by determining the stoichiometry of the reacting components (sulfhydryl to iodine ratio) and by studying the reversibility of the reaction.

Solutions containing 5 to 7×10^{-6} M protein were reacted with iodine in 1- to 12-fold molar ratios in 0.1 M phosphate buffer, pH 6.5, at 0 °C for five minutes. The reaction was followed by measuring the decrease of absorbancy of KI_3 at 355 mμ (Cunningham, Nuenke, 1959) and after completion of the reaction the number of unreacted sulfhydryl groups was determined.

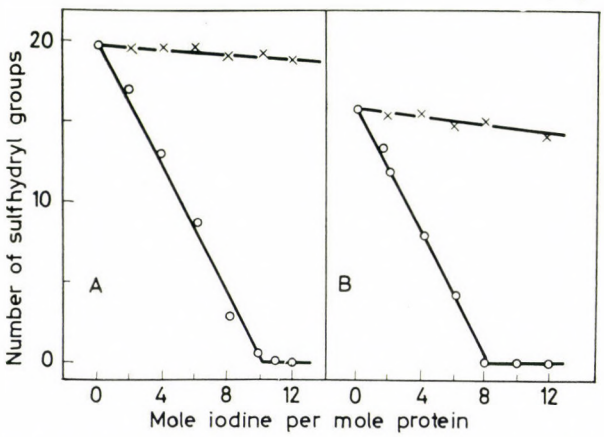


Fig. 2. Reaction of sulfhydryl groups of LDH (A) and GAPD (B) with iodine in 0.1 M phosphate buffer, pH 6.5, at 7×10^{-6} M protein concentration. o — change in the number of sulfhydryl groups upon iodine addition; × — number of sulfhydryl groups following incubation of the oxidized samples with 0.1 M β-mercaptoethanol in 6 M urea for two hours

Fig. 2 shows the relationship between the amount of iodine added and the number of sulfhydryls reacted. Upon addition of iodine a linear decrease in the number of sulfhydryl groups can be observed. All 16 sulfhydryl groups of GAPD and all 20 sulfhydryl groups of LDH disappeared when about 8 and 11 moles iodine per mole of protein were added, respectively. This corresponds to a sulfhydryl : iodine molar ratio of about 2 : 1 for both proteins. This ratio fits well Equ. (2), i.e. the conversion of sulfhydryls to disulfide.

The oxidation of sulfhydryl groups can be reversed by the addition of β-mercaptoethanol. To samples reacted with 1 to 12 moles of iodine β-mercaptoethanol was added in 0.1 M final concentration in 6 M urea. After two hours of incubation at 0 °C excess mercaptoethanol was removed by gel-filtration on a Sephadex G-75 column equilibrated with 6 M urea. As it can be seen in Fig. 2, more than 90 per cent of the original sulfhydryl content was recovered in this way. Thus, both the stoichiometry of the reaction and the almost complete recovery of sulfhydryl groups by reduction support the idea that iodine converts sulfhydryl groups into disulfides.

Effect of iodine treatment on enzyme activity

Both dehydrogenases contain 4 sulfhydryl groups per mole directly involved in their catalytic action. The blocking of the essential sulfhydryl groups completely inactivates the enzymes (Racker, Krimsky, 1952; Holbrook, Pfleiderer, 1965). Dehydrogenases were treated with iodine in different molar ratios as indicated in Fig. 3. Enzyme activity of the iodine treated samples was tested within ten minutes after the addition of iodine to avoid the disulfide-sulfhydryl interchange reaction which may occur when both types of side chains are simultaneously present in the solution (Boross, 1969).

As it can be seen in Fig. 3, iodine inactivates these enzymes and complete inhibition can be observed when 8 sulfhydryl groups per mole, presumably 2 per

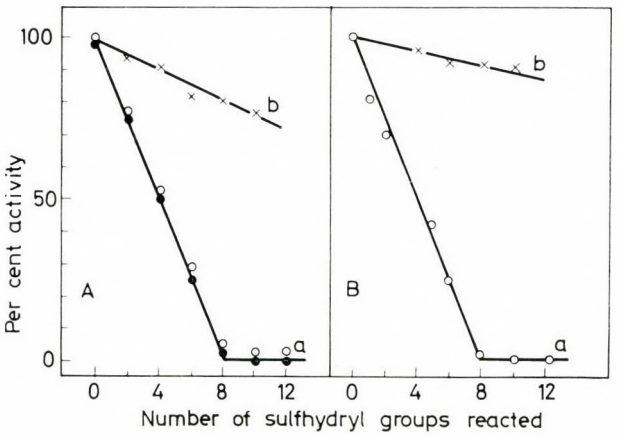


Fig. 3. Rate of inactivation of LDH (A) and GAPD (B) as a function of the number of sulfhydryl groups oxidized with iodine. A — Decrease of enzyme activity of LDH (curve a); ○ — with lactate and ● — with pyruvate. B — decrease of GAPD activity (○). Curve b — reactivation of oxidized enzymes by incubation with 0.1 M β-mercaptoethanol for two hours

subunit, reacted in GAPD or LDH. A related phenomenon was described by Harris (1964; Harris, Perham, 1968) with GAPD upon oxidation of two sulfhydryl groups per subunit with o-iodosobenzoate.

The inhibition of both dehydrogenases by iodine can be considered as a specific reaction if it is due to the formation of a disulfide bond between the essential sulfhydryl group and another one in its neighborhood. This might be the reason why the oxidation of only two sulfhydryls per subunit is needed to cause complete inhibition.

The inhibitory effect of iodine is almost completely reversible. When GAPD and LDH were first reacted with iodine in different ratios for five minutes, and then they were incubated with β-mercaptoethanol for 2 to 3 hours, a significant amount of the activity was recovered (Fig. 3, curve b). These experiments indicate that the oxidation by iodine does not cause irreversible structural alterations in

these enzymes and the inhibition observed after oxidation by iodine is due to local changes, i.e. only disappearance of free, essential sulfhydryl groups.

The oxidation of sulfhydryl groups in proteins can result in the formation of intermolecular, inter-subunit or intra-chain disulfide bonds, which may be reflected in the size of the protein. The formation of *intermolecular* disulfide bridges increases the preparative molecular weight. *Inter-chain* (i.e. *inter-subunit*) disulfides may partially or completely prevent the dissociation of subunits in concentrated guanidine hydrochloride. Finally, *intra-chain* disulfide bonds do not affect the molecular weight or the dissociation into subunits. We attempted to decide which of the three possibilities applies to the dehydrogenases under study.

The sedimentation constant of dehydrogenases did not change upon treatment with iodine, even when all sulfhydryls were oxidized (Table 1). The original 140 000 molecular weight was preserved, i.e. no association occurred. The formation of intermolecular disulfides can thus be excluded.

Table 1

Sedimentation constants of dehydrogenases in 0.1 M phosphate buffer, pH 7, at 5 mg per ml protein concentration

	S_{20}	
	control	oxidized enzyme*
GAPD	7.58	7.70
LDH	7.10	7.03

* All sulfhydryl groups of the enzyme were oxidized with iodine.

In addition, we compared native and oxidized GAPDs and LDHs by optical rotatory dispersion between 330 and 540 mμ, by the absorption spectra between 260 and 310 mμ, and by determining the intrinsic viscosity in 0.1 M phosphate buffer, pH 6.5. No significant differences were detected between the oxidized and untreated samples indicating that iodine does not markedly influence the gross steric structure of these enzymes.

Dissociation of oxidized GAPD

GAPD and LDH consist of four polypeptide chains which readily dissociate in the presence of denaturing agents, such as concentrated guanidine hydrochloride (Appella, Markert, 1961; Harrington, Karr, 1965). The dissociation of iodine treated dehydrogenases into subunits was studied to decide whether inter-subunit or intra-subunit disulfide formation occurred. The molecular weight of subunits was determined viscometrically (Fig. 4) in 6 M guanidine hydrochloride as suggested by Tanford et al. (1967).

It has to be mentioned that the determination of molecular weight by measuring intrinsic viscosity can be generally applied only for proteins of random coil conformation lacking secondary intra-chain covalent bonds, such as disulfides.

GAPD and LDH contain a relatively small number of sulfhydryl groups per subunit, i.e. four and five per 36 000 molecular weight, respectively. Therefore the possible number of disulfide bonds that can be formed upon iodine oxidation is negligibly low and this does not markedly influence the intrinsic viscosity as it was shown by Tanford et al. (1967). Thus the oxidized GAPD and LDH in 6 M guanidine hydrochloride can also be considered as a randomly coiled chain.

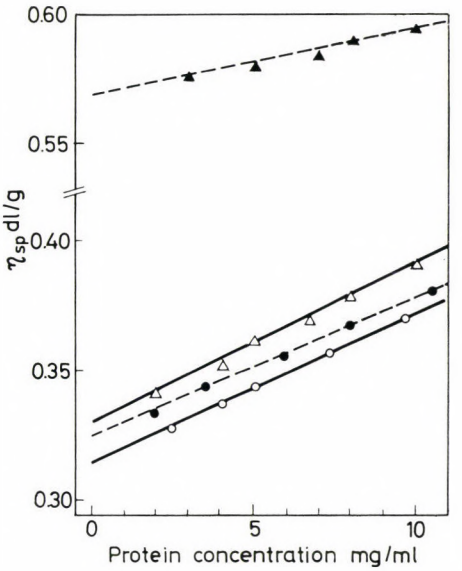


Fig. 4. Viscosity measurements in 6 M guanidine hydrochloride at 25 °C. ○ — GAPD, △ — LDH. Open symbols — control in the presence of 0.1 M mercaptoethanol; solid symbols — protein oxidized with iodine in 0.1 M phosphate buffer, pH 6.5

Table 2

Intrinsic viscosity and molecular weight of untreated and oxidized GAPD and LDH in 6 M guanidine hydrochloride

Experimental conditions	GAPD		LDH	
	[η] dl/g	Molecular weight	[η] dl/g	Molecular weight
Untreated control in the presence of 0.1 M β-mercaptoethanol	0.315±0.01 0.345*	33 700±1 600 36 300±1 500*	0.330±0.01 —	37 100±1 700 35 000±2 000**
Iodine treated				
All sulfhydryls oxidized	0.325±0.01	35 300±1 600	0.57 ±0.1	85 000±22 000
Two sulfhydryls oxidized per subunit	—	—	0.325±0.01	36 200±1 700

* from Harrington, Karr (1965)
** from Appella, Markert (1961)

When all sulfhydryl groups of GAPD were oxidized with iodine in 0.1 M phosphate buffer, pH 6.5, a molecular weight of 35 300±1 600 was found (Table 2). This value agrees fairly well with that for a subunit (Harrington, Karr, 1965). The complete dissociation of the oxidized GAPD indicates that oxidation of sulfhydryl groups resulted only in the formation of intra-chain disulfides.

It is known that two sulfhydryl groups of GAPD per subunit are separated in the amino acid sequence only by three residues (Harris et al., 1963) and they form a disulfide bond upon iodobenzoate oxidation (Harris, 1964). Our results indicate that the other pair of sulfhydryl groups is also situated in a steric position enabling the formation of a second intra-chain disulfide bond upon iodine treatment.

Dissociation of oxidized LDH

The molecular weight of LDH in 6 M guanidine hydrochloride was found to be 37 100±1 700 (Table 2). This value is close to that measured by Appella and Markert (1961).

The five sulfhydryl groups of LDH per subunit are not equal with respect to disulfide formation. Oxidation of two sulfhydryl groups per subunit (or 8 per mole) which causes complete inhibition of enzyme activity (see Fig. 3), does not alter the dissociation of LDH into subunits (Table 2), as it was demonstrated by the following experiment. Treatment of the protein first with one mole of iodine per subunit resulted in the disappearance of two sulfhydryl groups (Fig. 2). The additional three sulfhydryl groups were carboxymethylated in 6 M guanidine hydrochloride at pH 7, by the addition of a fivefold molar excess of iodoacetate per sulfhydryl group. The partially oxidized LDH dissociated freely to subunits in 6 M guanidine hydrochloride, as it was indicated by its molecular weight of 36 200±1 700. Consequently, the first disulfide bond is formed within each polypeptide chain (intra-chain disulfide).

When all sulfhydryl groups of LDH were oxidized in 0.1 M phosphate buffer, pH 6.5, the molecular weight increased to 85 000±22 000 (Table 2), indicating that LDH cannot dissociate anymore freely into subunits in 6 M guanidine hydrochloride. This finding suggests that inter-chain disulfides have also been formed in LDH. In contrast to the molecular weight data listed in Table 2, the reproducibility of the latter value is poor. It can be assumed that the oxidation of sulfhydryl groups occurs randomly, with the exception of those two which react first. Therefore, the completely oxidized LDH can be regarded as a heterogeneous mixture containing aggregates composed of different numbers of subunits.

From the data presented it can be concluded that iodine reacts with the sulfhydryl groups of GAPD and LDH at neutral pH. This reaction results in intra-chain disulfide formation in GAPD, while in LDH both intra- and inter-chain disulfides can be formed. The reaction of other groups than sulfhydryl can be neglected under the experimental conditions applied in these investigations. The increase in iodine concentration and pH results in the reaction of other side chains as e.g. tyrosine, etc. The investigations of the reaction of tyrosine with iodine will be presented elsewhere (Libor, Elődi, 1969).

As it was found in the present investigations, iodine treatment does not significantly change the conformation of the polypeptide chains of the dehydrogenases studied. Therefore, the formation of intra-chain disulfides from sulfhydryl groups may be connected with the fact that these are situated reasonably near each other. The identification of these groups is in progress in our laboratory.

The authors gratefully acknowledge the skillful technical assistance of Mrs P. Tóth and Miss A. Bertók. Discussions with Dr L. Boross were most helpful in the course of the work.

References

Appella, E., Markert, C. L. (1961) Biochem. Biophys. Res. Commun, *6* 171
Beney, L., Elődi, P. (1965) Biochim. Biophys. Acta *110* 210
Boross, L. (1969) Acta Biochim. Biophys. Acad. Sci. Hung. *4* 57
Cunningham, L. W., (1964) Biochemistry *3* 1629
Cunningham, L. W., Nuenke, B. J. (1959) J. Biol. Chem. *234* 1447
Cunningham, L. W., Nuenke, B. J. (1960) J. Biol. Chem. *235* 1711
Ellman, G. L. (1959) Arch. Biochem. Biophys. *82* 70
Elődi, P., Szörényi, E. (1956) Acta Physiol. Acad. Sci. Hung. *9* 339
Fraenkel-Conrat, H. (1959), in: Symposium on Sulfur in Proteins. Acad. Press, New York, p. 339
Harrington, W. F., Karr, G. M. (1965) J. Mol. Biol. *13* 885
Harris, J. I. (1964), in: Goodwin, T. W., Harris, J. I., Hartley, B. S. (editors) Structure and Activity of Enzymes. Acad. Press, London—New York, p. 97
Harris, J. I., Meriwether, B. P., Park, J. H. (1963) Nature *198* 354
Harris, J. I., Perham, R. N. (1968) Nature *219* 1025
Holbrook, J. J., Pfleiderer, G. (1965) Biochem. Z. *342* 111
Jécsai, G. (1961) Acta Physiol. Acad. Sci. Hung. *20* 339
Jécsai, G., Elődi, P. (1963) Acta Physiol. Acad. Sci. Hung. *24* 29
Libor, S., Elődi, P., Nagy, Z. (1965) Biochim. Biophys. Acta *110* 484
Libor, S., Elődi, P. (1969) in preparation
Racker, E., Krimsky, I. (1952) Nature *169* 1043
Szewczuk, A., Wolny, E., Wolny, M., Baranowski, T. (1961) Acta Biochim. Polon. *8* 201
Tanford, C., Kawahara, K., Lapanje, S. (1967) J. Amer. Chem. Soc. *89* 729

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (2), pp. 161—171 (1969)

Effect of Dimethyl Sulfoxide on Barley and Yeast Glucose-6-Phosphate Dehydrogenase

G. L. FARKAS, Maria GŐBEL

Institute of Plant Physiology, L. Eötvös University, Budapest

(Received February 4, 1969)

Cell-free extracts prepared from a number of plant tissues catalyse the enzymatic reduction of nicotinamide adenine dinucleotide phosphate (NADP). It was found that the addition of 2—30 per cent (v/v) dimethyl sulfoxide (DMSO) to cell-free crude extracts increased the rate of reduction of NADP by several hundred per cent. Evidence was obtained that the DMSO-stimulated reduction of NADP is enzymatic in nature. Dimethylsulfone ($DMSO_2$) had a similar, albeit lesser effect. One of the NADP-dependent enzymes affected by DMSO in crude extracts was shown to be glucose-6-phosphate (G-6-P) dehydrogenase. The enzyme was partially purified from barley leaves. The rate of NADP-reduction by the purified enzyme preparation was also enhanced in the presence of 10 per cent DMSO (v/v). As shown by kinetic analysis, the maximal reaction rate (V_{max}) was increased and the apparent Michaelis constants (K_m) for both NADP and G-6-P were lowered in the presence of DMSO. In contrast to the enzyme from barley, low DMSO-concentrations had no stimulatory effect on yeast G-6-P dehydrogenase, except at NADP levels far below saturation. DMSO-concentrations above 10 per cent markedly inhibited the yeast enzyme. Yeast G-6-P dehydrogenase was competitively inhibited by $NADPH_2$ and the inhibition was partially overcome by DMSO at low and medium NADP levels. The possible mode of action of DMSO on plant G-6-P dehydrogenase is discussed.

Introduction

The biological actions of dimethyl sulfoxide (DMSO) have attracted great interest in recent years in view of the possible medical application of the compound (cf. Weyer, 1967). Efficacy has been shown in musculoskeletal injuries, inflammations, urologic disorders, ear, nose and throat pathology, dermatology and several other major fields in clinical medicine (Leake, Rosenbaum, 1967). Agricultural applications are scarce but promising (Keil, 1967). Although the literature on the effect of DMSO on various biological processes is rapidly increasing (Hellman et al., 1967; Weyer, 1967; Gerhads, Gibian, 1968; Dickinson, Cochran, 1968), the mode of action of the compound remains to be elucidated. The present paper reports data to show that DMSO dramatically enhances the enzymatic reduction of NADP in the presence of plant tissue extracts. One of the enzymes affected proved to be glucose-6-phosphate (G-6-P) dehydrogenase. Additional data on the effect of DMSO on yeast G-6-P dehydrogenase are also included.

Materials and Methods

Plant material

Cucumber (Cucumis sativus L.), red pepper (Capsicum annuum L.), bean (Phaseolus vulgaris L.), barley (Hordeum vulgare L.) and tobacco (Nicotiana tabacum L. cv. Xanthi) plants were grown under ordinary greenhouse conditions. Leaf tissues of seedlings were used as starting material for the preparation of cell-free extracts.

Preparation of cell-free extracts

Leaf tissues were homogenized in 0.1 M Tris buffer, pH 7.5, in a pre-chilled mortar in the presence of quartz sand (ratio: 1 g of leaf tissue/1 ml of buffer). The resulting slurry was passed through 4 layers of cheese-cloth and centrifuged at 16 000 *g* for 15 minutes in a Janetzki Model K 60 refrigerated centrifuge. The supernatant served as a crude extract.

Assays and analyses

G-6-P dehydrogenase activity was assayed spectrophotometrically by measuring the increase in absorbance at 340 mμ upon addition of substrate and coenzyme to the reaction mixture. Unless stated otherwise, the reaction system contained 10 μmoles of G-6-P, 10 μmoles of $MgCl_2$, 0.3 μmoles of nicotinamide-adenine-dinucleotide phosphate (NADP), 150 μmoles of tris(hydroxymethyl)aminomethane buffer, pH 7.5, and a suitable amount of enzyme preparation to obtain a reaction rate closely linear with time for at least 3 minutes. Control cuvettes contained the complete assay system minus G-6-P or the complete system minus NADP. Since considerable heat is evolved when DMSO is mixed with aqueous solutions, the temperature of the cuvettes containing DMSO and buffer was brought to 25 °C before adding the enzyme to the reaction mixture.

Protein determination was carried out according to Lowry et al. (1951) using bovine serum albumine as standard.

Enzyme purification

For the partial purification of G-6-P dehydrogenase crude extract was prepared from the first leaves of 5-day old barley seedlings as described above. The 16 000 *g* supernatant was centrifuged in a Spinco Model L-50 preparative ultracentrifuge at 100 000 *g* for 1 hour. The supernatant was subjected to fractionation by $(NH_4)_2SO_4$. The fractions rich in G-6-P dehydrogenase (between 40 per cent and 70 per cent ammonium sulfate saturation) were collected by low speed centrifugation in the cold. The sediment was dissolved in 4 ml of 0.01 M Tris buffer, pH 7.5, and applied to a 32×3 cm Sephadex G-200 column equilibrated with 0.05 M Tris buffer, pH 7.5. Chromatography with ascending liquid flow was carried out using an LKB ReCyChrom apparatus. Fractions were collected in a Buchler refrigerated fraction collector, which supplied water for the cooling jacket of the

column during operation. The effluent was monitored at 280 mμ by using an LKB UVICORD-II UV-analyzer. Enzyme determinations were done on all protein-containing fractions. The fractions rich in G-6-P dehydrogenase were pooled and used as a partially purified preparation. A 15- to 20-fold purification was achieved.

Chemicals

G-6-P (disodium salt), 6-phosphogluconate (6-P-G, barium salt), isocitric acid, $NADPH_2$ and yeast G-6-P dehydrogenase (type VI) were obtained from Sigma Chemical Co. (St. Louis, Mo.), NADP was purchased from Boehringer and Soehne GmbH (Mannheim), shikimic acid and tris(hydroxymethyl)aminomethane was supplied by Fluka AG (Buchs), Sephadex G-200 was a product of Pharmacia Fine Chemicals (Uppsala), DMSO and all other reagents of analytical grade were obtained from Reanal, Hungarian Fine Chemical Co. Dimethylsulfone ($DMSO_2$) was kindly prepared by Dr. I. Kapovits, Department of Organic Chemistry, L. Eötvös University, Budapest.

Results

Effect of DMSO on the reduction of NADP in crude extracts

NADP added to crude extracts from various plant tissues (cucumber, red pepper, bean, barley and tobacco) was enzymatically reduced at the expense of endogenous substrate(s). This was indicated by the observation that (a) the absorbancy at 340 μm of the crude extracts increased upon addition of NADP to the reaction mixture, (b) the activity was lost upon dialysis, (c) the small molecular weight substances could be enriched by lyophilization and dialyzed extracts could

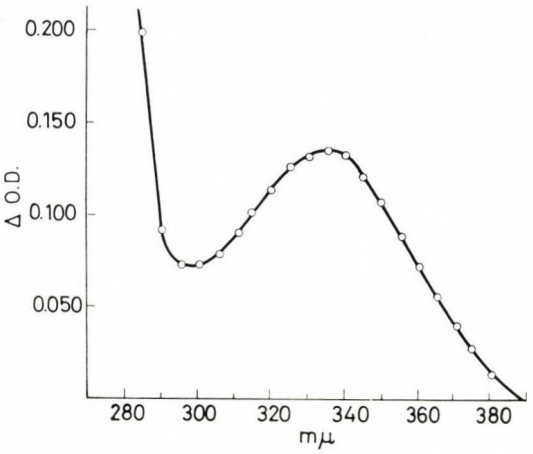


Fig. 1. Evidence for the formation of $NADPH_2$ in cell-free extracts from tobacco leaves upon addition of NADP and DMSO. Difference spectrum (0-time versus completed reaction) taken in a standard reaction mixture minus G-6-P. 10 per cent DMSO (v/v) and 0.25 ml crude extract from tobacco leaves was added to the cuvette in a final volume of 3.0 ml

be replenished by the dialyzate, (d) the activity of the extracts was destroyed by heating, (e) the small molecular weight substance(s) was (were) heat stable.

The addition of DMSO (10 per cent v/v) enhanced the enzymatic reduction of NADP at the expense of endogenous, small molecular weight substrate(s) contained in the crude extracts. The following observations are pertinent concerning this conclusion: (a) upon addition of DMSO, no change in absorption at 340 mμ occurred when NADP was omitted from the reaction mixture, (b) the UV spectrum of the compound formed during the reaction was identical with that of $NADPH_2$ and no evidence was obtained for the formation of other UV-absorbing products (Fig. 1), (c) the reduction of NADP by the crude extract both in the presence and absence of DMSO exhibited a typical pH-optimum characteristic of enzymatic reactions (Fig. 2), (d) NAD was reduced by the crude extracts neither in the absence nor in the presence of DMSO.

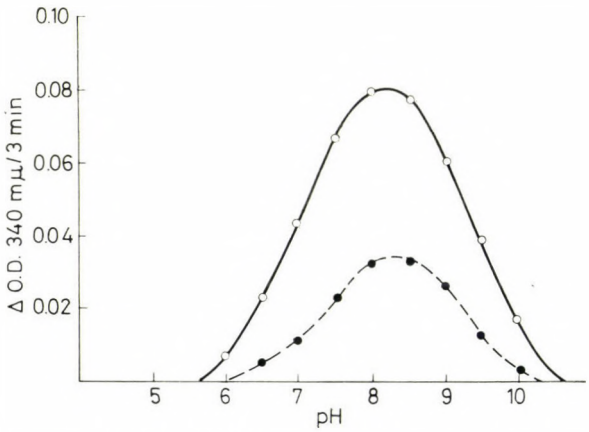


Fig. 2. pH-dependence of the reduction of NADP by cell-free crude extracts from tobacco leaves in the absence (● - - - ●) and presence (○——○) of 10 per cent DMSO (v/v). Composition of the system: standard reaction mixture minus G-6-P. As enzyme source 0.25 ml crude extract was used in a final volume of 3.0 ml

Effect of $DMSO_2$ on the reduction of NADP in crude extracts

Experiments similar to those described above were carried out with $DMSO_2$ instead of DMSO. $DMSO_2$ also stimulated the enzymatic reduction of NADP by plant extracts. The effect was, however, about 50 per cent less than with DMSO.

The concentration dependence of the DMSO-effect

As shown in Fig. 3 the extent of the stimulation of dehydrogenase activity in crude extracts from tobacco leaf tissues increased with increasing DMSO concentrations. Above a certain DMSO-level (approx. 25 per cent) proteins tended to precipitate and the protein coagulation interfered with the assay.

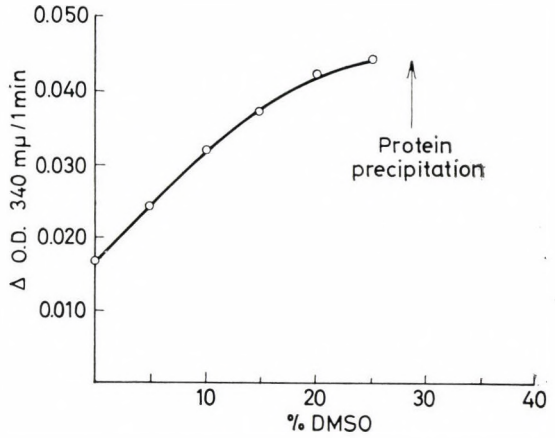


Fig. 3. Effect of various DMSO-concentrations on the activity of NADP-dependent dehydrogenases in crude extracts from tobacco leaf tissues. The standard assay system minus G-6-P was used. 0.25 ml crude extract served as enzyme source in a final volume of 3.0 ml

Effect of DMSO on NADP-dependent dehydrogenases of $(NH_4)_2SO_4$-fractions

To establish the nature of dehydrogenase(s) affected by DMSO, proteins were precipitated from the 100 000 *g* supernatant of crude extracts by $(NH_4)_2SO_4$ at 80 per cent saturation. The precipitate was dissolved in 0.01 M Tris buffer, pH 7.5, and aliquots were assayed for all the major NADP-dependent dehydrogenases known to be widespread in plant tissues. With all enzymes tested the assay system

Table 1

Effect of DMSO on NADP-dependent dehydrogenase activities in proteins precipitated from crude extracts of barley leaf tissues by ammonium sulfate

10 grams of tobacco leaf tissues were homogenized in 30 ml of 0.1 M Tris buffer, pH 7.5, containing 1 mg cysteine and 1 mg ascorbic acid per ml. After centrifugation of the crude extract at 16 000 *g* for 15 minutes and at 100 000 *g* for 1 hour the proteins were precipitated by $(NH_4)_2SO_4$ at 80 per cent saturation. The precipitate was collected by centrifugation and dissolved in 5 ml of 0.01 M Tris buffer, pH 7.5. 0.25 ml of this solution was used as an enzyme preparation in the standard assay described in Methods

Enzyme	Enzyme activity: Δ O.D. 340 mμ/1 min	
	In the absence of DMSO	In the presence of DMSO
Glucose-6-phosphate dehydrogenase	0.036	0.076
6-Phosphogluconate dehydrogenase	0.020	0.024
Isocitrate dehydrogenase	0.046	0.044
Alanine dehydrogenase	0.028	0.000
Glutamate dehydrogenase	0.010	0.008
Shikimate dehydrogenase	0.084	0.079

was identical with that described in Methods except that the substrate was varied (with the shikimate dehydrogenase borate buffer, pH 9.8, was used). The presence of the following NADP-dependent dehydrogenase activities was detected in the $(NH_4)_2SO_4$-precipitate: G-6-P dehydrogenase, 6-phosphogluconate (6-P-G) dehydrogenase, isocitric acid dehydrogenase, alanine dehydrogenase, glutamic acid dehydrogenase (traces), shikimate dehydrogenase. As shown in Table 1, only G-6-P dehydrogenase activity was markedly enhanced in the presence of 10 per cent DMSO (v/v). Slight stimulation of 6-P-G-dehydrogenase activity was also experienced, whereas alanine dehydrogenase was completely inhibited by 10 per cent DMSO (v/v).

Effect of DMSO on G-6-P dehydrogenase partially purified from barley leaves

G-6-P dehydrogenase, partially purified from barley leaves, was also affected by DMSO. This enzyme preparation showed normal Michaelis–Menten kinetics both in the absence and presence of DMSO. As shown in Figs 4 and 5 the presence of DMSO in the reaction mixture lowered the values of the apparent Michaelis constants of the enzyme preparation for both G-6-P and NADP. At the same time the V_{max} values were increased.

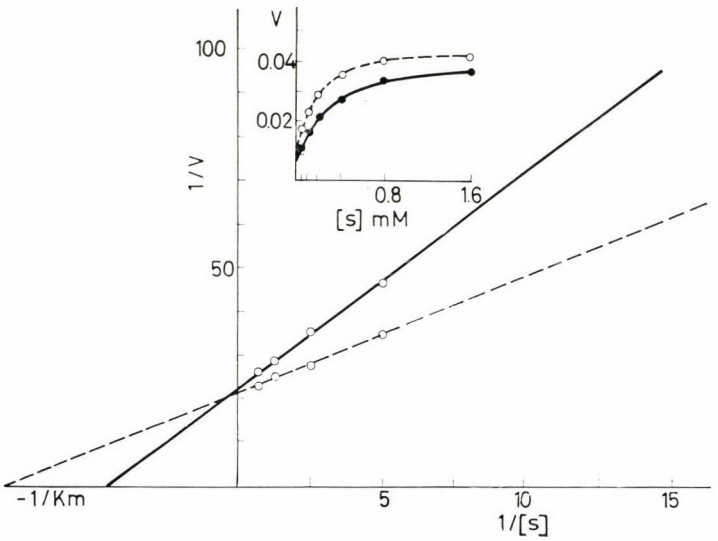


Fig. 4. The effect of DMSO on the kinetics of G-6-P dehydrogenase from barley. Lineweaver–Burk plot of the rate of reduction of NADP as a function of G-6-P concentration. Control: ●—●. 10 per cent DMSO in the reaction mixture: o - - - o. For the composition of the system see Methods

Effect of DMSO on G-6-P dehydrogenase from yeast

In contrast to the enzyme from barley, no significant stimulation of the activity of a highly purified commercial yeast G-6-P dehydrogenase was obtained at any DMSO-level if the NADP-concentration was kept close to saturation. High

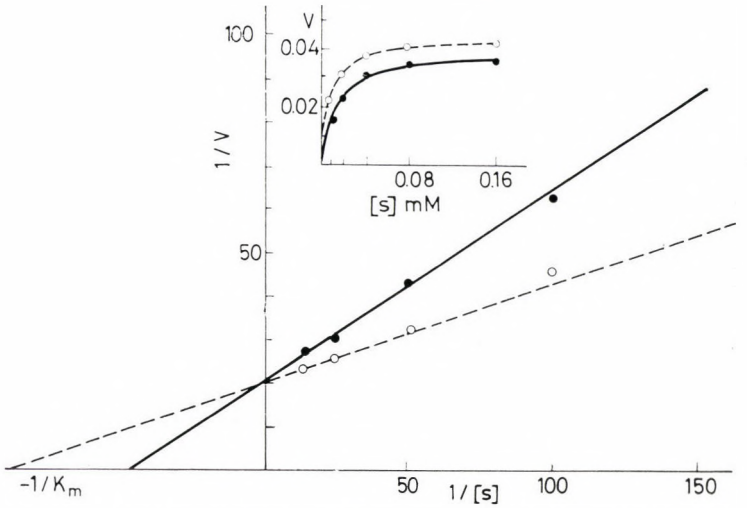


Fig. 5. The effect of DMSO on the kinetics of G-6-P dehydrogenase from barley. Lineweaver–Burk plot of enzyme activity as a function of NADP-concentration. Control: ●——● 10 per cent DMSO in the reaction mixture: o - - o. For the composition of the standard reaction mixture see Methods

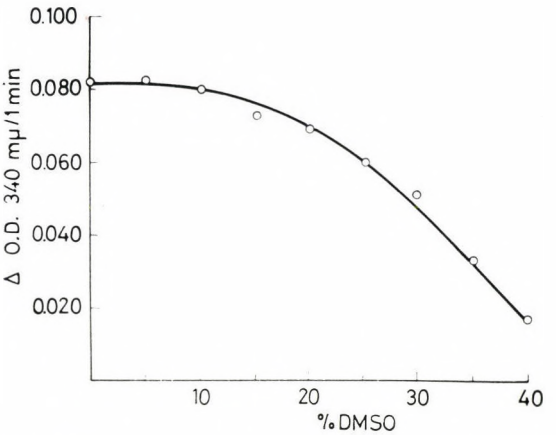


Fig. 6. Effect of DMSO-concentration on the activity of yeast G-6-P dehydrogenase. The assay system contained the standard ingredients described in Methods

DMSO-concentrations [20 per cent (v/v) and above] invariably inhibited the enzyme (Fig. 6). However, as shown in Fig. 7, the effect of DMSO on yeast G-6-P dehydrogenase activity depended not only on the concentration of DMSO in the reaction mixture but also on the amount of NADP present. DMSO in the same concentration (20 per cent v/v) stimulated the enzyme activity at low NADP levels and inhibited it at high NADP levels.

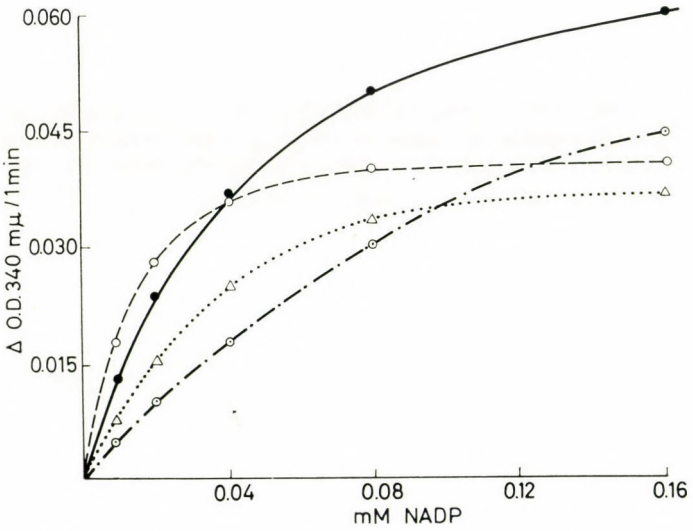


Fig. 7. Effect of DMSO on the kinetics of yeast G-6-P dehydrogenase. Effect of NADP concentration on the reaction rate in the presence of various ingredients. Complete system (control): ●——●. Complete system + 20 per cent DMSO (v/v): ○--○. Complete system + 0.05 μmole of $NADPH_2$/ml: ⊙—·—⊙. Complete system + 20 per cent DMSO + 0.05 μmole of $NADPH_2$/ml: △····△. For the composition of the complete system see Methods

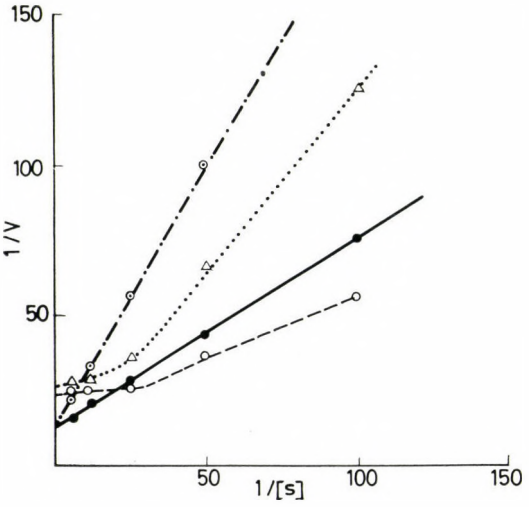


Fig. 8. Effect of DMSO on the kinetics of yeast G-6-P dehydrogenase. Lineweaver–Burk plot of the effect of NADP concentration on the reaction rate in the presence of various ingredients. Complete system (control): ●——●. Complete system + 20 per cent DMSO (v/v): ○--○. Complete system + 0.05 μmole $NADPH_2$/ml: ⊙---⊙. Complete system + 20 per cent DMSO + 0.05 μmole of $NADPH_2$/ml: △···△. For the composition of the complete system see Methods

Yeast G-6-P dehydrogenase was shown to be competitively inhibited by $NADPH_2$ (Figs 7 and 8). The enzyme was partially relieved from the inhibition in the presence of DMSO at relatively low NADP levels (Fig. 7). This might explain the observation that in the presence of DMSO the reaction rate of yeast G-6-P dehydrogenase remains constant for a longer time than in the absence of DMSO. This phenomenon was apparent particularly at relatively low NADP levels (Fig. 9).

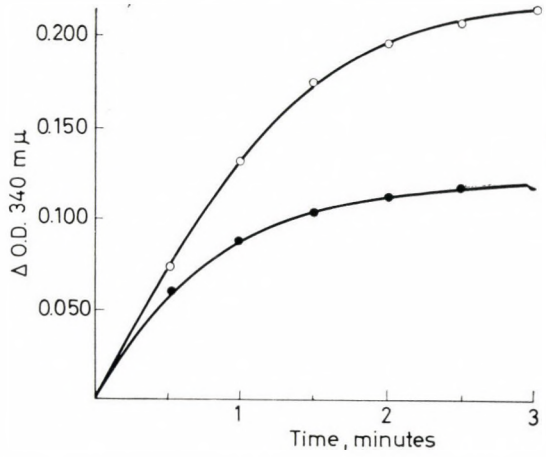


Fig. 9. Rate of reduction of NADP by yeast G-6-P dehydrogenase in the absence (●——●) and presence (o—o) of 20 per cent DMSO (v/v) in the reaction mixture. The system contained 10 μmoles of G-6-P, 10 μmoles of $MgCl_2$, 0.1 μmole of NADP, 150 μmoles of Tris-HCl buffer at pH 7.5 and a suitable amount of enzyme preparation

Discussion

In contrast to the large amount of literature available on the physical and chemical properties of DMSO and on its medical application (cf. Weyer, 1967; Gerhards, Gibian, 1968), detailed reports on the effect of DMSO in well defined biochemical systems are relatively scarce. As shown in rotatory dispersion studies DMSO is able to induce major configurational changes in proteins (Hamaguchi, 1964). This might explain the effect of DMSO on enzyme activities. Inhibition by DMSO of phosphomonoesterase (E. coli), β-galactosidase (E. coli), peroxidase (horse radish), catalase, glutamate dehydrogenase, lactate and alcohol dehydrogenase activities has recently been reported (Rammler, 1967; Perlman, Wolff, 1968). Only alcohol dehydrogenase has been investigated in detail. It appears that in the direction of aldehyde reduction the enzyme activity is competitively inhibited by DMSO. Aldehyde and DMSO compete for the substrate binding site (Perlman, Wolff 1968). Only preliminary reports on the stimulation by DMSO of some enzyme activities are available. The activity of a crude preparation of snail phosphomonoesterase (Rammler, 1967) and pancreatic deoxyribonuclease (Monder, 1967) has been shown to be enhanced by DMSO. Most interesting is, however, a report on the alteration by DMSO of the specificity of an allosteric

enzyme. The alanine dehydrogenase activity of glutamate dehydrogenase has been reported to increase dramatically in the presence of DMSO. At the same time the glutamate dehydrogenase activity of the preparation decreased (Rammler, 1967).

We observed a marked stimulation of enzymatic NADP-reduction in crude extracts from plant tissues upon addition of DMSO. The effect was traced back to G-6-P dehydrogenase. It seems that the effect is fairly specific since none of the other NADP-dependent dehydrogenases, known to occur widely in plant tissues, was stimulated by DMSO to any large extent. As shown by kinetic analysis on barley G-6-P dehydrogenase the "stimulation" of enzyme activity can be attributed primarily to a decrease of the K_m values for both G-6-P and NADP, with a simultaneous increase in V_{max} (Figs 4 and 5). The enzyme activity follows normal Michaelis–Menten kinetics both in the absence and presence of DMSO. Yeast G-6-P dehydrogenase was found to behave differently. On the one hand, no stimulation of yeast G-6-P dehydrogenase activity was obtained at any DMSO-concentration when the NADP-level was kept close to saturation. On the other hand, the enzyme activity did not follow normal Michaelis–Menten kinetics in the presence of DMSO.

Much remains to be learned about the various G-6-P dehydrogenases before the above observations can adequately be explained. Perhaps we can tentatively conclude that most G-6-P dehydrogenases are present in the tissues of higher plants in a form (state) the catalytic activity of which is partly "latent". The higher activity of the enzyme preparations in the presence of DMSO might be due to a "favorable" change in the conformation of the enzyme molecule. This interpretation is strongly supported by the fact that DMSO affected the enzyme from barley in the same way whether purified or present in a crude preparation. However, the alternative possibility that in the presence of DMSO an inhibitor is dissociated from the enzyme molecule cannot be entirely excluded.

It remains to be elucidated whether the ubiquitous increase in G-6-P dehydrogenase activity in diseased or injured plant tissues (Király, Farkas, 1962; Solymosy, Farkas, 1963; Németh et al., 1969) can be explained by the transformation of G-6-P dehydrogenase into a "more active form". In contrast to many other cases the parasitically induced increase in G-6-P dehydrogenase activity does not seem to be based on new protein synthesis (Farkas, Stahmann, 1966). The results described in the present paper are compatible with the idea that the G-6-P dehydrogenase(s) of higher plant tissues has (have) more than one catalytic form (conformation) and the molecule normally found in plant extracts is not the most active one.

References

Dickinson, D. B., Cochran, D. (1968) Plant Physiol. *43* 411
Farkas, G. L., Stahmann, M. A. (1966) Phytopathology *56* 669
Gerhards, E., Gibian, H. (1968) Naturwissenschaften *55* 435
Hamaguchi, K. (1964) J. Biochem. (Tokyo) *56* 441
Hellman, A., Farrelly, J. G., Martin, D. H. (1967) Nature *213* 982
Keil, H. L. (1967) Ann. N. Y. Acad. Sci. *141* 131

Király, Z., Farkas, G. L. (1962) Phytopathology *52* 657
Leake, C. D., Rosenbaum, E. E. (1967) Ann. N. Y. Acad. Sci. *141* 670
Lowry, G. H., Rosebrough, W. J., Farr, A. L., Randall, R. J. (1951) J. Biol. Chem. *193* 265
Monder, C. (1967) Ann. N. Y. Acad. Sci. *141* 300
Németh, J., Klement, Z., Farkas, G. L. (1969) Phytopath. Z. (in press)
Perlman, R. L., Wolff, J. (1968) Science *160* 317
Rammler, D. H. (1967) Ann. N. Y. Acad. Sci. *141* 291
Solymosy, F., Farkas, G. L. (1963) Virology *21* 210
Weyer, E. M. (ed.) (1967) Biological Actions of Dimethyl Sulfoxide. Ann. N. Y. Acad. Sci. *141* pp. 1—671.

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (2), pp. 173–176 (1969)

Studies on the Mechanism of the Effect of Trypsin on the Shape Maintenance of Erythrocytes

Preliminary Communication

ILMA SZÁSZ, VERA N. SŐTÉR, I. ÁRKY, G. GÁRDOS

Departments of Cell Metabolism and Haemoglobin Research, Central Institute of Haematology and Blood Transfusion, Budapest

(Received April 30, 1969)

Ponder (1951) has established that trypsin has no influence on the shape of erythrocytes or on the shape transformations induced by glass capillaries or lecithin, saponin, bile acids and anionic detergents. We have found that the typical disk-sphere transformation caused by ATP-breakdown is significantly delayed by trypsin treatment (Szász et al., 1967). In our present work the metabolic basis of this effect has been studied to obtain data on the mechanism of action.

It was found that when an almost total breakdown of ATP* was induced by 8–32 mM NaF, after 0–24 hour fluoride pretreatment the process of disk-sphere transformation slowed down steadily and significantly on the addition of trypsin. The initial stage of this process can be seen in Fig. 1 during the period of 3 to 4 hours. When, however, in addition to glycolysis inhibition by NaF arsenolysis was also induced by adding 2.5 mM of Na_2HAsO_4 eliminating thus even the traces of ATP, after 3 hours of such a pretreatment trypsinization exerted no advantageous effect on morphology any more (Fig. 2). We have also investigated whether the simultaneous presence of ATP and trypsin is necessary for the development of the effect or does trypsin treatment alter even ATP-free cells permanently. In latter case the morphological consequence may become manifest in the course of fermentative ATP resynthesis following the washing out of trypsin and metabolic poisons. Fig. 2 indicates that the morphological regeneration accompanying fermentative ATP resynthesis was significantly faster in cells pretreated with trypsin in an ATP-free state than in the not trypsinized controls. Consequently, trypsin exerted a steady effect which later resulted in the maintenance of the biconcave shape when ATP was generated in the cell. The morphological regeneration of trypsin-pretreated cells was the same irrespective of whether trypsin treatment was carried out in the presence or absence of ATP (compare Figs 1 and 2). Thus presumably the trypsin effect on the shape maintaining mechanism, unlike the trypsin effect on transport ATPase (Somogyi, 1967) is not significantly influenced by the fact whether or not ATP is bound to the active center.

* The exact measurement of these slight amounts of ATP was not possible by the available ion exchange chromatographic and phosphoglyceraldehyde dehydrogenase-phosphoglycerate kinase enzymatic methods. After a 3-hour incubation with NaF, however, the ATP level was always below 0.1 μmole/ml erythrocyte.

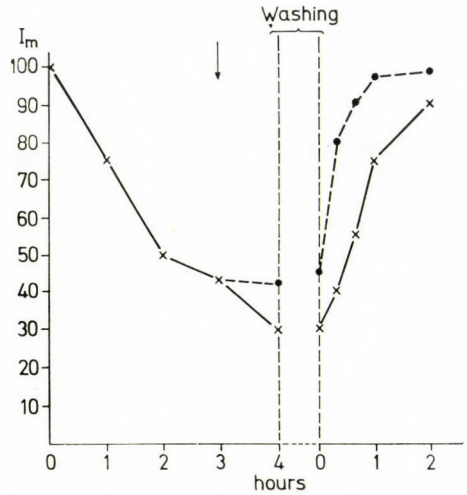


Fig. 1. Disk-sphere transformation caused by 32 mM NaF and its reversal by fermentative ATP resynthesis. Solid lines represent I_m, the "morphological index" (Gárdos et al., 1966) of the control cells, broken lines that of the trypsinized cells. 0.1 mg trypsin/ml RBC was added after a 3-hour incubation at 37 °C

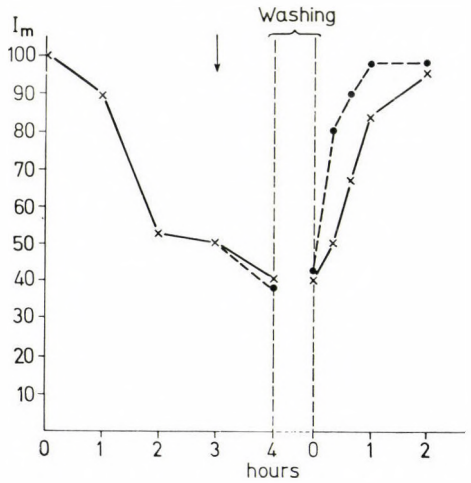


Fig. 2. Disk-sphere transformation caused by 8 mM NaF + 2.5 mM Na_2HAsO_4. For symbols see Fig. 1

From these experiments it cannot be established whether trypsinization promotes the enzymatic splitting of ATP or its binding to the receptor. Some theories on muscle contraction claim that the negative charges of ATP bound to the muscle fibers play a role by neutralizing the positive charges of the protein abolishing thereby its electrostatically secured unfolded conformation (Morales et al., 1955). At the same time, it is also known that trypsin releases 30–50 per cent of sialic acid, which carries the main ionogenic group of the erythrocyte membrane,

while neuraminidase causes an almost total breakdown of the mebrane sialic acid (Winzler et al., 1961). The question arises whether or not the trypsin effect is brought about by the elimination of the negative charges which otherwise inhibit the binding of ATP. Therefore washed erythrocytes were treated at 37 °C for 60 minutes with trypsin (Boehringer) and other proteolytic enzymes: papain (Merck), chymotrypsin (Reanal) and bromeline (Merck) as well as with neuraminidase (Koch Laboratories). Sialic acid determinations (Warren, 1959) indicated that treatment with proteolytic enzymes caused a partial release of the sialic acid of the erythrocytes, while the neuraminidase effect was almost complete.

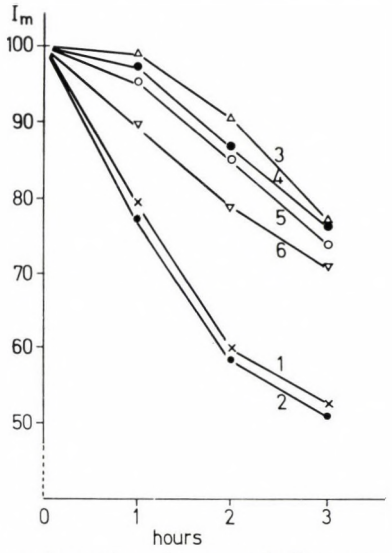


Fig. 3. Disk-sphere transformation of enzymatically pretreated erythrocytes in the presence of 16 mM NaF. Pretreatments: 1: None; 2: 20 IU neuraminidase/ml RBC; 3: 0.5 mg bromeline/ml RBC; 4: 0.1 mg trypsin/ml RBC; 5: 1.5 mg papain/ml RBC; 6: 0.1 mg chymotrypsin/ml RBC

(The starting sialic acid content was 0.09, the values after the proteolytic treatments varied between 0.04–0.06, after neuraminidase treatment it was 0.001 nmole per 10^{10} erythrocytes.) In spite of this change morphological degeneration was not affected by neuraminidase, while all the proteolytic enzymes slowed it down significantly (Fig. 3).* Thus, out of the electrostatic forces, at least the failure of the ATP repulsing effect of the negative charges of sialic acid cannot be supposed to be the basis of the trypsin effect.

On the other hand, on trypsinization the enhancement of ATP breakdown can be demonstrated by a method developed for intact erythrocytes (Whittam,

* The same type of differences are obtained in the presence of 8–32 mM NaF, but — due to the ATPase inhibitory effect of NaF (Gárdos et al., 1966) — the extents of the morphological changes are different. NaF concentrations producing the most illustrative effects are used in the experiments presented in the figures.

Ager, 1965). (ATPase activation was around 120 – 150 per cent, with 50 – 500 μg trypsin per ml erythrocyte.) Most probably the synthesis of the structural basis of the shape maintaining mechanism is an energy requiring enzymatic process, and in case of ATP breakdown morphological degeneration develops as far as the renewal of this structure becomes impossible. Trypsin seems to activate this renewal process to a significant extent.

For the skilful technical assistance thanks are due to Mrs Eva Irmai.

References

Gárdos, G., Szász, I., Árky, I. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 253
Morales, M. F., Botts, J., Blum, J. J., Hill, L. (1955) Physiol. Rev. *35* 475
Ponder, E. (1951) Blood *6* 350
Somogyi, J. (1967) Biochim. Biophys. Acta *151* 421
Szász, I., Árky, I., Gárdos, G. (1967) Haematologia *1* 287
Warren, L. (1959) J. Biol. Chem. *234* 1971
Whittam, R., Ager, M. E. (1965) Biochem. J. *97* 214
Winzler, R. J., Harris, E. D., Pekas, D. J., Johnson, A., Weber, P. (1961) Biochemistry *6* 2195

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (2), pp. 177—186 (1969)

Longitudinal Structure of the Striated Fibril

E. ERNST, K. KOVÁCS, G. METZGER-TÖRÖK, C. TROMBITÁS

Biophysical Institute, Medical University, Pécs

(Received August 3 and December 14, 1968)

The question has been analyzed what is the basis of the longitudinal mechanical coherence of the striped fibril. Experiments performed with different methods indicate: 1. Splits running longitudinally in the fibril frozen in CO_2 + ether are bordered with straight lines passing through several sarcomers. 2. Thin ribbons from single fibrils torn in a Zeiss micromanipulator by glass needles can be stretched to about treble their original lengths. 3. The A-substance (of the "thick filaments") can migrate along preformed longitudinal elements situated in the sarcomers and passing uninterruptedly from Z to Z. Conclusions: 1. Fibrils contain longitudinal elements passing through the whole fibril. 2. Muscle models being discussed nowadays are not sufficient for a contraction theory.

Introduction

Scrutinizing the question of what the basis of the longitudinal coherence of the striated fibril is, i.e. the basis of the common fact that the fibril is mechanically held together longitudinally, one cannot help facing an old problem, debated for a century. The debate has been about whether the sarcous elements (= sarcomers) are the native elementary structural units of the striated muscle fiber (Bowman, 1840), or the fibrils (Barry, 1851). The question has been settled: both units are native elements of the fibre, the longitudinal coherence of which is due (beside the sarcolemma) to the fibrils passing through the whole length of the fibre.

Putting the similar question of what the mechanical basis of the longitudinal coherence of a single fibril is we encounter the same situation. Soon after Bowman's "sarcous elements" of the *fibres* the single sarcomers of the *fibrils* were considered as the native elements of the latter: "Die Elemente der Muskeln (sc. aus Thorax der Insecten) sind kleine Würfel oder Cylinder, welche sich zu Fibrillen oder Scheiben zusammenlegen" (Auber, 1853). 1. Isolated sarcomers have also recently been described as transversal elements of single fibrils (Draper, Hodge, 1949; Ashley et al., 1951; see Ernst, 1958), 2. but it was simultaneously emphasized that the fibrils contained longitudinal elements passing through the whole fibril. Accordingly the longitudinal mechanical coherence of the single fibrils can be ascribed to these longitudinal elements passing through the sarcomers without interruption.

Huxley's model is known to contrast with this description, therefore we performed experiments in this field.

Methods and Results

Being of the opinion that the real three-dimensional submicrostructure can scarcely be recognized by a single method giving two-dimensional pictures (Ernst, 1963) we tried to use some different methods for this purpose. Accordingly, in contrast to the usual form of the papers in this periodical, the single methods and the results produced by them will be described in successive chapters.

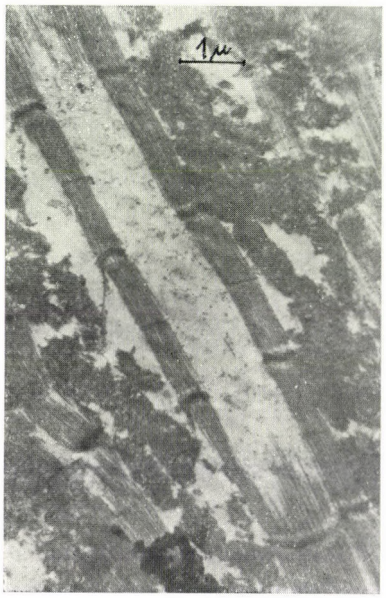
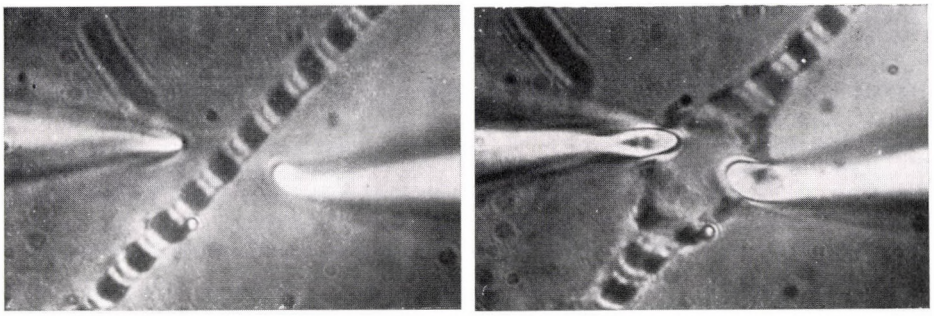


Fig. 1. Fibril (honey bee) with a longitudinal split bordered by straight lines

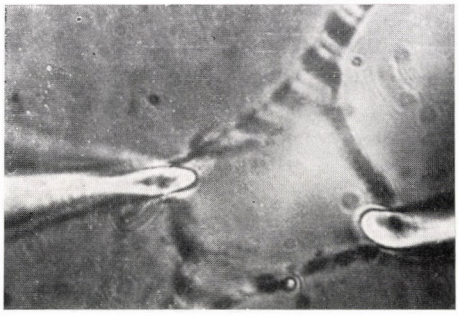
1. Many data in the literature form a contrast to Huxley's "two filament model" (e.g. 1956) of the sarcomer containing longitudinal elements no longer than a sarcomer, thus e.g. the "single filament model" (Hodge, 1955; Fan Shih-Fang, Hong Ming-Xia, 1962), or the "three filament model" (Guba, 1964; Hoyle, 1968).

Furthermore, many papers and figures give evidence that certain longitudinal elements pass through the sarcomers along the fibril (Draper, Hodge, 1949; Ashley et al., 1951; Sjöstrand, Anderson-Cedergren, 1957; Smith, 1961; Fawcett, Revel, 1961; Ernst, Benedetzky, 1962; Sjöstrand, 1962; Garamvölgyi, 1963, 1965; Adamjan, Krolenko, 1966; Pepe, 1967; Kelly, 1967).

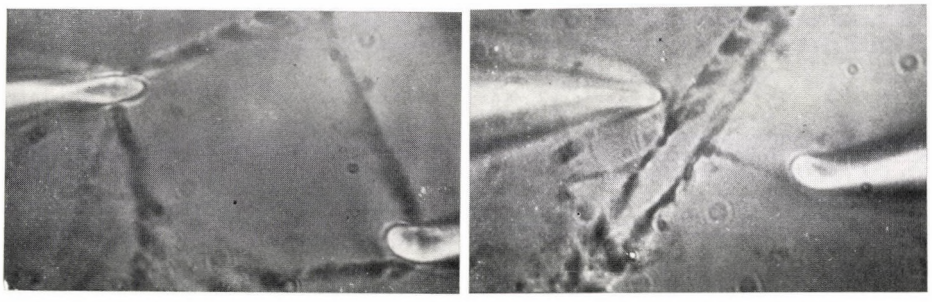
According to our conception that the structure should be investigated also with mechanical means (Ernst, Kellner, 1936) we again used the method of "micromechanical blasting". The muscle (Apis mellifica) was put in a mixture of dry ice + ether (−70 °C), in consequence of which it froze very fast and many splits developed inside the fibrils. Fig. 1 demonstrates such a fibril with a longitudinal split bordered by *quite straight lines passing through some sarcomers.* This picture corroborates the statement that there are longitudinal elements inside the



a *b*



c



d *e*

Fig. 2. *a*—*e*. Fibril (honey bee) torn by glass needles in the micromanipulator; *a:* fibril with the glass needles; *b*, *c* and *d:* torn transversely to different degrees; *e:* needles unhooked ×3000

fibril which, passing through the length of the latter, bring about its mechanical longitudinal coherence.

2. In the mechanical lengthwise coherence the role of the longitudinal elements, passing from sarcomer to sarcomer through the length of the fibril, seems to be of special importance in the light of the circumstance that the fibril has no fibrolemma to which a certain role in the longitudinal coherence could be ascribed (e.g. Hodge et al., 1954). Though some authors earlier debated this question (e.g.

Schaeffer, 1891: supporting; Meigs, 1912: denying), today it seems to be settled that the *striated fibrils have no fibrolemma.*

3. Considering what is described in the two preceding chapters we decided to investigate how the *native* fibril behaves when it is torn in the micromanipulator by needles. Single fibrils from the indirect flight muscles of honey bees were put in the hanging drop under the objective of the phase-contrast microscope equipped with a Zeiss micromanipulator (Ernst, Garamvölgyi, 1956). The fibril was torn with two fine glass needles the thinnest tips of which were $< 1\ \mu$. Figs 2*a*, *b*, *c*, *d*, *e* demonstrate such an experiment; *a:* the isolated fibril between the two glass needles; *b*, *c*, *d:* the hooky tips of the needles were stuck into the fibril, and the

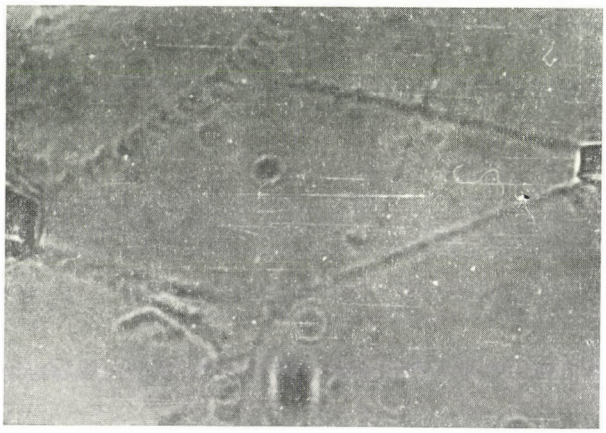


Fig. 3. Thin ribbon-like part of the fibril excessively stretched. × 3000 (see Fig. 2)

latter was torn continuously; *e:* the hooks of the needles were unhooked from the fibril, the stretched parts of which returned to their original form and place.

Fig. 3 shows a picture from such an experiment demonstrating a single fibril torn by needles; it can be seen that a longitudinal thin ribbon of the fibril — namely the right side of it — has been stretched to about treble its original length. This significant extensibility seems to be of no peculiarity for research workers who think they have succeeded in showing that fibrils contain longitudinal elements passing lengthwise through many sarcomers along the fibril; on the other hand, authors presuming longitudinal elements no longer than one sarcomer inside the fibril may try to explain this experimental result.

4. When explaining the great extensibility of thin longitudinal ribbons of the fibril one cannot evade the difficulty caused by the "two filament model", according to which the so-called *thick filaments* have no direct connection with the Z-discs. Furthermore, supposing the existence of such isolated thick filaments, one encounters the difficult question of how and where the "*A-substance*" is situated during its *migration* brought about by different means.

Disregarding this time the older literature (e.g. contraction band, Merkel, 1873) we want to mention that many papers in the last two decades have described

the migration of the A-substance (Draper, Hodge, 1949; Ashley et al., 1951; Hodge, 1955; Tigyi-Sebes, 1962, 1966, Garamvölgyi, Kerner, 1966; Zobel et al., 1967; Pepe, 1967). Now, the A-substance, after being mobilized, could migrate — according to the supposition of isolated thick filaments — at random without any distinct direction. In contrast, the experiments indicate that the mobilized A-substance migrates towards the Z-discs. And what is more, a paper published from our institute (Garamvölgyi, Kerner, 1966) gives evidence that the A-substance migrates along longitudinal elements (see Figs 3, 4 and 6 of that paper) preformed in the sarcomer.

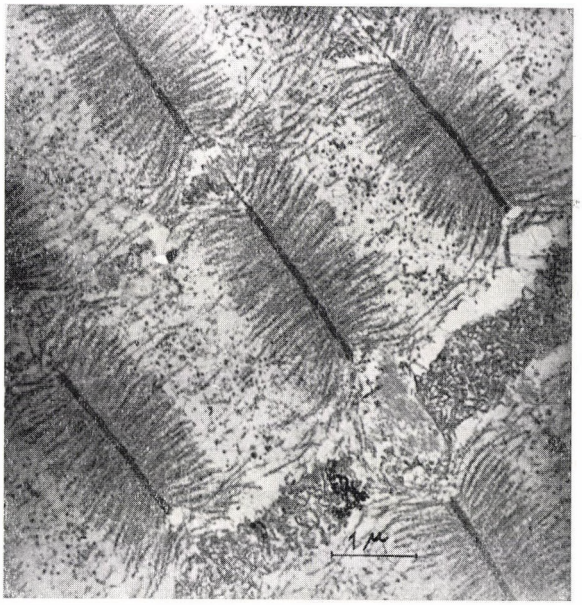
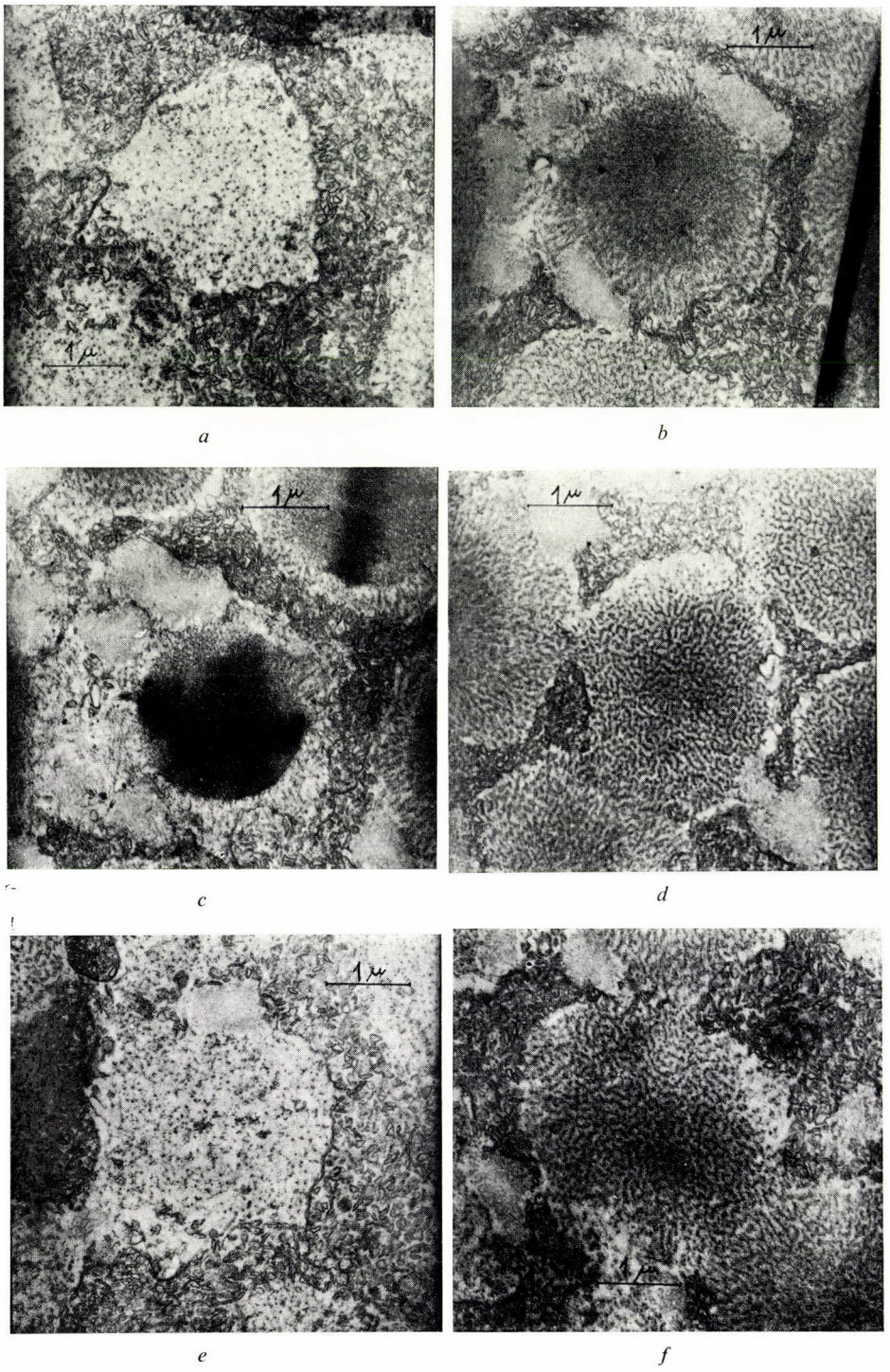


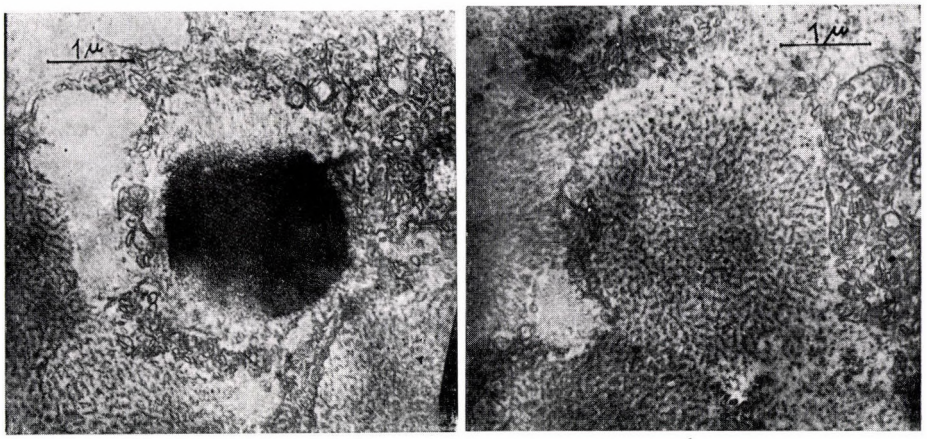
Fig. 4. Fibrils from a fibre (honey bee) stretched and put in Edsall—Weber's solution; migration of the A-substance towards Z; longitudinal section

Fig. 4 shows the electron micrograph of a longitudinal section of fibrils from a fibre (honey bee) stretched and put in Edsall–Weber's extracting solution. It can be seen very clearly that the dark substance has migrated along some lines reaching the Z-discs. But the continuity of these lines inside the sarcomer between neighbouring Z-discs is not demonstrated by this Figure.

Investigating this question we prepared several series of transverse sections from the same fibre which served for the longitudinal section shown in Fig. 4. For this purpose the grip was used (Ernst, Benedetzky, 1962) by means of which the block containing the fibre could be cut in different directions. Fig. 5 shows 8 pictures from a series of 90 successive transverse sections. Fig. 5*a* demonstrates the 1st cross section of a sarcomer cut nearly in the middle; in the shapeless transverse section many cross sections of longitudinal elements are to be seen. Fig. 5*b* is the 14th, 5*c* the 16th transverse section showing gradually increasing density,



a b c d e f



g *h*

Fig. 5. *a*—*h*. Transverse sections from the same block as in Fig. 4; see text

the greatest degree of which is reached in the latter (Z-disc). The following transverse sections again show decreasing density (*d* = the 30th section) the lowest value of which is seen in Fig. 5*e* (middle of the sarcomer, the 44th section) containing, notwithstanding, many cross sections of longitudinal elements. The successive sections again demonstrate increasing density (Fig. 5*f* = the 59th section), the highest value of which is contained in the next Z-disc (Fig. 5*g* = the 71st section); and the density decreases again in the following sections (Fig. 5*h* = the 84th section).

The reality of these pictures is indicated by the fact that between the two limiting Z-discs of a sarcomer, i.e. between the 16th and 71st transverse sections, there were altogether 55 successive transverse sections, each of ~300 Å width. That means that this sarcomer had a length of about 2 μ. Thus, the following two inferences can be considered as based on experimental facts: a) the A-substance (of the thick filaments) is able to migrate inside the sarcomer, b) the A-substance (of the thick filaments) migrates along preformed longitudinal elements situated in the sarcomer and passing uninterruptedly from Z to Z.

Discussion

What has just been described agrees well with the conception of Draper and Hodge (1949), according to which — briefly — the *thick filaments* represent parts of the thin filaments on which the A-substance is accumulated. But with this suggestion contrast many papers with figures of transverse sections showing cross sections of thick and thin longitudinal elements in a conspicuous geometrical order.

On the other hand, from the immense literature many other pictures should also be mentioned indicating that cross sections of the thick filaments can show very different forms, such as a circle, triangle or even quite an irregular shape.

Besides, Huxley himself described (1953) that thick filaments have an empty interior, and similar data can be found in many other papers (Hodge, 1955; Stranger, Spiro, 1961; Smith, 1961; Francini-Armstrong, Porter, 1964; Tice, Smith, 1965; Grimstone, Cleveland, 1965; Bacetti, 1965; Brandt et al., 1965; Carlsen et al., 1965; Hoyle et al., 1965; Page, 1965; Audley, Cockbain, 1966; Ashhurst, 1967; Sjöstrand, Jagendorf-Elfvin, 1967; Pepe, 1967; Hagopian, Spiro, 1968). The circumstance that the interior of the cross sections of thick filaments often appears to be empty can perhaps be explained by an old experience. Namely, half a century ago the same circumstances had been described in the case of fibrils (Marcus, 1920), showing an "empty" interior which turned out to have been caused by the fact that the interior had remained unstained (see Pease, Baker, 1949).

The often encountered picture showing an empty interior of the thick filaments is accompanied by the other strange phenomenon that the cross sections of thick filaments consist of many "subfilaments". The number of these "subunits" is given as 9–11 (Bacetti, 1965) or even 13–18 (Gilev, 1966).

Considering the question of the so-called *thin filaments* one encounters a similar uncertainty even in the number of them. In contrast to Huxley's model, showing that six thin filaments surround the thick filaments in a very regular hexagonal array, their number and localization cannot be considered as settled. Their number can be about 8–9 (Hagopian, Spiro, 1968), or 12 (Moritz, 1965), or still more (e.g. Toselli, Pepe, 1967; see also Rosenbluth, 1965). Neither can their localization be considered as regular as it is sometimes pretended to be (see e.g. Elliot, 1964; Bacetti, 1965).

Continuing this train of thoughts we recall the pictures of transverse sections (fibril from the dorsal-longitudinal flight muscle of Letocerus maximus) cut obliquely "at the level of the Z-line. Between the A-band and the Z-line lattices a region of I-filaments only is present" (quoted from Ashhurst, 1967). We counted the number of the cross sections of the filaments per equal areas in plates I and II of that paper. It turned out that e.g. in the upper right part of the picture on plate I there are altogether 36 cm^{-2} cross sections of filaments in the A-band and 38 cm^{-2} in the I-band quite near the Z-line, though in the A-band larger (thick filament) + smaller (thin filament) cross sections were found, whereas in the I-band only smaller ones occur. Besides, we projected the pictures of plates I and II and counted the cross sections of the filaments per equal areas of 5×5 cm; e.g. on plate I F_{A+I}* = 124, F_I = 129; on plate II F_{A+I} = 109, F_I = 105.** This seems to be in contrast with what would be expected on the basis of Huxley's model according to which equal areas in the transverse sections of the A-band and the I-band should contain cross sections of filaments in the ratio of $F_{A+I} : F_I = 3 : 2$, because in the A-band 6 thin filaments belong to every thick one in the manner that every thin filament is shared by 3 thick ones.

* F = Frequency.

** The author's interpretation given in Fig. 1 of her paper is at variance with the electron micrographs of plates I and II.

Considering the fact, generally known for a century, that the A-bands can shorten very much during contraction, one cannot help concluding that the recent muscle models with sarcomers containing the longitudinal elements as being discussed today are not sufficient for a contraction theory.

References

Адамян, С. Я., Кроленко, С. А (1966) Электронная Микроскопия *8* 106
Ashhurst, D. E. (1967) J. Mol. Biol. *27* 385
Ashley, C. A., Porter, K. R., Philpott, D. E., Haas, G. M. (1951) J. Exp. Med. *94* 9
Auber, Dr. (1853) Zschr. wissensch. Zool. *4* 388
Audley, B. G., Cockbain, E. G. (1966) J. Mol. Biol. *18* 321
Bacetti, B. (1965) J. Ultrastr. Res. *13* 245
Barry, (1854) quoted from: German translation by J. E. Purkinje: Opera omnia. Vol. VI, p. 333, Prague.
Bowman, W. (1840) Philos. Trans. II, p. 457
Brandt, P. H. W., Reuben, J. P., Girardier, L., Grundfest, H. (1965) J. Cell Biol. *25* 233
Carlsen, F., Fuchs, F., Knappeis, G. G. (1965) J. Cell Biol. *27* 25
Draper, M. H., Hodge, A. J. (1949) Austr. J. exp. Biol. Med. Sci. *27* 465
Elliott, G. F. (1964) J. Mol. Biol. *10* 89
Ernst, E. (1958) Die Muskeltätigkeit. Versuch einer Biophysik des quergestreiften Muskels. Verl. Ung. Akad. Wiss.
Ernst, E., Benedetzky, St. (1962) Acta Physiol. Acad. Sci. Hung. *212* 211
Ernst, E. (1963) Publications of the Biol. Section, Hung. Acad. Sci. *6* 1 (in Hungarian)
Ernst, E., Garamvölgyi, N. (1956) Acta Physiol. Acad. Sci. Hung. *9* 41
Ernst, E., Kellner, B. (1936) Z. Zellforsch. mikr. Anat. *25* 377
Fan Shih-Fang, Hong Ming-Xia (1962) Acta Biochem. Biophys. Sinica *2* 79
Fawcett, D. W., Revel, J. P. (1961) J. Biochem. Biophys. Cytol. *10* 89
Francini-Armstrong, C., Porter, K. R. (1964) J. Cell Biol. *22* 675
Garamvölgyi, N. (1963) J. de Mikroskopie *2* 107
Garamvölgyi, N. (1965) J. Ultrastr. Res. *13* 409
Garamvölgyi, N., Kerner, J. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 81
Gilev, V. P. (1966) Biochim. Biophys. Acta *112* 340
Grimstone, A. V., Cleveland, L. R. (1965) J. Cell Biol. *24* 387
Guba, F. (1964) Electron Microscopy, Vol. A. Czechosl. Acad. Sci., Prague, p. 77
Hagopian, M., Spiro, D. (1968) J. Cell Biol. *36* 433
Hanson, J., Huxley, H. E. (1956) Symp. Soc. Exp. Biol. *9* 228
Hodge, A. J. (1955) J. Biochem. Biophys. Cytol. *1* II 361
Hodge, A. J., Huxley, H. E., Spiro, D. (1954) J. Exp. Med. *99* 201
Hoyle, G. (1968) Symposium on Muscle. Akad. Kiadó, Budapest, p. 34
Hoyle, G., McAlear, J. H., Gelverston, A. (1965) J. Cell Biol. *26* 621
Huxley, H. E (1953) Biochim. Biophys. Acta *12* 387
Kelly, D. E. (1967) J. Cell Biol. *34* 827
Marcus, H. (1919) Anatomischer Anzeiger, Centralblatt für die ges. Wissenschaftl. Anat. *52* 410
Meigs, E. B. (1912) J. Exp. Zool. *13* 497
Merkel, Fr. (1873) Arch. für. mikroskopische Anatomie *9* 293
Moritz, M. (1965) J. Ultrastr. Res. *13* 383
Page, S. G. (1965) J. Cell Biol. *26* 477
Pease, D. C., Baker, R. F. (1949) Amer. J. Anat. *84* 175
Pepe, F. A. (1967) J. Mol. Biol. *27* 203
Rosenbluth, J. (1965) J. Cell Biol. *25* 495
Schaeffer, E. A. (1891) Proc. Roy. Soc. London B. *49* 280
Sjöstrand, F. (1962) J. Ultrastr. Res. *7* 225

Sjöstrand, F., Anderson-Cedergren, E. (1957) J. Ultrastr. Res. *1* 74
Sjöstrand, F. S., Jagendorf-Elfvin, M. (1967) J. Ultrastr. Res. *17* 348
Smith, D. S. (1961) J. Biophys. Biochem. Cytol. *10* Suppl. 123
Stenger, R. J., Spiro, D. (1961) J. Cell Biol. *9* 325
Tice, L. W., Smith, D. S. (1965) J. Cell Biol. *25* 121
Tigyi-Sebes, A. (1962) Acta Physiol. Acad. Sci. Hung. *22* 243
Tigyi-Sebes, A. (1966) Acta Biochem. Biophys. Acad. Sci. Hung. *1* 407
Toselli, P. A., Pepe, F. A. (1967) J. Cell Biol. *35* 278
Zobel, C. R., Baskin, R. J., Wolfe, S. L. (1967) J. Ultrastr. Res. *18* 637

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (2), pp. 187—194 (1969)

Transversal Structure of the Striated Fibril

E. ERNST, K. KOVÁCS, G. METZGER-TÖRÖK, C. TROMBITÁS

Biophysical Institute, Medical University, Pécs

(Received August 3 and December 14, 1968)

The question whether the native structure of the fibril could be considered as containing preformed transversal elements served as the starting point of our investigations. Some of the results are: 1. Longitudinal sections of stretched fibrils show a transverse line system crossing the longitudinal lines in the I- and A-bands along the whole fibril. 2. Transverse section of a fibril mutilated by treatment with the method of "submicro-blasting" can show long splits bordered by cross sections (of longitudinal elements) which are arranged along straight lines. 3. Other mutilations are also bordered in the same manner. 4. Cross sections (of longitudinal elements) in a straight row displaced to a certain degree from the transverse section of a fibril are connected by straight lines with the cross sections of the next row, which has remained in its normal place. 5. Mechanical pressure brings about equal effects in the rows of cross sections (of the longitudinal elements). 6. A fibril extended transversally regains its normal width when released, i.e. it shows transversal elasticity. 7. The longitudinal section shows some transversal structure when the direction of its shadowing (with aluminium) turned out well. The experimental results are explained by considering the submicro structure of the fibril as a network consisting of preformed longitudinal and transversal elements.

Introduction

Starting from the same sphere of thought as that of the preceding paper we put the question: what is the basis of the transverse mechanical coherence of the fibril? Accepting that the fibril has nothing like a sarcolemma we wish to investigate whether the fibril containing the Z- and M-discs has any other cross-elements which participate in its transversal coherence. By that are meant transversal elements crossing the whole width of the fibril uninterruptedly; hence, the so-called cross bridges provisionally binding together neighbouring filaments are left out of account.

Having used different methods we again describe in successive chapters the single methods together with the results obtained by them.

Methods and Results

1. That the fibril contains some periodic transversal elements passing through its whole width has been demonstrated e.g. by Fig. 6 in the paper of Draper and Hodge (1949), in which a sarcomer of a fibril (not a longitudinal section!) shows regular submicro cross striations with the period of some 400 Å and perpendicular

to the length of the fibril. Since then several similar data have been published about submicro cross striations on longitudinal sections (see e.g. Ernst, 1963).

Fig. 1 also demonstrates such transverse structural elements on a longitudinal section of the sartorius muscle (Rana esculenta) considerably stretched before fixation. In this picture there are cross lines in the stretched I- and A-bands. On the other hand, many data in the literature describe similar uninterrupted lines in the I-bands (e.g. Huxley, 1960; Hanson, Lowy, 1964; Pepe, 1966; Achátz, 1967; Walker, Schrodt, 1967; Huxley, Brown, 1967) *crossing the thin fila-*

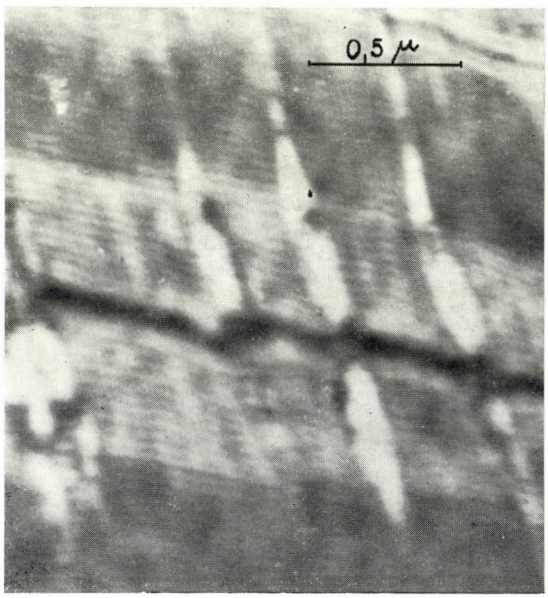


Fig. 1. Longitudinal section of the sartorius muscle (Rana esculenta) stretched very considerably; cross lines in the I- and A-bands

ments, or in the A-bands (e.g. Francini-Armstrong, Porter, 1964; Page, 1965; Pepe, 1967) *crossing the thick filaments*. Should these transversal elements be identified with the cross bridges, then two possibilities could come into consideration: 1. crossing exclusively the thin filaments in the I-band and exclusively the thick filaments in the A-band, these transversal elements lose the function ascribed to them as clamps between thin *and* thick filaments; 2. the cross bridges could be considered as remainders of transversal elements crossing the fibril uninterruptedly in the native state but torn by the methodological procedure.

Disregarding the above implications we assume these transversal lines to demonstrate native transversal elements crossing uninterruptedly the width of the fibril in its whole length.

2. The fact should, however, also be mentioned that in the immense literature pictures of transverse sections of fibrils can be found where not even a trace of transverse organization can be seen (e.g. Hagopian, Spiro, 1968). Fig. 2

shows a part of Fig. 4 (of that paper); neighbouring cross sections of thick and thin filaments have been connected with thicker and thinner lines drawn by us with Indian ink.* This expresses the suggestion *that transversal elements connect the longitudinal ones* in the native state of the fibril.

3. The following chapters will experimentally investigate whether different methods can produce some results in favour of this suggestion.

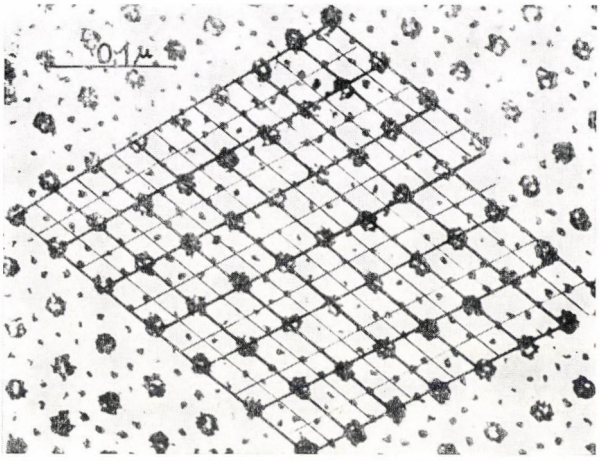


Fig. 2. Cross sections of thick and thin filaments in the transverse section of a fibril (flight muscle of Leucophaea moderae); the cross sections have been connected with thick and thin lines drawn by us with Indian ink

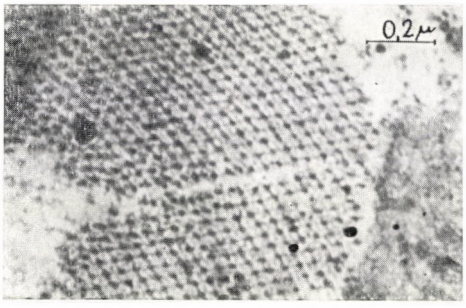


Fig. 3. Transverse section of a fibril (Apis mellifica) frozen at $\sim -70\,^{\circ}C$; a long thin split (see text)

3.1. The fibrils (Apis mellifica) treated by the method called "submicro-blasting" (see the preceding paper) were frozen at $\sim -70\,^{\circ}C$; Fig. 3 shows a transversal section of such a fibril. On the left side a large hole caused by the method can be seen; this hole continues into a long thin split to the right. This

* In this picture another system of connecting lines could have been drawn, namely vertical and horizontal lines intersecting at right angles. The following text explains why the oblique-angled line system was chosen.

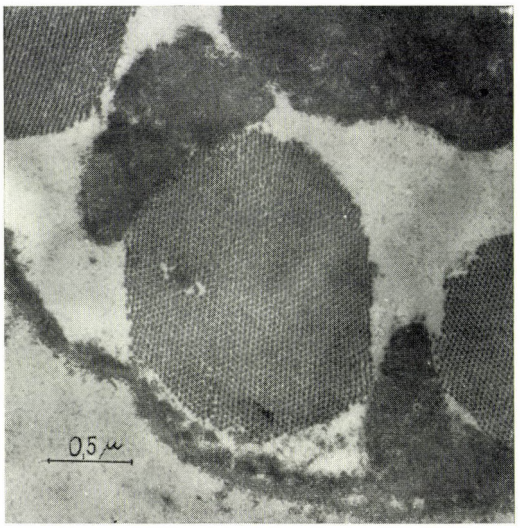


Fig. 4. Transversal section of fibrils (bee) frozen and mutilated; bordering straight lines intersecting at angles similar to those in Fig. 2

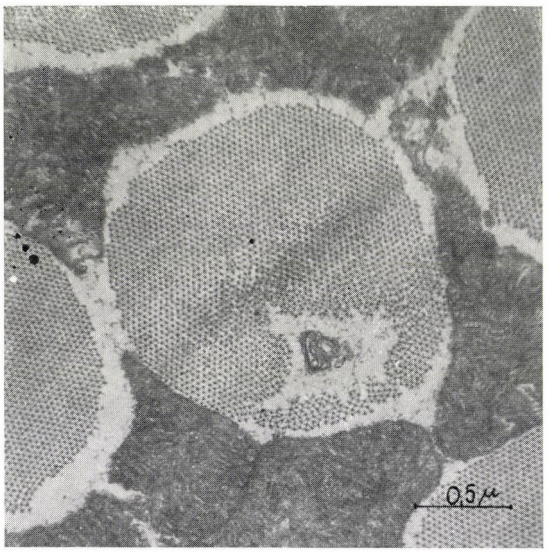


Fig. 5. Transversal section of a fibril (bee) frozen and mutilated on the periphery; bordering straight lines intersecting at angles similar to those in Fig. 2

split is bordered on both sides by cross sections of the longitudinal elements, and these *cross sections are arranged along straight lines.* This result is to be expected according to the supposition that in the native state of the fibril the longitudinal elements are bound together by transversal elements. On the other hand, it can hardly be explained on the basis of the known model containing exclusively

longitudinal elements because, in that case, only irregular groups of cross sections of the longitudinal elements would be expected at submicro-blasting.

3.2. Fig. 4 demonstrates also the transversal section of a fibril (honey bee) treated with the method of submicro-blasting. This time, the marginal parts of the fibril became mutilated in consequence of which the transverse section, instead of showing the form of a circle, is bordered by *straight lines.* And what is more, these lines intersect at *angles very similar to those in Fig. 2;* should the line system drawn by us in Fig. 2 have a concern with the real structure of the fibril?

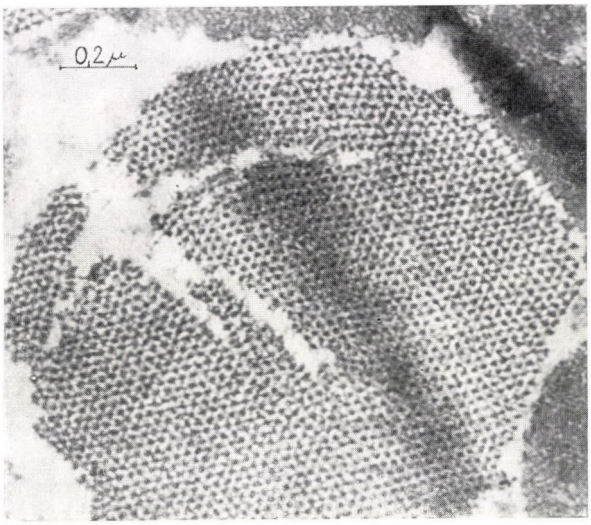


Fig. 6. Transversal section of a fibril (bee) frozen and mutilated; cross sections (of the longitudinal elements) in a straight row are connected with the cross sections standing in the next row (upper right corner)

3.3. Further illustrating this question, Fig. 5 may follow; it shows a transverse section of a fibril treated by the method of submicro-blasting. Several mutilations on the periphery are to be seen bordered with longer or shorter *straight lines* intersecting, again, *at angles similar to those in Fig. 2.*

3.4. Fig. 6 demonstrates also some mutilated parts on the periphery of the transverse section and, besides, *several cross sections* (of longitudinal elements) *forming a straight line.* Though at a certain distance from the next one, this row of cross sections has *remained connected with the former by short transverse lines.* These short lines, connecting the cross sections in the displaced row with those in the next one, may be considered as parts of the continuous transversal elements connecting the longitudinal elements in the native state of the fibril.

3.5. The assumption of transverse elements which, crossing the fibril uninterruptedly and tying the longitudinal elements together, keep the fibril together, helps to explain the mechanism of how the structure shown in Fig. 7 could be produced. Namely, the fibril was treated by the method of submicro-blasting, and accordingly a hole in the transverse section (upper left corner) can be seen.

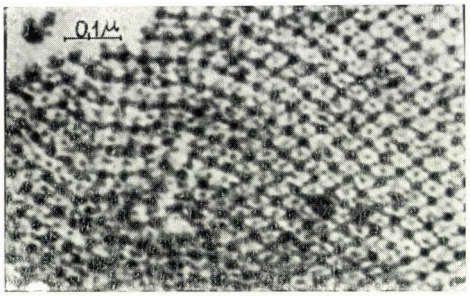
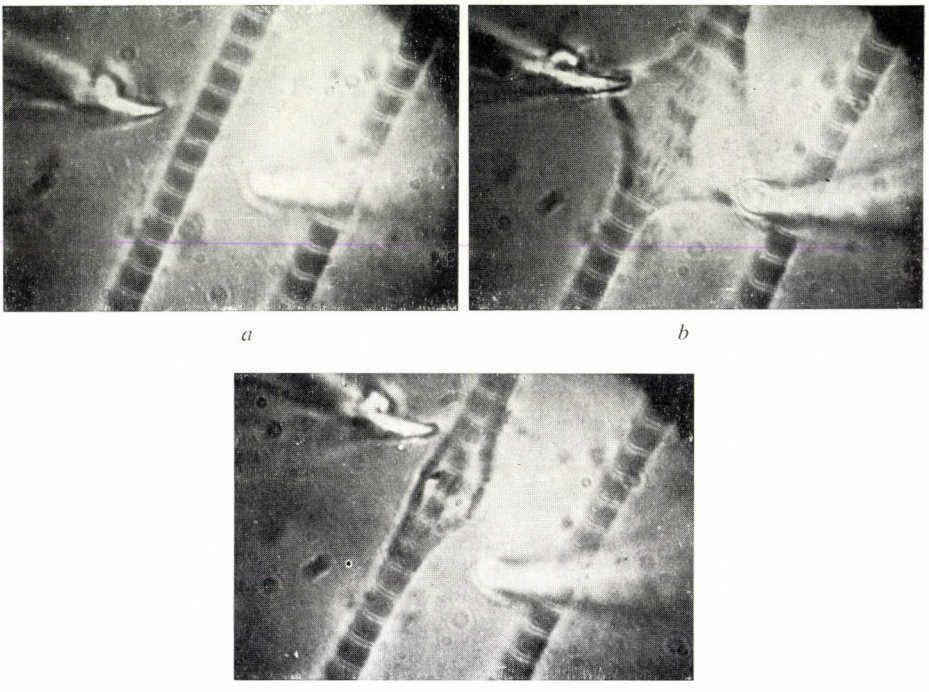


Fig. 7. Transverse sections of a fibril (bee) frozen; the hole (upper left) shows the mechanical mutilation causing displacement in the rows of cross sections of the longitudinal elements



a *b*

c

Fig. 8. Fibril (bee) torn in a micromanipulator; *a:* fibril with two glass needles; *b:* fibril extended transversally (and torn at two places); *c:* glass needles unhooked, the middle contracted to its former width. × 3000

Starting from this the rows of cross sections (of the longitudinal elements) *show lines a) curved in S-form and b) parallel to each other.* a) The uninterrupted S-form of the single rows of cross sections indicates that these cross sections i.e. the longitudinal elements, the cross sections of which are in the rows, were bound together by transversal elements before they were impressed by the mechanical effect of the method. b) The fact that the neighbouring *rows of the cross sections* (of the longi-

tudinal elements) had been *equally affected* by the method causing mechanical pressure renders the assumption probable that these rows were bound together in the native state before having suffered the mechanical effect. In contrast with that self-evident explanation, the believers in the model accepting exclusively longitudinal elements in the fibril would encounter great difficulty in understanding the picture shown in Fig. 7.

4. Supposing the existence of transversal elements which hold together the native fibrils and strengthen their mechanical coherence transversally, we wished to investigate the latter aspect also directly. For this purpose the Zeiss micromanipulator was used as described in the preceding paper (Ernst et al., 1969). Figs 8*a*, *b*, *c* are taken from such an experiment; *a:* fibril (honey bee) between the

Fig. 9. Longitudinal section of stretched sartorius muscle (frog); the sections are extracted with amylacetate and shadowed with aluminium; some transverse structure is to be seen

two glass needles; *b:* the fibril is transversally extended and torn at two places; *c:* the glass needles unhooked, the fibril *contracted transversally to its former width.* The transversal elasticity investigated and qualitatively proved by these experimental results indicates that the native fibril contains transverse structural elements crossing its width.

5. Fig. 9 serves to demonstrate the existence of a transversal structure in the fibril. Sartorius muscle (frog) was stretched, fixed, embedded in acrylate, unstained, extracted to a certain degree with amylacetate, and shadowed with aluminium (Trombitás, in press). Due to the circumstance that the direction of the shadowing turned out well, the transverse structure or its remainders are quite conspicuous.

Discussion

Investigating a structure one bears in mind a function and thus, considering some data about the structural details of the muscle, one tries to use them as a basis on which a contraction theory can be built up. The building stones are required to be perfectly reliable, on the one hand, and, in our case, they can be obtained exclusively by experiments, on the other. Now, experiments on muscle structure are performed — as it mostly happens — on material deformed by methodological treatment, therefore they very seldom produce data free of distortion or, at least, of some changes. Furthermore, when preparing longitudinal or transverse sections from a three-dimensional body one tries to elucidate the real structure from two-dimensional pictures, which can often be accompanied by optical mistakes (e.g. Moiré patterns, see Achátz, 1968).

That is why Hoyle's admonition should never be forgotten (1968): "It may be unwise to assume that the features found on the first fibre to be investigated in detail should be expected to serve as a basis for a universal hypothesis, and it seems desirable to proceed by making comparative studies." In this context e.g. the data may be mentioned according to which 7–9 transversal lines appear in the A-band due to anti-myosin staining (Pepe, 1967); but quite similar pictures can be seen on longitudinal sections of a sartorius muscle (frog) without any similar treatment (Trombitás, in press).

Considering caution obligatory in hypothesis or even in statements, we wish to regard our investigations described above as making an attempt at the conception that the fibril's submicro structure consists of a network of longitudinal and transversal elements. And even if this conception proves verifiable, still much work will lead to an acceptable hypothesis of the mechanism of contraction, perhaps by using the conception e.g. of Katchalsky (1949; 1954) or Kuhn (1962) or some other ideas referring to electronic effects.

References

Achátz, I. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 225
Achátz, I. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 183
Draper, M. H., Hodge, A. J. (1949) Austr. J. Exp. Biol. Med. Sci. *27* 465
Ernst, E. (1963) Biophysics of the Striated Muscle. Publishing House of the Hung. Acad. Sci., Budapest
Ernst, E., Kovács, K., Metzger-Török, G., Trombitás, C. (1969) Acta Biochim. Biophys. Acad. Sci. Hung. *4* 191
Francini-Armstrong, C., Porter, K. R. (1964) J. Cell Biol. *22* 675
Hagopian, M., Spiro, D. (1968) J. Cell Biol. *36* 433
Hanson, J., Lowy, J. (1964) Circulation Res. *14—15* Suppl. II-4
Hoyle, G. (1968) Symposium on Muscle, Publ. House of the Hung. Acad. Sci., Budapest, p. 34
Huxley, H. E. (1960) The Cell. Brachet, J. et Mirsky, A. E. (eds) Acad. Press New York, *4* 369
Huxley, H. E., Brown, W. (1967) J. Mol. Biol. *30* 383
Katchalsky, A. (1949) Experientia *5* 319
Katchalsky, A. (1954) Progress in Biophysics *4* 1
Kuhn, W., Tóth, I., Kuhn, H. J. (1962) Helv. Chim. Acta *45* 2325
Page, S. G. (1965) J. Cell Biol. *26* 477
Pepe, F. A. (1966) J. Cell Biol. *28* 505
Pepe, F. A. (1967) J. Mol. Biol. *27* 203
Trombitás, C. (1969) Acta Biochim. Biophys. Acad. Sci. Hung. (in press)
Walker, S. M., Schrodt, G. R. (1967) Abstracts Biophys. Soc. p. 114

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (2), pp. 195—199 (1969)

Development of Media Suitable for Dose Measurement

Dose Measurement Media

I. NIKL

"Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest

(Received January 25, 1969)

The paper discusses the use and development of small animal media designed for dose measurement. Homogeneous phantoms of animals in various postures and systems, paraffine-impregnated animal cadavers and media fixed by refrigeration are described.

Introduction

The problem of the actually absorbed dose and actual exposure at various points of the animal's body, organs or in its total body often arises when performing animal experiments with various radiation sources. This problem may arise when evaluating the results, eventually in an intermediate phase of the experiments or even as early as when outlining the experimental project. In the majority of cases the answer will be given by the results obtained by dose measurements. A suitable medium is one of the basic requirements for dose measurements.

Three various media were developed for measuring X-ray and gamma-doses: homogeneous phantom, prepared and frozen animal cadaver.

As a rule, one cannot be satisfied with obtaining measurement results but once in the various points of the volume in question. It is hardly disputable that it is rather difficult, sometimes even unfeasible, to repeatedly fix the detecting device to the same points within the body of the animal. Maybe, the animal will not even be able to endure several interventions of this kind. Should the above difficulties not exist, the animal cannot practically be brought into the very same position. Being convinced that, in spite of all its difficulties, *in vivo* dose measurement cannot be spared in practice, as a next step we started to study and to construct various phantoms.

Solid state, gel or liquid mixtures may, as a rule, be used as phantom materials. The degree to which such mixtures may simulate mammalian tissues depends on the quality and energy of the radiation.

Water is one of the most ancient and even nowadays frequently used phantom materials. Often the dosimeter itself is used as a phantom (HAP dosimeter, plastic scintillator, Fricke's dosimeter).

As regards its shape, the phantom may accurately simulate or approach by some regular geometrical formation the shape of the animal. When using a regular geometrical formation, the "equivalent" sizes have to be determined by special comparative measurements.

Experimental

1. Within the scope of our research project, homogeneous phantoms of different systems simulating small animals in different postures were produced from two types of solid phantom materials (Mix D and M 3). Apart from a deviation of a few per cent, their weights and volumes agreed with those of the living models (Fig. 1). Some characteristics of these materials (Markus, 1956) are given in Tables 1a, b, the geometry and weight data of the phantoms in Table 2.

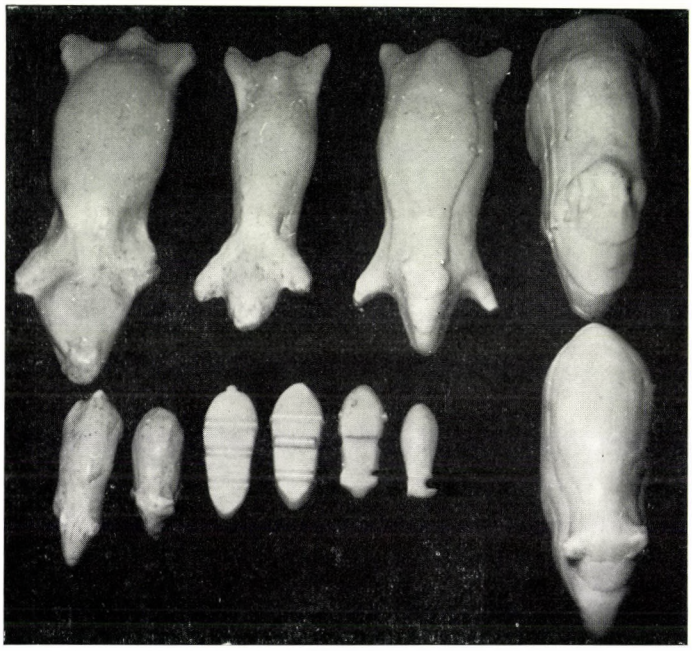


Fig. 1. Homogeneous animal phantoms

Simplicity and durability are the most characteristic parameters of a phantom, qualities mainly accounting for its application. On the other hand, simplicity in simulation represents a drawback. In the majority of cases, the varying tissues of the mammalian organism are substituted by water or by some water-equivalent or muscle-tissue approaching substance (homogeneous phantom). The substitution of tissues that very much differ from the others (bone, lung, fat) represents a special problem. Even if they are available, their building together is difficult and tiresome.

2. Trying to find some other method, easier than to construct an inhomogeneous phantom, we studied preparation preserved by paraffine embedding (Gellért et al., 1967). The process of embedding was the following: depending on their size, the various objects were fixed in 4 per cent formol, for 2 or more weeks. Washing in tap water for 24 hours followed, i.e. preceded the proper dehydration process in 50, 70, 80 and 96 per cent alcohol, depending again on the size and

Table 1

a) Mass absorption coefficients

		10 KeV	50 KeV	100 KeV	200 KeV	1 MeV	5 MeV	100 MeV
H_2O	$\mu'=\tau+\sigma_a+\pi_a$	5.243	0.0580	0.0280	0.03007	0.03090	0.01942	0.01640
Mix D	$\frac{\left(\frac{\mu'}{\rho}\right)_I}{\left(\frac{\mu'}{\rho}\right)_{H_2O}}$	0.84	0.93	0.99	1.02	1.05	1.00	0.85
M 3	$\frac{\left(\frac{\mu'}{\rho}\right)_{II}}{\left(\frac{\mu'}{\rho}\right)_{H_2O}}$	0.98	0.99	0.99	0.99	0.99	0.99	0.91

b) Electron densities

	g/cm³	N (electron)g
H_2O	1.00	$3.34 \cdot 10^{23}$
Mix D	0.99	$3.40 \cdot 10^{23}$
M 3	1.06	$3.30 \cdot 10^{23}$

Table 2

Geometry and weight data of homogeneous animal phantoms

	Posture	Maximum		volume, mol	weight, g
		height, mm	length, mm		
Mouse phantom	natural posture	26.4	69	25	23
Rat phantom	natural posture	51	150	230	215
Rat phantom	stretched body posture	36	195	250	232
Guinea-pig phantom	natural posture	78	194	470	460

nature of the object. The alcohol concentration was permanently checked and the liquid changed, if necessary. Prior to impregnation with paraffine, the specimen was placed into benzine used as intermedium, for 2 to 12 days, according to necessity Benzine was renewed twice. Embedding was performed in vacuum. Paraffine s re.placing the lacking part in tissues preserved in this manner. As an informative experiment, several animal organs (ox thigh, liver, kidneys, brains, spine, rabbit spine) were embedded into paraffine. The weight loss of the prepared organs ranged from 11 to 68 per cent, related to their crude weights representing 100 per cent. All these organs shrinked strongly and their densities also decreased.

Also their permeability in the range of therapeutic X-ray doses decreased by 4 to 11 per cent. On the basis of our experiences the process of preparation was modified so as to promote paraffine embedding by the prolongation of "vacuum time".

Weight and volume values in crude and paraffinized states, resp., representing the mean of 10 mice, are indicated in Table 3. Change in density as related to the entire body of the mouse is minimum. According to all probability, the various organs take up paraffine to various extents. This may result in shifts, as compared to conditions in crude or living state. Weight data for rabbits are also indicated in this Table. As regards preparation, further improvement and reducing the rate of shrinking should be achieved, eventually by attempting some other method of preparation.

Table 3

Data for crude and prepared animal cadavers

Mouse	Crude	Paraffinized	
Weight	22.3 g	21.5 g	
Volume	25.0 ml	24.5 ml	
Density	0.89 g/ml	0.88 g/ml	
Rabbit	**Crude**	**Paraffinized**	**Weight loss %**
I	2500 g	1955 g	21.6
II	2300 g	2265 g	1.5

The idea of applying an appropriately preserved human cadaver for dosimetry purposes turns up automatically from the above. Gellért (1935) preserved several cadavers by embedding into paraffine for the purpose of demonstration. Hodes and Ramsay (1964) describe a cadaver preserved in some other manner, designed for practising the taking-up of X-rays. However, none of these authors has studied the difference between crude and preserved tissues from the aspect of dosimetry. Depending on the type of the tissue, the various preservation methods may modify the original tissues to various extents.

3. As a next step, the killed animals were deep-frozen. With this procedure the change in the volume of the tissues is minimum, their composition remains practically unchanged, the animal is being fixed in a given posture. Following appropriate deep-freezing such phantoms warm up to room-temperature rather slowly (e.g. a guinea-pig weighing 450 g took 100 minutes to warm up from -50 °C to -3 °C, and another 20 minutes to warm up from -3 °C to -1.5 °C). This means that sufficient time will be left for all the preparatory operations preceding dose measurement (e.g. for cutting into pieces, boring, etc.) and also for repeated dose measurements. Apart from other factors, both the rate of freezing and that of warming-up depend also on the volume of the animal. In case of a quicker warming-up, or when longer preservation is required, the cadaver may be refrigerated again whenever necessary (Fig. 2).

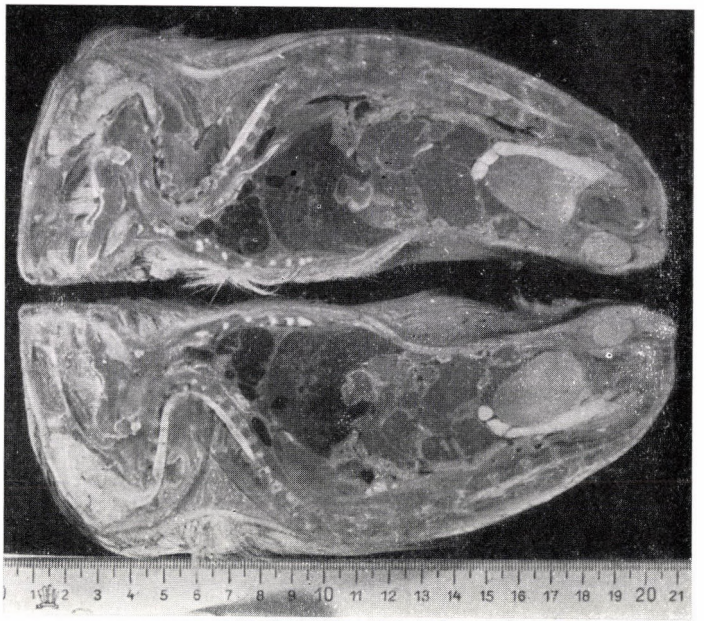


Fig. 2. Section of a frozen guinea-pig

Discussion

The frozen animal cadaver seems to be best for simulation. Indeed, it is more than simulation. It enables the satisfactory reproduction of the measurement sites. Its use is limited only by the low temperature and the possibility of preservation.

The frozen animal has the undisputable advantage that, provided shrinkage is not too strong, the tissues simulate the truth according to shape and may be differentiated. The extent to which the frozen animal, i.e. prepared animal, approaches the living one under given conditions should be determined by direct comparative measurements and its use should be decided thereafter.

The homogeneous or inhomogeneous animal phantom gives but an average of the shapes and material composition of the living organism or it approaches that to a certain extent only. When evaluating the results obtained on the phantom one should rely on the results of the direct comparative measurements.

References

Gellért, A. (1935) Szegedi Leíró és Tájbonct. Int. Közl. *20* 1
Gellért, A., Kozma, M., Nikl, I. (1967) Magyar Rad. *6* 373
Hodes, P. J., Ramsay, A. J. (1964) Am. J. Roentg. *92* 215
ICRU Report (1962) No. 10 e
Koczkás, Gy., Dósay, K. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 305
Markus, B. (1956) Strahlenther. *101* 111
Potsaid, M. S., Irie, G. (1961) Radiology *77* 61
Potsaid, M. S., Umezawa, T. (1965) Am. J. Roentg. *93* 725

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (2), pp. 201—209 (1969)

The Use and Comparative Study of Dose Measurement Media in Animal Experiments

Various Media in Animal Irradiations

I. NIKL

"Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest

(Received January 25, 1969)

Irradiation exposure in several points of the irradiated volume is measured in a homogeneous animal phantom, frozen animal cadaver and *in vivo*, following the irradiation by an external source of small animals. The results obtained in various media and under various geometrical conditions are compared. It is established that the parameters obtained for the homogeneous phantom, frozen cadaver and living animal by the same exposure are in a good agreement. This, however, approaches the actual conditions by a certain error only. The author establishes that a considerably better homogeneity may be achieved by multilateral irradiation, the degree of irradiation homogeneity changing as a function of the posture of the animal of the same size.

Introduction

Report No. 10e of the I.C.R.U. sets up three classes to define the "homogeneity" of animal irradiations. Accordingly, irradiation should be considered as uniform in all cases when the maximum absorbed dose (M) is not more than 1.1-fold of the minimum dose (m) (Class A) in the irradiated volume; as moderately uniform up to 1.3 (Class B) and as inhomogeneous above 1.3 (Class C).

The principles laid down in the I.C.R.U. Report attempt to standardize the various experimental irradiations as well as to reach a dose distribution as homogeneous as possible in the irradiated volume when irradiating biological objects. All these would restrict the wide spectrum of the parameters of radiobiological experiments.

Theoretical Part

Depending on the actual conditions of irradiation (type of source, geometry between the source and the animal, size of the animal, etc.) the rate of homogeneity may vary between wide limits and should be determined in each case separately. Once the rate of homogeneity is established one knows to what extent the exposure arrangement should be modified to achieve homogeneity.

The performance of measurements reflecting homogeneity conditions is no easy task. The tissues in mammalian organism are rather inhomogeneous (fat, muscle, bone, lung). In the category of small and medium size animals the volume of contiguous tissues of the same sort is rather small. Accordingly, the size of the

detector has to be adequately small. The size of the conventional, most frequently used ionization chamber exceeds that of the contiguous tissue volume, it is sometimes as big as the volume of the animal's body (e.g. mouse and X-ray thimble chamber). In a case like that one cannot even think of "scanning" irradiation exposure in various points of the experimental animal and, knowing the absorption coefficients for the various tissues, of determining the absorbed dose for point to point. Such absorbed dose data would enable the calculation of the M/m value defined by the I.C.R.U.

The performance of measurements is not simple even if adequately small size detectors are available. Namely, the border surface between the various tissues is relatively large in the experimental animal and no electron equilibrium is completed on such surfaces. It is perhaps just the detector itself that upsets irradiation equilibrium in the otherwise homogeneous tissue.

When replacing the mammalian organism by some homogeneous matter, differentiation of the various tissues is automatically given up. The degree of the so obtained "error" depends on whether the phantom material is an average for all the tissues of the living organism or only for some of them (e.g. the water phantom simulates but the muscle tissue, it does not simulate bone tissue). Such an averaging is also energy dependent (e.g. a Cobalt-60 source senses less the difference between bone and muscle tissues than an X-radiation excited by 200 kV).

As it appears from the above, it is rather difficult to define the degree of homogeneity established by the I.C.R.U. as M/m in practice.

Experimental

Some measurement results obtained in homogeneous animal phantoms, in animal cadavers preserved by deep-freezing and *in vivo* will be reported on as follows. The cylindric condenser chamber of the PTW Kondiometer, 7 mm in diameter and 16 mm long, was the smallest detector at our disposal. As compared to the volume of small animals, this detector is not ideally small. The ionization chambers were fitted into the holes made into the homogeneous phantom or in the frozen animal cadaver. The empty parts remaining after the introduction of the condenser chamber and the other empty bore-holes were filled in with stoppers made of the same substance as the phantom. As regards the bore-holes in the frozen animal cadavers, they were also filled in with stoppers made out of Mix D phantom material, i.e. by a substance not identical with the animal tissues.

Under such conditions, since the volume of the detector used is sufficiently large as compared with the irradiated volume, the measured value represents integration. The change of the spectrum was not taken into account in case of the results obtained in various depths. When performing measurements in animal cadavers, the surface of the detector touches several such tissue borders, where no electron equilibrium exists. Owing to the homogeneous stoppers of the phantom material introduced into the bore-holes in the frozen animal, the detected count approaches the result obtained for the homogeneous phantom. Owing to the low number of measuring sites, the volume in question could not be scanned precisely enough. The M/m value calculated from the results is not the one which

would correspond to the I.C.R.U. definition. Considering the type of radiation used as well as the energy, this represents an appreciable difference as compared with the actual conditions. Accordingly, the conclusions drawn from the results are only of an approximative, informative character. Still, they are characteristic of the development of homogeneity conditions. Considering the given measurement possibilities, we could not aim at completeness.

An X-ray unit was used as source. Results in the Tables are given in exposure (R). Exposure times vary from medium to medium. They were always chosen so as to ensure the best measurement range for the unit.

Results

a) A mouse phantom weighing 23 g, volume 25 ml, was whole body irradiated in the midpoint of the radiation field. The focus distance (FD) indicates the distance between the surface of the irradiation table and the focus. Fig. 1

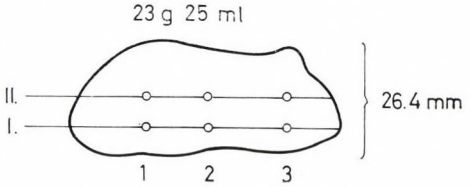


Fig. 1. Scheme of measurement points in a homogeneous mouse phantom in natural posture

indicates the sites of the measurement points, Table 1 the exposures. M/m calculated from 6 exposures is 1.11.

Table 1

Mouse phantom: 23 g, 25 ml (natural posture), 180 kV, 0.5 mm Cu, FD = 60 cm, projection: P.A.

	1	2	3
II	64.5 R	64.8 R	65.7 R
I	58.9 R	60.0 R	60.1 R

b) A rat phantom weighing 215 g, of a volume of 230 ml, was whole body irradiated in an arrangement similar to the above. Radiation exposures obtained in the points indicated in Fig. 2 are given in Table 2. The M/m value of the 12 exposures runs to 1.43. Accordingly, irradiation is inhomogeneous in the volume defined by the measurement points.

c) When totally irradiating a rat phantom stretched on its abdominal side, we found the M/m value calculated from 12 measurement points (Fig. 3 and Table 3) to run to 1.21. As compared with the rat phantom in a natural posture, homogeneity conditions are more advantageous. This is due to the changed geometrical arrangement between the source and the medium.

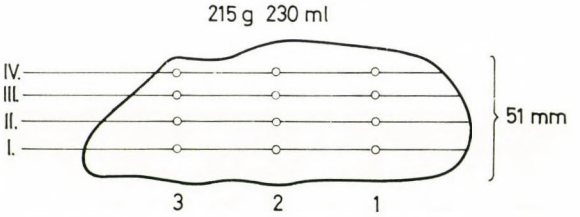


Fig. 2. Scheme of measurement points in a rat phantom in natural posture

Table 2

Rat phantom: 215 g, 230 ml (natural posture), 200 kV, 0.5 mm Cu, projection: P.A., FD = 60 cm

	1	2	3
IV	98.7 R	98.6 R	94.6 R
III	93.3 R	92.0 R	89.0 R
II	82.7 R	83.6 R	80.5 R
I	72.7 R	73.3 R	68.9 R

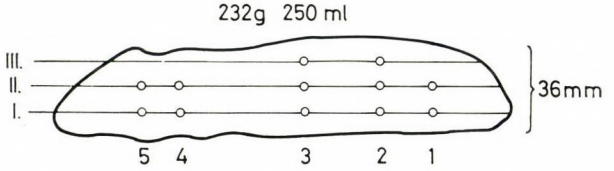


Fig. 3. Scheme of measurement points in rat phantom stretched on the abdominal side

Table 3

Rat phantom: 232 g, 250 ml (stretched body posture), 200 kV, 0.5 mm Cu, FD = 60 cm, projection: P.A.

	1	2	3	4	5
III	—	72.9 R	73.0 R	—	—
II	66.5 R	67.8 R	67.5 R	69.4 R	67.1 R
I	59.9 R	60.9 R	61.0 R	62.7 R	61.2 R

d) Exposures measured in a guinea-pig phantom weighing 460 g and dismountable into horizontal segments, and in a 450 g frozen guinea-pig cadaver cut up in a similar way, are compared in Fig. 4a. The temperature of the frozen medium was kept at $-1.5\,^{\circ}C$. The scheme of the measurement points is given in Fig. 4b, and exposures are given in Tables 4 and 5. Exposure values measured in the corresponding points of the two media are rather close to each other. The

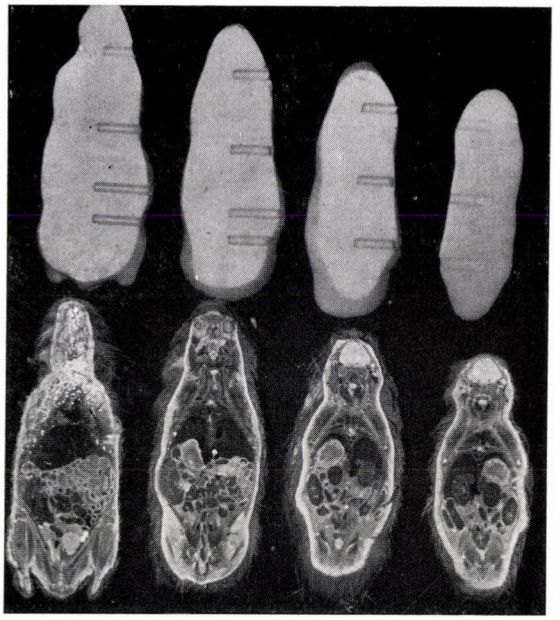


Fig. 4a. Measurement planes in a homogeneous guinea-pig phantom and frozen guinea-pig cadaver, resp.

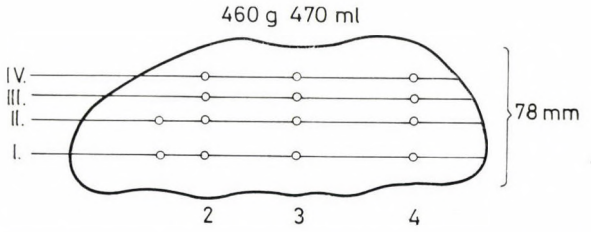


Fig. 4b. Scheme of measurement points in a homogeneous guinea-pig phantom and in a frozen cadaver, resp., in natural posture

maximum difference between them runs to 11 per cent. However, even though the values approach each other, they do not actually agree.

e) The guinea-pig phantom specified in Paragraph *d)* was used for the comparison of homogeneity conditions after uni- and multilateral irradiations. The measurement points agree with the above. The distance between focus and the medium was measured from Segment II of the phantom (FD). A 2.5 mm thick plexi glass was used as irradiation table. Total irradiation was first performed from the P.A. direction (Table 6). Then the source was turned by 180° and irradiation was repeated from the A.P. direction (Table 7). As it clearly appears from both Tables, homogeneity conditions are most disadvantageous after both kinds of irradiation (M/m: 1.8 and 1.9).

Table 4

Guinea-pig phantom: 460 g, 470 ml (natural posture), 200 kV, 1.0 mm Cu, FD = 60 cm, projection: P.A.

	1	2	3	4
IV	—	71.8 R	72.1 R	64.3 R
III	—	66.9 R	67.6 R	57.9 R
II	63.0 R	59.7 R	60.2 R	50.6 R
I	53.7 R	51.3 R	50.7 R	41.4 R

Table 5

Frozen guinea-pig cadaver: 450 g, 476 ml (natural posture), 200 kV, 1.0 mm Cu, FD = 60 cm, projection: P.A.

	1	2	3
IV	—	71.4 R	—
III	66.2 R	—	68.0 R
II	60.0 R	—	62.6 R
I	53.2 R	—	56.2 R

Table 6

Guinea-pig phantom: 460 g, 470 ml (natural posture), 200 kV, 0.5 mm, FD = 64 cm, projection: P.A.

	1	2	3	4	
IV	—	44.0 R	44.6 R	39.0 R	
III	—	41.1 R	41.5 R	34.6 R	
II	39.1 R	36.6 R	36.2 R	30.4 R	M/m = 1.8
I	32.6 R	30.6 R	30.0 R	24.0 R	

Provided half of the unilateral exposure was delivered from the A.P., the other half from the P.A. direction (Table 8), rather advantageous homogeneity conditions — approaching those of the small volume mouse — were obtained. M/m ran to 1.12 as compared with the M/m values of 1.8 to 1.9 obtained after unilateral irradiation. Similar examples could be quoted for bi- or multilateral exposures, too. When considering which type of irradiation should be used, the size, number and arrangement of the animals should be decisive. It is beyond dispute that the practice of multilateral irradiation represents an advantage when attempting to create an almost homogeneous exposure in the irradiated volume of the animal. However, whenever performing multilateral irradiations the technical conditions should be chosen carefully and be maintained up to the end of irradiation. On the other hand, one should never forget that the same exposure may induce various biological effects if delivered uni-, bi- or multilaterally (Wald, Várterész, Bozóky, 1951).

Table 7

Guinea-pig phantom: 460 g, 470 ml (natural posture), 200 kV, 0.5 mm Cu, FD = 64 cm, projection: A.P.

	1	2	3	4	
IV	—	21.6 R	23.0 R	23.6 R	
III	—	26.2 R	28.7 R	28.4 R	
II	31.0 R	32.2 R	32.7 R	33.5 R	M/m = 1.9
I	38.9 R	39.9 R	40.7 R	41.7 R	

f) When irradiating several animals simultaneously, depending on their sizes the exposures within the volume of the animal may be modified due to scattered radiation originating from the neighbouring medium, eventually irradiation will not even be symmetrical (Figs 5, 6).

g) Measurement results obtained in a homogeneous phantom and *in vivo* are compared in the following two cases.

a) Dosimeters of the same type were simultaneously introduced into the abdominal cavity of a mouse phantom and of two living mice. The media were put in a container designed for the irradiation of mice. The remaining compartments were filled with living mice. Total irradiation was performed with 200 kV, 0.5 Cu filter. The results given in Fig. 7a were obtained.

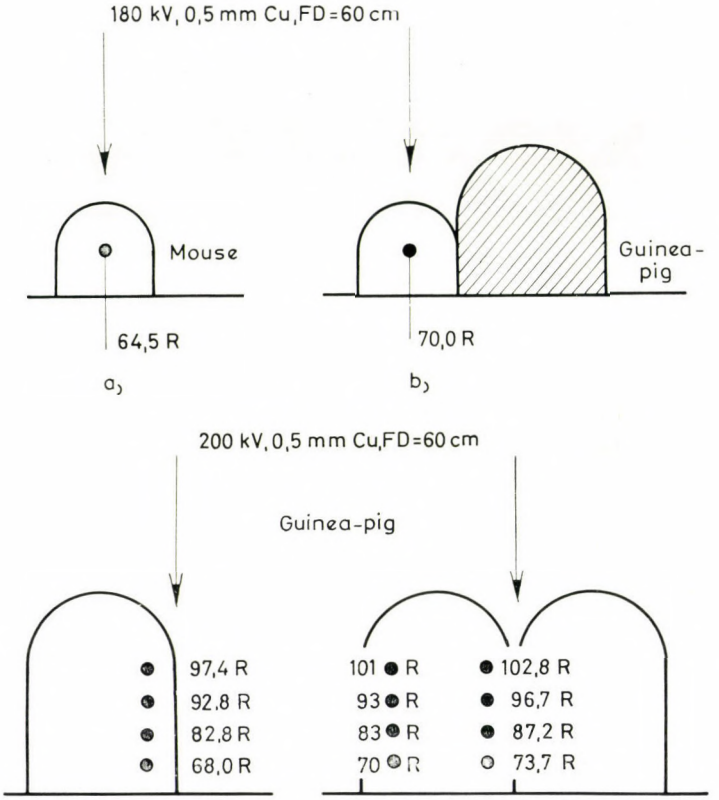
b) A dosimeter was introduced into the stomach of each of two living rats weighing 250 g each. At the same time two dosimeters were placed into a rat phantom of the same size: one of them into its "stomach", the other somewhat higher. All these were irradiated in the arrangement as shown in Fig. 7b. When dissecting the rats, it has turned out that the stomach of the rat given 118 R was situated appreciably higher in the animal's body than that of the other animal.

Table 8

Guinea-pig phantom: 460 g, 470 ml (natural posture), 200 kV, 0.5 mm Cu, FD = 64 cm, projection: A.P. + P.A.

	1	2	3	4	
IV	—	32.8 R	33.8 R	31.3 R	
III	—	33.7 R	45.1 R	31.5 R	M/m = 1.12
II	35.0 R	34.4 R	34.5 R	31.9 R	
I	35.8 R	35.2 R	35.4 R	32.8 R	

The dosimeters could be introduced into the body of the animals by a rather rough intervention, by abdominal section only. Small size dosimeters are more appropriate for these measurements.



Figs 5 and 6. Changes in irradiation dose due to scattered radiation from neighbouring media

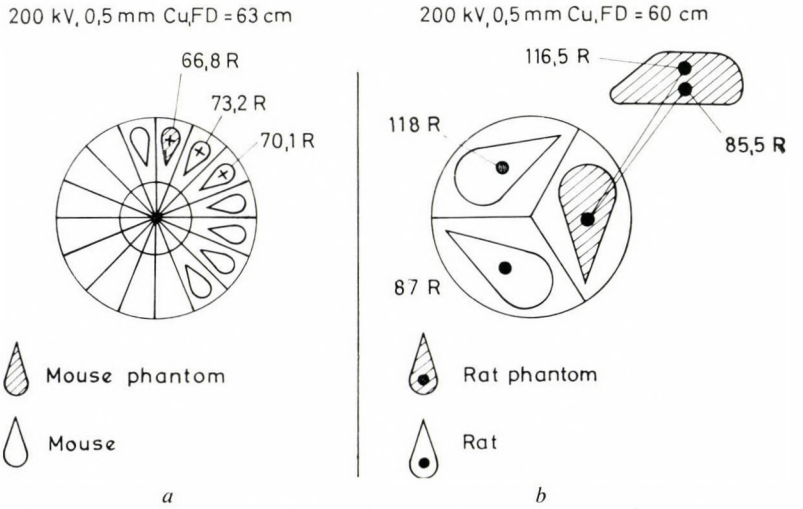


Fig. 7a and b. Comparison of irradiation doses of living animals and animal phantoms

Discussion

Under the above cautels, one may conclude from the data cited above that, in the case of the applied unilateral irradiations being inhomogeneous, an appreciable improvement may be achieved by delivering the same dose in form of a multilateral irradiation. In animals of the same size the homogeneity rate of irradiation is a function of the posture of the animal. The parameters measured by the same exposure and compared for the homogeneous phantom, frozen cadaver and living animals are in a good agreement. However, they reflect actual conditions with a certain error only. On this basis, provided the appropriate instrument and medium are available, one may project a systematic survey by measuring the absorbed dose or some quantity proportional to it.

References

Benner, S., Johansson, J. M., Lindskoug, B., Nyman, P. T. (1966) Solid State and Chemical Rad. Dosimetry in Med. and Biology (Proc. of a Symp., Vienna), p. 65
I.C.R.U. Report (1962) 10e.
Reichel, G., Nicht, M., Rösler, B. (1967) Strahlenther. *133* 390
Reichel, G., Rösler, B. (1968) Rad. biol. ther. *9* 197

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (2), pp. 211—216 (1969)

Effect of Radioactive Radiations on the Lifetime of Sperms

A. NIEDETZKY, CS. LAJTAI

Biophysical Institute, Medical University, Pécs

(Received January 18, 1969)

The effect of β and γ radiation on the motility of ripe sperms has been examined. It has been shown that β radiation with dosages of 20 to 1200 rad decrease the motility of sperms significantly. γ radiation is efficient but in dosages over 800 rad. The experiments have shown that β radiation exerts a given effect in dosages of one order of magnitude lower than does γ radiation. A correlation can be demonstrated between the effect and the dosage of irradiation. The radiation-resistance against β and γ radiation of ripe sperms is smaller than the values found in the literature concerning X-ray radiation.

Introduction

The great radiosensitivity of the testis is well known from literary data. The radiosensitivity of different cell forms of the spermatogenesis is rather varying. According to the data at our disposal spermatogonia represent the most radiosensitive cell forms and dosages as low as 5 R of X-ray radiation, 3 rad of γ radiation or 2 rad of neutron radiation already decrease their number significantly (Oakberg, 1955, 1957, 1959, 1962). Contrasting to this, sperms are radiation-resistant to a high degree (Bloom, 1948; Oakberg, 1955; Hollaender, 1954; Siegel, 1966), and ripe sperms are not damaged even by irradiation with 80 000 R (Fritz-Niggli, 1959). It is also known that the radiosensitivity of sperms is not constant (Spear, 1953) but their motility is not influenced by irradiation. However, according to the data of Herpen and Rikmenspoel (1968), a half of the bull-sperms is killed under the effect of an X-ray dosage of 10 000 R, and also the motility of the remaining sperms decreases to 45 per cent of the original value.

The data concerning the question refer almost without exception to the effect of X-ray radiation. On the other hand, the effect of irradiation has been examined after irradiation of the whole body of the animals. However, if we want to examine the effect of irradiation on a definite kind of cells, this method is disadvantageous because of the simultaneous interaction of very complicated circumstances and other factors.

We examined, therefore, in our experiments the effect of outer β and γ radiation on the motility and lifetime, respectively, of isolated ripe frog-sperms. It is known that motility is closely related to the fertilizing ability.

Methods

Two testes of the decapitated frog were isolated, cut into several pieces, put into 25 ml of Holtfreter fluid medium and smashed up by a glass rod.

The Holtfreter fiuid medium applied in the experiments contained 0.350 g NaCl, 0.005 g KCl, 0.01 g $CaCl_2$, 0.020 g $NaHCO_3$ in 100 ml of bidistilled water. The frog testes were smashed in a 1 : 4 mixture of this stock solution and tap water. The pH of the solutions was between 7.2 and 7.4. 20 ml of the sediment-free upper part of the solution was carefully poured down after the settling of the tissue fragments of the testes, so we obtained a relatively clean spermium suspension. This suspension was divided into two parts (10 ml each). One part was irradiated by β or γ rays, the other part served as control. During the experiment we always took good care that the irradiated and the control samples be always under the same environmental conditions. The very high sensitivity of sperms against heat and light is known. Thus it was first of all important to ensure the same heat and light circumstances. Therefore both samples were stored in a cold room in the darkness at a temperature of +2 °C. The β irradiation was done in darkness at a temperature of +2 °C, the γ irradiation was performed in darkness, at room temperature. Low temperature does not damage the sperms (Shettles, 1940), but it lengthens their lifetime, which is favourable for the effect examined by us.

In our experiments the effect of β and γ radiation was examined.

A ^{90}Sr—^{90}Y preparation of 86 mCi activity was used as a source of β radiation. (The radiation energy is 0.54 and 2.25 MeV resp.) The sample to be irradiated and the control one were placed into a glass-stoppered bottle of a volume of 30 ml. The bottom of the bottle was formed by a mica window of 5.5 mg/cm^2 thickness which was fixed to the bottle — the bottom of which had been cut off — by araldit. The size of the surface of the mica window was 7 cm^2. Taking the volume of 10 ml into consideration the thickness of the layer of the irradiated sperm-suspension was about 1.4 cm. The samples were placed on the ^{90}Sr—^{90}Y β radiation source, and irradiated through the mica window. The dose rate was 48 rad/h at the contact surface. The effect of radiation was examined in the range from 20 to 1200 rad.

A ^{60}Co radioactive preparation equivalent to 35 g Ra was used as a source of γ radiation (gamma energy: 1.17 and 1.33 MeV). The irradiation was done in a bottle with a 3.5 mm thick wall; thus, the absorption of the β radiation of ^{60}Co was assured. The dose rate of γ irradiation was 3100 rad/h at the place of the irradiation. The effect of γ irradiation was examined in the range from 0 to 1600 rad.

The number of moving sperms (N) was determined in a Bürker chamber before the irradiation (N_0) and once every day after the irradiation. The number of moving sperms was expressed in a value relative of the pre-irradiation starting value (K). The decrease in time of the number of moving forms was plotted in a coordinate system. The period of time during which the number of moving sperms diminished to half of the starting value was determined by interpolation on the basis of the experimental curves. (Further on, this value is denoted with T.) After

this, the T-values of the control group and of the irradiated one were compared by statistical methods.

Applying different times of irradiation in the case of both β and γ radiations we examined what kind of relationships could be shown to exist between the dosage applied and the T-values.

The measurement of the dosage was performed by the chemical dosimetrical method of Fricke (Fricke, Morse, 1927).

Results

The effect of β radiation of 20 to 1200 rad was examined in 36 experiments. The experimental results are shown in Fig. 1. The time in hours are shown by the abscissa, and the relative number of moving forms (K) by the ordinate. The 0 point of the abscissa indicates the beginning of the irradiation. The figure summarizes the average values of the 36 experiments. The number of moving forms decreased roughly exponentially with the time. The period of time (T) until the num-

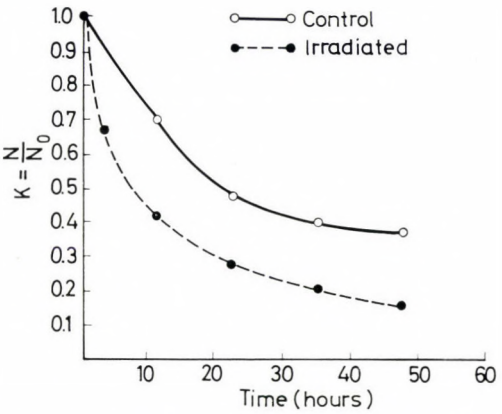


Fig. 1. Change in the number of moving sperms under the effect of β radiation

ber of moving sperms decreased to its half (K = 0.5) was about 22 hours with non-irradiated control samples (this is indicated by a solid line in the figure). The same value was 7 hours with the irradiated samples (indicated by a dotted line in the figure). Thus, the motility of the sperms remarkably decreased under the effect of β radiation in dosages used by us. So, the lifespan of sperms in the irradiated samples decreased considerably. The difference of the T-values of the irradiated and control group was checked by the t-test. Taking P = 0.05 for probability level we find a significant difference between irradiated and control groups as regards the time values necessary for the number of moving sperms to decrease by 50 per cent (T).

It was examined how different dosages of β radiation affected the period of time passing until the number of moving sperms decreased to its half. The sum-

marized results of these experiments are indicated in Fig. 2. The abscissa shows the applied dosage in rad units and the ordinate the T-values in hours. The figure indicates that relatively small doses already diminish the value of T considerably. There exists a positive correlation between the dosage measured in rad and the periods of time necessary for diminishing the number of moving forms to the

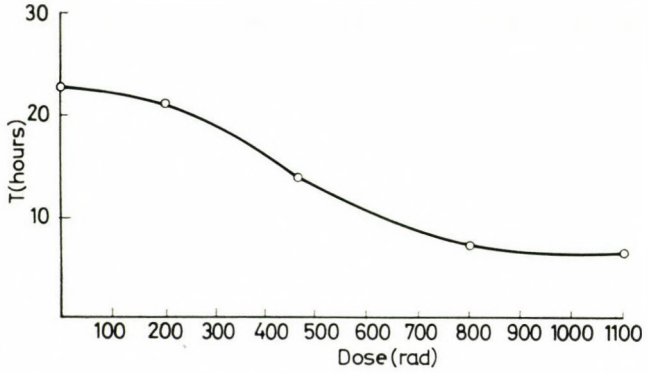


Fig. 2. Relationship between the dosage of β radiation and the period of time until the number of moving sperms decreases to its half (T)

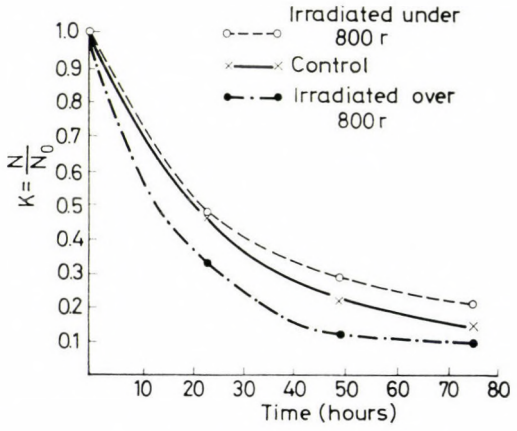


Fig. 3. Change in the number of moving sperms under the effect of γ radiation

half. The value of the correlation coefficient is $+0.62 \pm 0.09$ from the experimental data.

The effect of γ irradiation was examined in 45 experiments by using dosages from 0 to 1600 rad. Fig. 3 shows the correlation obtained from the data of the average values of 45 experiments. The abscissa shows the time in hours and the ordinate the relative values of the number of moving forms at the given time. The solid line indicates the values of the non-irradiated control group. The curve above the control curve shows the average values of samples irradiated

with dosages under 800 rad; the curve below the control curve shows the average values of samples irradiated with dosages over 800 rad. The period of time (T) passing until the number of moving sperms diminished to its half (K = 0.5) was about 20 hours with the non-irradiated control samples. With samples irradiated with dosages smaller than 800 rad the value of T was also 20 hours. If dosages over 800 rad were used, this value was 13 hours.

The relationship between different dosages of γ radiation and the period of time passing until the number of moving sperms decreased to its half (T) was also examined. The summarized results of these data are shown in Fig. 4. The abscissa shows the applied dosage in rad units, and the ordinate the values of T

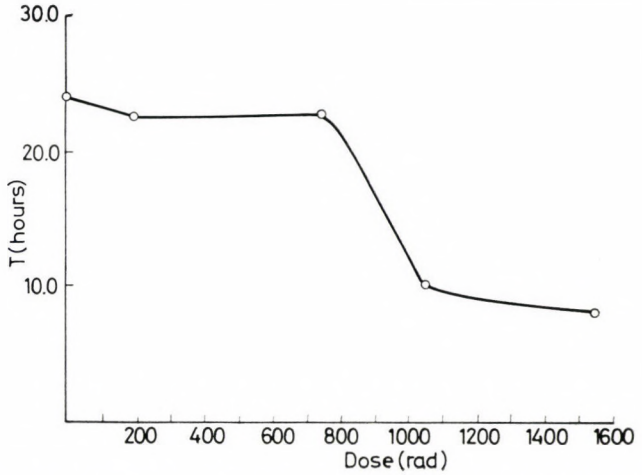


Fig. 4. Relationship between the dosage of γ radiation and the period of time until the number of moving sperms decreases to its half (T)

in hours. The figure indicates that with dosages under 800 rad the T-values of the irradiated group do not differ significantly from those of the control group. But in the case of dosages over 800 rad the above-mentioned value decreases with increasing dosages. Thus, γ dosages under 800 rad do not significantly influence the motility of sperms. When γ dosages over 800 rad were applied the motility and the lifespan decreased significantly, as compared with the control group at a probability level of $P = 0.05$. With dosages over 800 rad a negative correlation can be demonstrated between the dosage and the time periods passing until the number of moving sperms decreases to its half (T). On the basis of the experimental results the value of the correlation coefficient is -0.56 ± 0.04.

Discussion

Our experiments indicate that the resistance of ripe sperms against β and γ radiation is not as great as stated in literary data concerning experiments performed with X-ray irradiation. The period of time passing until the number of moving sperms decreases to its half is significantly diminished by relatively small β dosages

(20 to 1200 rad). A definite correlation between the applied dosage and the effect can be demonstrated within the above-mentioned dose ranges. A marked effect of γ irradiation can be shown but in dosages over 800 rad. On the basis of these facts it seems that β radiation is about ten times more effective than γ radiation in decreasing the motility of ripe sperms. In the case of K-values smaller than 0.5 the comparison of experiments indicated in Fig. 1 and Fig. 3 is made uncertain by the fact that the samples in experiments performed with β radiation were kept at a temperature of +2 °C throughout the experiments, but γ irradiation was performed at room temperature.

On the other hand, our experimental data — when compared with literary data on the effect of X-ray irradiation — indicate that the motility of ripe sperms is markedly influenced by β and γ radiation in dosages under 1000 rad. But the same dosages of an X-ray radiation are ineffective according to literary data (Fritz-Niggli, 1959). On the basis of all these considerations the radiosensitivity of ripe sperms seems to be greater than it has been stated by literary data.

References

Bloom, W. (1948) Histopathology of Irradiation from External and Internal Sources. McGraw-Hill Book Company, New York, p. 753
Fricke, H., Morse, St. (1927) Strahlentherapie *26* 749, 757
Fritz-Niggli, H. (1959) Strahlenbiologie. Thieme Verlag, Stuttgart, p. 307
Herpen, G., Rikmenspoel, R. (1968) Abstr. of Biophysical Journal *A-121*
Hollaender, A. (1954) Radiation Biology, Vol. I, p. 998
Oakberg, E. F. (1955) Radiat. Res. *2* 369
Oakberg, E. F. (1957) Nature *180* 1137
Oakberg, E. F. (1959) Radiat. Res. *11* 700
Oakberg, E. F. (1962) Strahlentherapie *51* 103
Shettles, L. B. (1940) Amer. J. Physiol. *128* 408
Siegel, G. (1966) Die biologischen Wirkungen kleiner Dosen ionisierender Strahlung. Gustav Fischer Verlag, Jena, p. 42
Spear, F. G. (1953) Radiations and Living Cells. Chapman and Hall Ltd., London, p. 126

Conference on "Biology and Mathematics"*

As an item on the agenda "Mathematics and Biology" were discussed by IUPAB's Council in Cambridge (England) on June 30, 1968. It was decided: "The Council felt that it would be desirable to hold a symposium on this general problem during the course of the Congress in 1969 at M.I.T. The symposium was to be primarily concerned with a specific aspect of mathematics as applied to a reasonably well defined biological area." That was in accordance with my views advanced at this Council's meeting and emphasizing that the biological phenomenon should primarily be formulated precisely and, afterwards, the adequate mathematical method to deal with the biological system should be found or selected with the help of professional mathematicians. Wishing to promote this question also in our country I proposed a meeting to be organized by our Society; the conference took place under the chairmanship of Prof. J. Szentágothai and with the cooperation of Prof. F. B. Straub. Out of the 200 attendants, joining in the discussion after my introductory lecture,** biologists, mathematicians, physicists (altogether 22) agreed that their discussion was to be considered as a start in mathematizing biology — thus helping the latter to become an exact science. The following two points were accepted: 1. Biological results are to be described so precisely that they can be formulated mathematically. 2. Biological research teams should have professional mathematicians as team-mates from the outset.

E. Ernst

* Organized by the Hungarian Biophysical Society under the auspices of the Biological Section of the Hungarian Academy of Sciences, Budapest, February 25, 1969.

** Sent by mail to the attendants in advance.

Hoppe-Seyler's Zeitschrift für Physiologische Chemie

Editors in Chief

A. BUTENANDT · K. THOMAS · G. WEITZEL

Subscription Rates

For one volume (12 parts) DM 360,—

Vol. 349, No. 12 December 1968

Contents

Walter de Gruyter & Co · Berlin 30

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója Műszaki szerkesztő: Farkas Sándor

A kézirat nyomdába érkezett: 1969. III. 15. — Terjedelem: 9,25 (A/5) ív, 77 ábra

69.67347 Akadémiai Nyomda, Budapest. — Felelős vezető: Bernát György

Reviews of the Hungarian Academy of Sciences are obtainable at the following addresses:

ALBANIA

Ndermarja Shtetnore e Botimeve
Tirana

AUSTRALIA

A. Keesing
Box 4886, GPO
Sydney

AUSTRIA

Globus Buchvertrieb
Salzgries 16
Wien I

BELGIUM

Office International de Librairie
30, Avenue Marnix
Bruxelles 5
Du Monde Entier
5, Place St. Jean
Bruxelles

BULGARIA

Raznoiznos
1, Tzar Assen
Sofia

CANADA

Pannonia Books
2. Spadina Road
Toronto 4, Ont.

CHINA

Waiwen Shudian
Peking
P. O. B. 88

CZECHOSLOVAKIA

Artia
Ve Směčkách 30
Praha 2
Poštovná Novinová Služba
Dovoz tisku
Vinohradská 46
Praha 2
Mad'arská Kultura
Václavské nám. 2
Praha I
Poštová Novinová Služba
Dovoz tlače
Leningradská 14
Bratislava

DENMARK

Ejnar Munksgaard
Nörregade 6
Copenhagen

FINLAND

Akateeminen Kirjakauppa
Keskuskatu 2
Helsinki

FRANCE

Office International de Documentation et Librairie
48, rue Gay Lussac
Paris 5

GERMAN DEMOCRATIC REPUBLIC

Deutscher Buch-Export und Import
Leninstraße 16
Leipzig 701

Zeitungsvertriebsamt
Fruchtstrasse 3—4
1004 Berlin

GERMAN FEDERAL REPUBLIC

Kunst und Wissen
Erich Bieber
Postfach 46
7 Stuttgart S.

GREAT BRITAIN

Collet's Holdings Ltd.
Dennington Estate
London Rd.
Wellingborough. Northants.
Robert Maxwell and Co. Ltd.
Waynflete Bldg. The Plain
Oxford

HOLLAND

Swetz and Zeitlinger
Keizersgracht 471—487
Amsterdam C.
Martinus Nijhof
Lange Voorhout 9
The Hague

INDIA

Current Technical Literature
Co. Private Ltd.
India House OPP
GPO Post Box 1374
Bombay I

ITALY

Santo Vanasia
Via M. Macchi 71
Milano
Libreria Commissionaria Sansoni
Via La Marmora 45
Firenze

JAPAN

Nauka Ltd.
92, Ikebukur O-Higashi 1-chome
Toshima-ku
Tokyo
Maruzen and Co. Ltd.
P. O. Box 605
Tokyo-Central
Far Eastern Booksellers
Kanda P. O. Box 72
Tokyo

KOREA

Chulpanmul
Phenjan

NORWAY

Johan Grundt Tanum
Karl Johansgatan 43
Oslo

POLAND

Ruch
ul. Wronia 23
Warszawa

ROUMANIA

Cartimex
Str. Aristide Briand 14—18
Bucuresti

SOVIET UNION

Mezhdunarodnaya Kniga
Moscow G—200

SWEDEN

Almquist and Wiksell
Gamla Brogatan 26
Stockholm

USA

Stechert Hafner Inc.
31, East 10th Street
New York, N. Y. 10003
Walter J Johnson
111, Fifth Avenue
New York, N. Y. 10003

VIETNAM

Xunhasaba
19, Tran Quoc Toan
Hanoi

YUGOSLAVIA

Forum
Vojvode Mišića broi 1
Novi Sad
Jugoslovenska Knjiga
Terazije 27
Beograd

40,— Ft

Contents

8. VIII. 1969

Index: 26.030

VOLUME 4
NUMBER 3
1969

Acta biochimica et biophysica

ACADEMIAE SCIENTIARUM HUNGARICAE

AKADÉMIAI KIADÓ, BUDAPEST

Acta Biochimica et Biophysica

Academiae Scientiarum Hungaricae

Szerkeszti:

STRAUB F. BRUNÓ és ERNST JENŐ

Technikai szerkesztők:

SAJGÓ MIHÁLY és NIEDETZKY ANTAL

Szerkesztőség címe: Budapest XI., Karolina út 29. (biokémia);
Pécs, Rákóczi út 80. (biofizika)

Az *Acta Biochimica et Biophysica*, a Magyar Tudományos Akadémia idegen nyelvű folyóirata, amely angol nyelven (esetleg német, francia vagy orosz nyelven is) eredeti tanulmányokat közöl a biokémia és a biofizika — fehérjék (struktúra és szintézis), enzimek, nukleinsavak, szabályozó és transzport-folyamatok, bioenergetika, izom-összehúzódás, radiobiológia, biokibernetika, funkcionális és ultrastruktúra stb. — tárgyköréből.

A folyóirat negyedévenként jelenik meg, a négy füzet évente egy kb. 400 oldalas kötetet alkot. Kiadja az Akadémiai Kiadó.

Előfizetési díja belföldre 120,— Ft, külföldre 165,— Ft kötetenként. Megrendelhető az Akadémiai Kiadónál (Bp. V., Alkotmány u. 21), a külföld részére pedig a Kultúra Könyv és Hírlap Külkereskedelmi Vállalatnál (Budapest I., Fő u. 32).

The *Acta Biochimica et Biophysica*, a periodical of the Hungarian Academy of Sciences, publishes original papers, in English, on biochemistry and biophysics. Its main topics are: proteins (structure and synthesis), enzymes, nucleic acids, regulatory and transport processes, bioenergetics, excitation, muscular contraction, radiobiology, biocybernetics, functional structure and ultrastructure.

The Acta Biochimica et Biophysica is a quarterly, the four issues make up a volume of some 400 pp, per annum. Manuscripts and correspondence with the editors and publishers should be sent to

Akadémiai Kiadó Budapest 24 P.O.B. 502.

The subscription rate is 165,— Ft per volume. Orders may be placed with *Kultúra* Trading Co. for Books and Newspapers (Budapest 62, P.O.B. 149) or with its representatives abroad, listed on p. 3 of the cover.

Acta Biochimica et Biophysica — журнал Академии Наук Венгрии, публикующий на английском языке (возможно и на немецком, французском и русском языках) оригинальные статьи по проблемам биохимии и биофизики — белков (структура и синтез), энзимов, нуклеиновых кислот, процессов регуляции и транспорта, биоэнергетики, мышечного сокращения, радиобиологии, биокибернетики, функциональной структуры и ультраструктуры и т. д.

Журнал выходит ежеквартально, выпуски каждого года составляют том объемом около 400 страниц. Журнал выпускает Издательство Академии Наук Венгрии.

Рукописи и корреспонденцию просим направлять по следующему адресу:

Akadémiai Kiadó Budapest 24 P.O.B. 502.

Подписная цена — 165 Фт за том. Заказы принимает:

Предприятие по внешней торговле книгами и газетами «Kultúra» (Budapest 62. P. O. B. 149) или его заграничные агентства.

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (3), pp. 219–236 (1969)

Activity and Structural Changes in Mg^{++}-dependent and $(Na^{+}+K^{+})$-activated Adenosine Triphosphatase Prepared from Rat Brain Following Detergent Treatment

J. SOMOGYI, Mária BUDAI, L. NYÍRŐ, G. A. KALUZA, W. NAGEL, F. WILLIG

Experimental Research Department and Radiology Clinic, University Medical School, Budapest and Institute of Physiological Chemistry, University of Heidelberg

(Received January 28, 1969)

Various disruptive agents had different effects on the membrane bound ATPases. Detergents, like triton-x-100, deoxycholate and digitonin increased total ATPase activity up to a certain concentration limit, but they lowered the activity at higher concentrations. After cetavlon treatment or sonication, the total activity decreased regularly. Mg^{++}-dependent ATPase activity could be increased following preincubation with digitonin alone, all other disruptive agents tested gradually inactivated the enzyme.

Among the detergents used only triton-x-100 proved suitable for the solubilization of the ATPase activities without any loss. Mg^{++}, Na^{+}, K^{+} as well as EDTA influenced the detergent action on the ATPases.

Reducing agents, like $Na_2S_2O_4$ diminished the ATPase activities but, when they acted together with detergents, a further increase in activities followed. Ascorbic acid, however, inhibited the ATPase activities in the lowest efficient concentration both in the presence and absence of triton-x-100. On the other hand, the effect of albumin as well as tris-chloride was different with and without triton-x-100.

The experiments strongly suggest that detergent treatment of the enzyme preparation gave rise to a change in enzyme conformation. The structural change of ATPase took place not only at a detergent concentration required for the solubilization or causing a loss of activity but also at a lower concentration which resulted in a maximal activation of $(Na^{+} + K^{+})$-activated ATPase. The present experiments carried out with disruptive agents did not support the assumption of an interconversion between Mg^{++}-dependent and $(Na^{+} + K^{+})$-activated ATPase. This problem requires further detailed investigations.

Introduction

Subcellular preparations containing fragments of cellular membranes exhibit ATPase activity. The ATPase is activated by Mg^{++} and can be further stimulated by $Na^{+}+K^{+}$. The $(Na^{+}+K^{+})$-activated, strophantine-inhibited ATPase (socalled transport ATPase) has a fundamental role in the process of active ion transport. Several recent reviews deal with the enzymatic properties and physiological function of this enzyme system (Csáki, 1965; Skou, 1965; Albers, 1967; Heinz, 1967; Rothstein, 1968).

Enzyme preparations obtained from tissue homogenates by differential centrifugation always contained Mg^{++}-dependent ATPase. This ATPase can neither be activated by $Na^{+}+K^{+}$ nor inhibited by strophantine. The $Mg^{++}+$

$+Na^{+}+K^{+}/Mg^{++}$ activity ratio might vary in different enzyme preparations obtained from various tissue sources (Skou, 1965) but its value depends also on the method used during the preparative procedure. Relative proportion of the Mg^{++}-dependent ATPase in the different preparations might be diminished in several ways: hypotonic treatment (Yoshida, Fujisawa, 1962), ageing (Dunham, Glynn, 1961; Deul, McIlwain, 1961; Somogyi, Vincze, 1961, 1962; Somogyi, 1963; Hokin, Reasa, 1964), dialysis (Landon, Norris, 1963), treatment with different detergents (Skou, 1962; Somogyi, 1963; Rendi, Uhr, 1964; Swanson et al., 1964; Järnefelt, 1964), application of NaI during preparation (Nakao et al., 1965), etc.

Various disruptive agents, first of all detergents, are able not only to modify the ratio between Mg^{++}-dependent and $(Na^{+}+K^{+})$-activated ATPases of a given enzyme preparation but also to interfere with the relation between the enzymes and the membrane structure (Bradford et al., 1964; Swanson et al., 1964; Cooper, McIlwain, 1967).

As the action of different non-ionic, anionic and cationic detergents is closely related to their chemical character, their effect upon the membrane structure and function may differ, accordingly. These were the reasons why we decided to study the effects of different detergents as well as those of sonication upon ATPases prepared from rat brain tissue. Our aim was also to investigate how Mg^{++}, Na^{+} and K^{+}, which are all needed for full ATPase activity, can modify the effects of different disruptive agents.

It is well known that protein conformation might be considerably affected by different detergents (Putnam, 1948; Markus, Karush, 1956; Keller, Gottwald, 1960; Jirgensons, 1961). Changes in protein conformation are by no means connected with simultaneous changes in enzyme activity. According to our previous data (Somogyi, Gaál, 1965), e.g. total ATPase activity of an enzyme preparation when preincubated with 0.05 per cent triton-x-100 was only insignificantly altered as compared to the control. However, the estimated quantity of the SH-groups was higher by about 75 per cent in the detergent treated preparation in comparison to the control. Half maximal inhibition of the $(Na^{+}+K^{+})$-activated ATPase could be achieved with 20 μM p-hydroxy mercurybenzoate in control preparations but 60 μM of the SH-blocking agent was needed when the enzyme was preincubated with 0.05 per cent triton-x-100. The present experiments give further evidence of structural changes in ATPase following detergent treatment.

Methods

The enzyme system was prepared from rat brain homogenates as described previously (see preparation of fraction "P", Somogyi, Vincze, 1961). Following the last centrifugation the enzyme preparation was suspended in 0.25 M sucrose. After determining its protein content with the biuret method (Gornall et al., 1949) it was diluted with 0.25 M sucrose to a concentration of 1 mg protein/0.6 ml. For diminishing turbidity of lipid substances, Na-laurylsulfate was applied.

Detergent treatment of enzyme preparations was carried out as follows: an appropriate volume of the detergent solution as well as that of other materials

indicated were pipetted in a conical centrifuge tube 10 mm in diameter. This was filled up with water to a final volume of 0.8 ml. After the mixture had been cooled down to 0 °C, 1.2 ml of enzyme suspension (corresponding to 2 mg protein) was added. ATPase activity of 0.2 ml of the enzyme-detergent mixture was determined immediately following preincubation at 0 °C for 20 min. This meant a 10-fold dilution of the detergent in the final test volume.

Sonication of the enzyme preparations was accomplished as follows: 3.0 ml of the enzyme suspension (corresponding to 5 mg protein) was added to 2.0 ml of water containing in some experiments ions as indicated. The samples were then sonicated at 0 °C by an MSE ultrasonic disintegrator (100 W, 20 kc./sec, 7 microns amplitude).

ATPase activity was measured in 2.0 ml test volume containing 50 mM tris-buffer (pH 7.4), 5 mM Mg^{++}, 5 mM ATP (tris-salt, pH 7.4), 100 mM Na^{+} +20 mM K^{+} as indicated and 0.2 ml preincubated enzyme suspension. Following a 10 min incubation at 37 °C, 1.0 ml of 20 per cent trichloroacetic acid was added to the test solutions.

Inorganic phosphate content of the deproteinized filtrate was determined according to Lohmann and Jendrassik (1926). In a few cases, in addition to the P_i determination the amount of unhydrolyzed ATP was also assayed by the aid of a coupled NAD dependent dehydrogenase reaction (ATP-test, Boehringer).

Detergents, like triton-x-100 (octylphenol-polyethylenglycolether, Serva), cetavlon (cethyltrimethyl-ammonium bromide, Serva), digitonin (B.D.H. AnalaR Grade) and deoxycholic acid (Fluka) were dissolved in all experiments in water. Deoxycholic acid was neutralized to pH 7.6 either with NaOH or with tris-base. Among the reducing agents used ascorbic acid and $Na_2S_2O_4$ were Merck's products, dehydroascorbic acid was purchased from Fluka and $NaHSO_3$ was obtained from Riedel. Albumin (Calbiochem) was dialyzed prior to use.

Results

Effect of detergents on ATPase activity

Triton-x-100: The non-ionic detergent triton-x-100 affected ATPase activity in a different manner when measurement was made in the presence of Mg^{++} and $Mg^{++}+Na^{+}+K^{+}$, respectively. Mg^{++}-dependent activity diminished parallel with an increasing concentration of the detergent. On the other hand, total activity (with $Mg^{++}+Na^{+}+K^{+}$) increased up to a 0.025 per cent triton-x-100 concentration in the preincubated medium but became lowered regularly at a higher concentration, approaching values of the Mg^{++}-dependent activity (Fig. 1). Preincubation of the individual enzyme preparations in a medium containing 0.025 per cent detergent caused about 10 per cent decrease in Mg^{++}-dependent activity but about 40 per cent increase in total activity in comparison to the control preparations preincubated in a detergent-free medium. Triton-x-100 in 0.05 per cent concentration diminished Mg^{++}-dependent ATPase activity by about 25 per cent but left total activity essentially unaffected in comparison to the control. Elevated activity of the $(Na^{+}+K^{+})$-activated ATPase, seen at a low

detergent concentration, appeared to be due not so much to the diminished level of the Mg^{++}-dependent activity but rather to the substantial increase in the total activity. ($Na^+ + K^+$-dependent activity equals the difference between total and Mg^{++}-dependent activity.) When preincubation was carried out in the presence of a detergent concentration (0.25 per cent) which as a rule caused a complete inactivation of the ($Na^+ + K^+$)-activated ATPase activity, two thirds of the original Mg^{++}-dependent activity could still be detected (Fig. 1).

It was also demonstrated in our previous experiments that preincubation of the enzyme preparation at 37 °C diminished Mg^{++}-dependent ATPase activity (Somogyi, Vincze, 1961, 1962; Somogyi, 1963). On the other hand, no significant

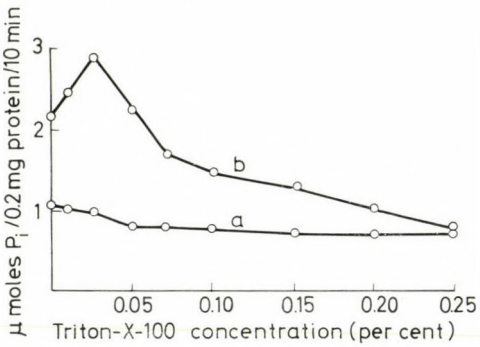


Fig. 1. Effect of triton-x-100 on ATPase activity. Enzyme preparations (1 mg protein/ml) were preincubated at 0 °C for 20 min with detergent at the concentration indicated. After preincubation the ATPase activity of 0.2 ml enzyme suspension was immediately determined at 37 °C in 2.0 ml test volume containing 50 mM tris-chloride (pH 7.4), 5mM Mg^{++}, 5 mM ATP (tris-salt, pH 7.4), and 100 mM Na^+ + 20 mM K^+ if indicated. For other experimental details see Methods. Curve a: ATPase activity in the presence of Mg^{++}; Curve b: ATPase activity in the presence of $Mg^{++} + Na^+ + K^+$

alteration in ($Na^+ + K^+$)-activated ATPase activity could be observed under similar conditions (Somogyi, 1963). Decrease in Mg^{++}-dependent ATPase activity was less pronounced in cases when preincubation was carried out in a medium containing also Mg^{++} (Somogyi, Vincze, 1962).

Based on these observations, it was then investigated what kind of change in ATPase activity could be elicited with enzyme preparations preincubated in a medium containing beside triton-x-100 also Mg^{++} or Na^+ or K^+ or EDTA. In these experiments triton-x-100 was used in a 0.05 per cent concentration. Though this concentration inhibited total ATPase activity in relation to the maximal value (preincubation with 0.025 per cent triton-x-100), the rest of the activity still remained in the order of the control values. If the preincubation medium contained Mg^{++}, Na^+ as well as K^+, in addition to 0.05 per cent triton-x-100, then Mg^{++}-dependent activity reached a substantially higher level than it did in a medium containing the detergent alone. It was especially conspicuous that while Mg^{++}-dependent activity of preparations preincubated with 0.05 per cent triton-x-100 and 5 mM Mg^{++} was higher by about 50 per cent than the activity of prep-

arations preincubated with the detergent alone, total activity failed to be elevated under similar conditions (Table 1).

It is also seen in Table 1 that when preincubation was carried out in the presence of detergent and Na^+ or K^+ or Mg^{++} together with EDTA, total ATPase activity was higher by about 50–80 per cent in comparison to the appropriate control values. The extent of increase in activity was dependent on Na^+ or on K^+ concentration of the preincubated medium. Maximal effect was obtained with

Table 1

Effect of 0.05 per cent triton-x-100 on ATPase activity in the presence of Mg^{++}, Na^+, K^+ and EDTA

Additions in the preincubation mixture	Concentration of triton-x-100			
	0		0.05 per cent	
	ATPase activity			
	Mg^{++}	$Mg^{++}+Na^++K^+$	Mg^{++}	$Mg^{++}+Na^++K^+$
none	1.16	2.36	0.89	2.40
5 mM Mg^{++}	1.06	2.08	1.36	2.36
100 mM Na^+	1.27	2.29	1.36	3.45
100 mM K^+	1.15	2.24	1.33	3.79
5 mM EDTA + 5 mM Mg^{++}	0.99	2.55	1.10	3.60
100 mM Na^+ + 5 mM Mg^{++} + + 5 mM EDTA	1.29	2.35	1.24	3.98
5 mM Mg^{++} + 100 mM K^+ + + 5 mM EDTA	0.99	2.50	1.07	4.18

Enzyme preparations (1 mg protein/ml) were preincubated at 0 °C for 20 min as indicated, with or without triton-x-100, immediately thereafter the ATPase activity of 0.2 ml enzyme suspension was determined as described in Fig. 1.

100 mM Na^+ or K^+, respectively. When Na^+ or K^+ content of the preincubation medium surpassed a limit of 200 mM concentration, total ATPase activity was lowered well below the control value.

Digitonin: Digitonin displayed a similar effect on total ATPase activity like triton-x-100 (Fig. 2). Though preincubation of the enzyme preparation with 0.1 per cent digitonin stimulated total ATPase activity to a maximal value, activity diminished with higher concentrations of the detergent. It is essential that digitonin, unlike all other detergents tested, substantially increased Mg^{++}-dependent ATPase activity. In 1 per cent concentration it caused a maximal increase of about 100 per cent in activity. Following preincubation with 2 per cent digitonin, Mg^{++}-dependent activity was hardly lower than the maximal value. On the other hand, $(Na^+ + K^+)$-dependent activity was almost completely abolished by this digitonin concentration.

Deoxycholate: Effect of deoxycholate corresponded qualitatively to that of triton-x-100 (Fig. 3). Total ATPase activity reached a maximum following preincubation with 0.08–0.1 per cent deoxycholate. Mg^{++}-dependent activity de-

creased to about 75 per cent of the control value following preincubation with 0.2 per cent deoxycholate. As a result of preincubation of the enzyme with 0.4 per cent detergent, $(Na^+ + K^+)$-activated ATPase has been completely abolished, but approximately 50 per cent of the original Mg^{++}-dependent activity remained unaffected.

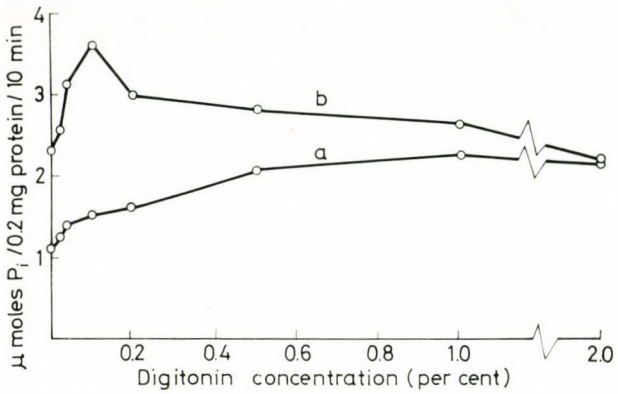


Fig. 2. Effect of digitonin on ATPase activity. For experimental details see Fig. 1

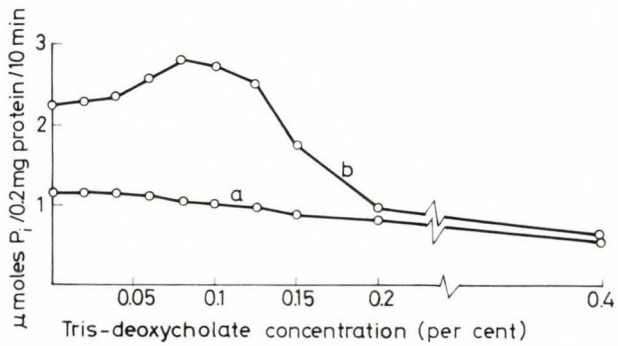


Fig. 3. Effect of deoxycholate on ATPase activity. For experimental details see Fig. 1

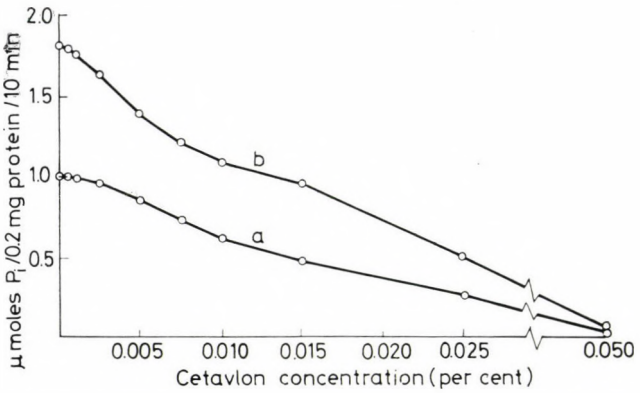


Fig. 4. Effect of cetavlon on ATPase activity. For experimental details see Fig. 1

Cetavlon: All the non-ionic and anionic detergents tested exerted a biphasic action on the $(Na^+ + K^+)$-activated ATPase: enzyme activity increased progressively with increasing detergent concentrations up to a given limit, but diminished or became completely blocked when higher detergent concentrations were applied. In contrast to this, cetavlon, used in the lowest efficient concentration, inhibited alike Mg^{++}-dependent and total ATPase activity (Fig. 4). Following a preincubation with 0.01 per cent cetavlon, enzyme activities were diminished by about 40 per cent and were completely eliminated by 0.05 per cent cetavlon.

Solubilization of ATPase activity

Digitonin, deoxycholate and cetavlon solubilize ATPase activity at a concentration which causes a simultaneous and substantial loss of enzyme activity. Among detergents triton-x-100 proved to be the most appropriate material for this purpose. However, concentration ranges of the detergents eliciting a maximal activation of $(Na^+ + K^+)$-dependent ATPase, on the one hand, and those ensuring an optimal solubilization, on the other, are far from being identical. 0.025 per cent triton-x-100 e.g. solubilizes ATPase activities to an insignificant extent only. Nevertheless, approximately 80 per cent of the protein content and ATPase activities could be solubilized by applying the detergent in a 0.05 per cent concentration. The proportion of the solubilized activity, however, could not be further increased by raising the detergent concentration up to a limit of 0.1 per cent. Centrifugation of the detergent treated enzyme either at 20 000 *g* or 100 000 *g* for 60 minutes did not cause any change in the activity of the supernatant fraction. The bulk of triton-x-100 could be separated from the solubilized protein by gel filtration using a Sephadex G-100 column.

Mg^{++} or EDTA applied simultaneously with triton-x-100 not only modified the detergent action of ATPase activity but altered also the extent of solubilization. As seen in Table 2, 0.05 per cent triton-x-100 solubilized 75 per cent of the

Table 2

Solubilization of ATPase activity by 0.05 per cent triton-x-100, effects of Mg^{++}, and EDTA

Additions during the preincubation with triton-x-100	ATPase activity per cent			
	Mg^{++}		$Mg^{++} + Na^+ + K^+$	
	S.	P.	S.	P.
none	76	18	79	20
5 mM Mg^{++}	29	68	12	78
5 mM EDTA	67	29	56	43
5 mM EDTA + 5 mM Mg^{++}	31	58	12	82

Enzyme preparations (1 mg protein/ml) were preincubated at 0 °C for 20 min as indicated. After preincubation the enzyme suspensions were centrifuged at 100 000 *g* for 60 min, and ATPase activity was assayed in the supernatant (S) and in the pellet (P), as described in Fig. 1. ATPase activity is expressed as percentage value of the unfractionated enzyme suspension.

Mg^{++}-dependent and about 80 per cent of the total ATPase activity. In contrast to this, EDTA in a 5 mM concentration decreased solubilized Mg^{++}-dependent activity to about 65 per cent. Less than 60 per cent of the total ATPase activity could be solubilized under similar conditions. Treatment of the enzyme preparation with detergent in the presence of 5 mM Mg^{++} or 5 mM Mg^{++} + 5 mM EDTA resulted in approximately 30 per cent solubilization of the Mg^{++}-dependent but hardly more than in a 10 per cent solubilization of the total ATPase activity.

Effect of sonical disintegration of ATPase activity

In contrast to the detergent treatment sonical disintegration elicited a nearly parallel inactivation of both Mg^{++}-dependent and total ATPase activities. Sonication for one minute resulted in an about 20 per cent decrease of enzyme

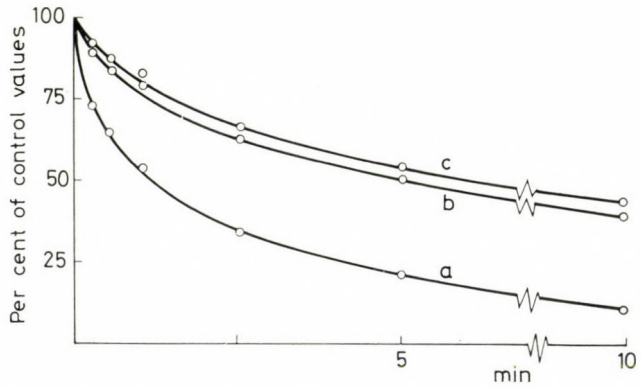


Fig. 5. Effect of ultrasonic disintegration on ATPase activity. The sonication of enzyme preparations (1 mg protein/ml) was carried out at 0 °C. For other experimental details see Methods and Fig. 1. Curve a: Absorbance of the enzyme preparation at 520 mμ; Curve b: ATPase activity in the presence of Mg^{++}; Curve c: ATPase activity in the presence of $Mg^{++} + Na^{+} + K^{+}$

activities. 50 per cent of the ATPase activities has been rendered inactive by a sonication for 5 minutes and 60 per cent by a sonication for 10 minutes, respectively (Fig. 5, curves b, c). Absorbance of the enzyme preparation measured at 520 mμ diminished to about half of the control value following a 1 minute sonication and merely 15 per cent of the control absorbance could be detected after a treatment for 10 minutes (Fig. 5, curve a).

On the contrary, X-irradiation of the enzyme preparation with 800 r (0.5mm copper filter, 20 kV, 50 cm preparation target distance) at 0 °C raised both Mg^{++}-dependent and total activity by about 15 per cent.

When the enzyme preparation was centrifuged at 20 000 *g* for 60 minutes, following the sonical disintegration the protein content of the supernatant fraction increased at a faster rate than enzyme activities did (Fig. 6). Following a 1 minute sonication, approximately 50 per cent of the protein content and about 25 per cent of the ATPase activities were found in the supernatant fraction. In the case

of a sonical disintegration for 5 minutes nearly 70 per cent of the protein content and about 50 per cent of the enzyme activities could not be sedimented. Finally, approximately 80 per cent of the protein content and 75 per cent of the ATPase activities were found in the supernatant fraction following sonication for 10 minutes.

Diminution of ATPase activity, due to sonication for 5 minutes, was only insignificantly affected by 5 mM Mg^{++} or 5 mM EDTA. In contrast, 5 mM

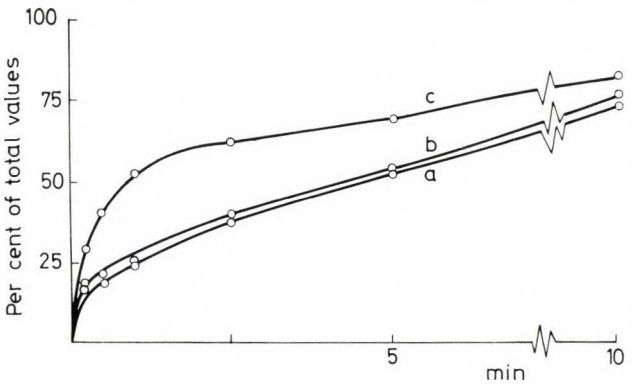


Fig. 6. Solubilization of protein and ATPase activity by ultrasonic disintegration. Ultrasonic disintegration of enzyme preparations (1 mg protein/ml) was performed at 0° C for the indicated time. After sonication the enzyme suspension was centrifuged at 20 000 *g* for 60 min, and the supernatant was analyzed for protein content and ATPase activity. The vules are expressed as the percentage values of unfractionated enzyme suspension. Curve a: ATPase activity in the presence of Mg^{++}; Curve b: ATPase activity in the presence of $Mg^{++} + Na^{+} + K^{+}$; Curve c: protein content

Mg^{++} +5 mM EDTA applied simultaneously essentially prevented the inactivation of total activity but failed to reduce the loss of Mg^{++}-dependent activity under similar conditions (Table 3).

Table 3

Activity of ATPase after 5 min sonication, effects of Mg^{++} and EDTA

Additions in the preincubation mixture	ATPase activity			
	before sonication		after sonication	
	Mg^{++}	$Mg^{++}+Na^{+}+K^{+}$	Mg^{++}	$Mg^{++}+Na^{+}+K^{+}$
none	0.94	2.18	0.52	1.26
5 mM Mg^{++}	0.90	1.94	0.50	1.29
5 mM EDTA	0.89	1.92	0.46	1.12
5 mM Mg^{++} + 5 mM EDTA	0.90	2.08	0.54	2.02

Enzyme preparations (1 mg protein/ml) were sonicated at 0 °C for 5 min as indicated. After sonication the ATPase activity of 0.2 ml enzyme suspension was determined as described in Fig. 1.

Distribution of ATPase activity and protein content between the pellet and the supernatant fraction was hardly affected when sonication for 5 minutes was carried out in the presence of 5 mM EDTA. Following sonical disintegration, performed in the presence of 5 mM Mg^{++}, a somewhat larger proportion of the activity could be detected in the pellet than in the former cases. On the other hand, approximately 75 per cent of the ATPase activity and the protein content could be recovered from the pellet following sonication performed in the simultaneous presence of 5 mM Mg^{++} and 5 mM EDTA (Table 4).

Table 4

Solubilization of protein and ATPase activity by sonication

Additions during sonication	Protein		ATPase activity			
	per cent					
			Mg^{++}		$Mg^{++}+Na^{+}+K^{+}$	
	S.	P.	S.	P.	S.	P.
none	68	31	52	44	53	42
5 mM Mg^{++}	48	51	41	52	34	56
5 mM EDTA	67	32	55	43	57	41
5 mM Mg^{++} + 5 mM EDTA	23	75	24	74	14	81

Enzyme preparations (1 mg protein/ml) were sonicated at 0 °C for 5 min as indicated. After sonication the suspensions were centrifuged at 20 000 *g* for 60 min, and the protein content and ATPase activity were determined in the supernatant (S.) and in the pellet (P.). Distribution of protein content and ATPase activity are expressed as percentage values of the unfractionated enzyme suspension.

Change in ATPase conformation due to detergent treatment

Effect of albumin on ATPase activity. It is demonstrated in Fig. 7 that Mg^{++}-dependent and total ATPase activities were equally unaffected by preincubation with albumin (5–15 mg/ml). Inhibition of Mg^{++}-dependent ATPase activity due to triton-x-100 could be completely prevented by 5 mg/ml albumin. When the albumin concentration was further increased, Mg^{++}-dependent activity never exceeded the control values. Total ATPase activity of a preparation preincubated simultaneously with 0.05 per cent triton-x-100 and 5 mg/ml albumin was higher by approximately 40 per cent than that of a preparation preincubated with detergent alone. Total ATPase activity was somewhat further increased by preincubation with detergent and 10 mg/ml albumin but it was somewhat lowered if 15 mg/ml albumin was applied.

Effect of reducing agents on ATPase activity. Reducing agents inhibit (Na^{+} + $+K^{+}$)-activated ATPase (Glynn, 1963; Cooper, McIlwain, 1967). This inhibition was not only reversed in our experiments but a substantially higher activity could also be observed when reducing agents such as $NaHSO_3$ or $Na_2S_2O_4$ acted together with the detergents.

Preincubation of enzyme preparations with 1 mM $NaHSO_3$ affected neither Mg^{++}-dependent nor total ATPase activity. Mg^{++}-dependent activity was hardly influenced by preincubation with 0.05 per cent triton-x-100 and 1 mM $NaHSO_3$. On the other hand, total activity increased approximately by 25 per cent under similar conditions.

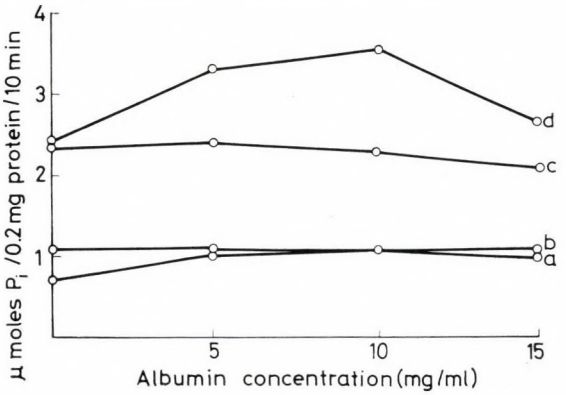


Fig. 7. Effect of albumin in the absence and presence of 0.05 per cent triton-x-100 on ATPase activity. Enzyme preparations (1 mg protein/ml) were preincubated at 0 °C for 20 min in the presence of albumin without detergent (curves b, c) and with 0.05 per cent triton-x-100 (curves, a, d), immediately thereafter the ATPase activity of 0.2 ml preincubated enzyme suspension was determined as described in Fig. 1. Curves a and b: ATPase activity in the presence of Mg^{++}; Curves c and d: ATPase activity in the presence of $Mg^{++} + Na^+ + K^+$

0.75 mM $Na_2S_2O_4$ caused a 10–20 per cent diminution in ATPase activities. However, when preincubation was carried out in the simultaneous presence of $Na_2S_2O_4$ and triton-x-100, Mg^{++}-dependent activity increased approximately by 50 per cent, while total activity by 40 per cent (Table 5).

Table 5

Effect of reducing agents on ATPase activity

Addition	Concentration of triton-x-100			
	0		0.05 per cent	
	ATPase activity			
	Mg^{++}	$Mg^{++}+Na^+ + K^+$	Mg^{++}	$Mg^{++}+Na^+ + K^+$
none	1.15	2.41	0.89	2.40
1 mM $NaHSO_3$	1.14	2.44	0.96	3.00
0.75 mM $Na_2S_2O_4$	1.07	2.12	1.32	3.34
0.5 mM ascorbate	0.93	1.40	0.61	1.05
0.5 mM dehydroascorbate	1.08	2.37	0.86	2.29

Enzyme preparations (1 mg protein/ml) were preincubated at 0 °C for 20 min with the indicated reducing agent either in the absence or in the presence of 0.05 per cent triton-x-100, immediately thereafter the ATPase activity of 0.2 ml preincubated enzyme suspension was determined as described in Fig. 1.

Ascorbic acid, unlike $NaHSO_3$ and $Na_2S_2O_4$, inhibited both Mg^{++}-dependent and total ATPase activities irrespective of whether preincubation of the enzyme preparation with ascorbic acid was accomplished in the presence or absence of triton-x-100. 0.5 mM ascorbic acid inhibited Mg^{++}-dependent ATPase activity by 20–30 per cent and total ATPase activity by 40–50 per cent. Dehydroascorbic acid, unlike ascorbic acid, was ineffective (Table 5). Similarly no change in the ATPase activities was found when ascorbic acid was added to the enzyme preparation in addition to a stoichiometric quantity of 2,6-dichlorophenol-indophenol. This indicated that the characteristic effect of ascorbic acid was linked to its dienol form. It appears unlikely that the effect of ascorbic acid and several other reducing agents on ATPase activity would be based upon a common mechanism. This is supported by the fact that in the presence of the smallest efficient concentration of ascorbic acid, ATPase activities decreased, both in the control and the detergent pretreated enzyme preparations.

Simultaneous effect of different detergents and $Na_2S_2O_4$ is shown in Table 6. All detergents were applied in concentrations which gave rise to a maximal activation of total ATPase. It is seen that total ATPase activities, already increased by the triton-x-100 or deoxycholate treatment, were further stimulated by 0.75 mM $Na_2S_2O_4$ (by 30 and 60 per cent, respectively). Digitonin-linked increase in activity was unaffected by $Na_2S_2O_4$. The inhibitory effect of triton-x-100 and that of deoxycholate on Mg^{++}-dependent activity was completely abolished by 0.75 mM $Na_2S_2O_4$.

Table 6

Effect of $Na_2S_2O_4$ on ATPase activity in the presence of different detergents

Added detergent in the preincubation mixture	Concentration of $Na_2S_2O_4$			
	0		0.75 mM	
	Mg^{++}	$Mg^{++}+Na^{+}+K^{+}$	Mg^{++}	$Mg^{++}+Na^{+}+K^{+}$
none	1.17	2.22	1.07	1.97
0.025 per cent triton-x-100	1.04	2.94	1.15	3.84
0.1 per cent Na-deoxycholate	1.02	2.68	1.35	4.27
0.1 per cent digitonin	1.34	3.70	1.53	3.82

Enzyme preparations (1 mg protein/ml) were preincubated at 0 °C for 20 min with the detergent indicated either in the absence or in the presence of 0.75 mM $Na_2S_2O_4$. After preincubation the ATPase activity of 0.2 ml enzyme suspension was immediately assayed as described in Fig. 1

The effect of different reducing agents upon the ATPase activities observed with and without detergents indicated that SH-groups might not be the sole target of the reducing agents.

Effect of tris-chloride on ATPase activity. When an enzyme preparation was preincubated with 25–150 mM tris-chloride (pH 7.4) no change in the activity of the Mg^{++}-dependent and in that of the total ATPase could be observed (Fig. 8, curves a, d). However, in the case of a preincubation carried out in the presence

of 0.05 per cent triton-x-100, Mg^{++}-dependent activity increased successively with increasing buffer concentrations, but total activity decreased simultaneously (Fig. 8, curves b, c). The activating effect of $Na^+ + K^+$ upon the ATPase was completely lost following preincubation with triton-x-100 and 150 mM tris-chloride. No difference in ATPase activity could be demonstrated whether determination of activity was based on measuring P_i liberated or ATP decomposed.

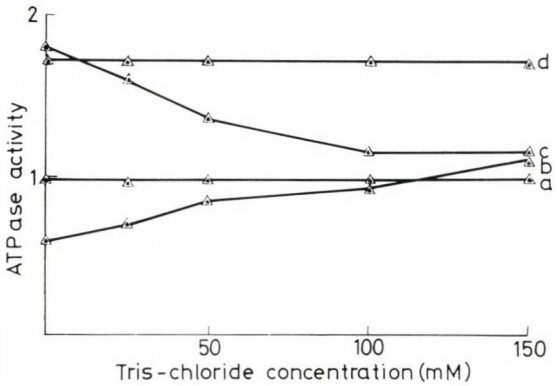


Fig. 8. Effect of tris-chloride on ATPase activity in the absence and presence of 0.05 per cent triton-x-100. Enzyme preparations (1 mg protein/ml) were preincubated at 0 °C for 20 min in the presence of tris-chloride (pH 7.4), without detergent (curves a, d) and with 0.05 per cent triton-x-100 (curves b, c). After preincubation the ATPase activity of 0.2 ml enzyme suspension was immediately assayed simultaneously as P_i liberated △—△ and as hydrolyzed ATP. Curves a and b: ATPase activity in the presence of Mg^{++}; Curves c and d: ATPase activity in the presence of $Mg^{++} + Na^+ + K^+$

Discussion

For an exact study of the enzymatic properties of the membrane bound, $(Na^+ + K^+)$-activated ATPase, the use of enzyme preparations of highest purity is a first order requirement. As a next step, the action of various enzyme solubilizing agents may be investigated. It seems also essential to get information on the changes in the structure and activity of the ATPase occurring during disintegration of the membrane constituents.

The various disruptive agents exerted different effects on the ATPase. Detergents, like triton-x-100, deoxycholate and digitonin displayed a biphasic action on the $(Na^+ + K^+)$-activated ATPase: up to a certain concentration of the detergent the activity increased but it decreased when higher concentrations were applied. Cetavlon as well as sonication caused a gradual loss of the $(Na^+ + K^+)$-activated ATPase activity. Only digitonin treatment gave a substantial increase in Mg^{++} dependent activity, however, Mg^{++}-dependent ATPase activity decreased following treatment with any other disruptive agent tested. On the other hand, among all the detergents investigated, only triton-x-100 proved to be suitable for solubilizing ATPase without a significant loss of activity. Kishimoto and Adelman

(1964) similarly demonstrated that detergent effects are linked to their chemical characters: both resting and acting potentials as well as sodium and potassium conductivity of the squid axon were affected divergently by the different detergents.

The first question concerned the mechanism of the activating effect of different detergents on the $(Na^+ + K^+)$-activated ATPase. We reached essentially similar conclusions as Swanson et al. (1964) did. They observed that solubilization was not an unconditional requirement for increasing $(Na^+ + K^+)$-activated ATPase activity seen following detergent treatment. Accordingly, a lower concentration of detergent was needed for securing a maximal ATPase activity than it was used for solubilizing the enzyme. A possible explanation for the detergent activation would be that detergents ascertain a greater steric accessibility for the substrate (Somogyi, 1964a). According to Swanson et al. (1964) it is also possible that the enzyme will be released from a normal inhibitory system by detergent treatment.

It has been demonstrated by several earlier investigations that membrane bound ATPase has a lipoprotein structure (Skou, 1961; Schatzmann, 1962; Ahmed, Judah, 1964; Swanson et al., 1964). Schatzmann (1962) supposed that lipoprotein structure of the enzyme could play a role in the binding process which takes place between substrate and enzyme. It might also be assumed that active ion transport across the membrane would be realized through fixing sodium and potassium on the binding sites of the enzyme and transforming thereby the ions into their lipoid soluble forms. It seems likely that as a result of detergent treatment the integrity of the ATPase lipoprotein structure is also affected. Gradual inactivation of the ATPase, observed at higher detergent concentrations, is likely connected with this phenomenon. However, there is some reason to suppose that the disorganization process takes place to a lesser extent at such detergent concentrations which elicit a maximal enzyme activity than at a concentration giving rise to a more complete solubilization of the enzyme. Nevertheless, further investigations are needed to verify what kind of linkages in the lipoprotein structure may be damaged without eliciting a consecutive change in ATPase activity.

It was demonstrated by Emmelot and Bos (1966) on rat liver membrane preparation that ATPase activity might be lowered substantially by oleic acid and that this inhibition could be prevented by albumin. Oleic acid inhibition of $(Na^+ + K^+)$-activated ATPase of rat brain preparation was observed also in our previous experiments (unpublished results). Albumin stabilizes also the mitochondrial structure, it prevents uncoupling of oxidative phosphorylation. This protective effect of albumin may be connected with its ability of binding liberated free fatty acid (Sacktor, 1954; Pullman, Racker, 1956; Azzone et al., 1961; Weinbach, Garbus, 1966).

The ATPase activities of detergent treated preparations could be also influenced by albumin. Albumin prevented the activity loss of Mg^{++}-dependent ATPase due to treatment with triton-x-100, furthermore, the total ATPase activity was much higher after detergent and albumin treatment than it was following incubation with triton-x-100 alone. It appears likely that the protective action of the albumin was associated in this case with its ability of preventing the formation of the lipoportein structure of the enzyme, while albumin itself was able to bind detergents.

However, in any kind of investigation carried out with ATPase prepared by using detergents one should never forget that detergents may affect also the conformation of the enzyme protein in one way or other. In our previous experiments triton-x-100 not only unmasked SH-groups of the enzyme preparations but in addition a substantially higher concentration of p-hydroxymercury benzoate was required for a half maximal inhibition of $(Na^+ + K^+)$-activated ATPase than in control preparations (Somogyi, Gaál, 1965). The present experiments strongly suggest that changes in ATPase conformation took place not only at a detergent concentration required for the solubilization or causing a loss of activity but also at a lower concentration which simultaneously resulted in a maximal activation of $(Na^+ + K^+)$-activated ATPase. This was indicated by the observation that $Na_2S_2O_4$ which diminished enzyme activity when used separately caused a further increase in ATPase activity if acting together with detergents.

The inhibitory effect of increasing the concentration of tris buffer together with triton-x-100 may also be explained by supposing a change in enzyme conformation. This action of tris-chloride on $(Na^+ + K^+)$-activated ATPase cannot be explained by supposing that the enzyme largely solubilized by 0.05 per cent triton-x-100, would be rendered much more susceptible to an increasing ionic strength than a structure bond enzyme would be. This is contradicted also by the observation that total enzyme activity following preincubation with 200 mM Na^+ or K^+ and 0.05 per cent triton-x-100 failed to decrease in comparison with the activity of a preparation preincubated with detergent alone.

Dayan and Wilson (1964) demonstrated that alkaline phosphatase of Escherichia coli formed tris-phosphate in the presence of higher concentration of tris-chloride. This assumption had been based on the observation that, using p-nitrophenol phosphate as substrate, alkaline phosphatase activity, determined as phosphate liberated, decreased with increasing tris-chloride concentrations, and that in turn no change in activity was observed when the amount of nitrophenol split was measured.

In the case of ATPase preparations, however, obviously no tris-phosphate was formed because no difference in ATPase activity could be demonstrated whether determination of activity was based on measuring P_i liberated or ATP decomposed. The exact explanation of this observation, however, requires further experiments.

Detergent treatment carried out in the presence of Na^+ or K^+ resulted in a more expressed increase in the $(Na^+ + K^+)$-activated ATPase activity than in their absence. This indicates that these ions may also influence the conformation of the enzyme. Protective action of cations prevailed not only against detergents but also against proteolytic enzymes acting on ATPase (Somogyi, 1968). ATPase solubilized by means of Lubrol W may be stabilized by Na^+ or K^+ (Medzihradsky et al., 1967).

In addition to Na^+ and K^+ also Mg^{++} displays a protective action. Mg ions prevent the inhibitory action of triton-x-100 upon Mg^{++}-dependent ATPase. Furthermore, Mg ions counteract partly the solubilizing effect of triton-x-100 and that of sonical treatment as well. This effect of Mg^{++} is especially pronounced in the presence of an equimolar amount of EDTA. Consequently, these observa-

tions strongly indicate that Mg^{++} is not only needed for ensuring the interaction between ATP and ATPase but it is also capable of affecting stability of the whole enzyme system. Further investigations are needed, however, to elucidate whether the protective action of Mg^{++} against a loss of Mg^{++}-dependent ATPase activity, on the one hand, and the counteraction of the solubilization of the enzyme, on the other, is to be attributed to the same cause or not. It seems unlikely that Mg^{++}-dependent protection could be ascribed solely to the ensuing aggregation of the membrane fragments (in which the ATPase was localised in our preparations) caused by the Mg ions.

There is no definitive answer as yet to the question whether or not Mg^{++}-dependent and $(Na^+ + K^+)$-activated ATPase activities, respectively, derive from the same enzyme systems. Järnefelt (1964) claims that the activating effect of deoxycholate on $(Na^+ + K^+)$-activated ATPase is due to the fact that a Mg^{++}-dependent enzyme which contains bound sodium and potassium loses the monovalent cations following deoxycholate treatment and by this means the enzyme is transformed into a sodium- and potassium-dependent form. This opinion was supported by the observation of Landon and Norris (1963) who demonstrated that ATP ase prepared from rat kidney could be activated by Mg^{++} alone, the enzyme, however, became $Na^+ + K^+$-dependent following dialysis. Askari and Fratantoni (1964) as well as Skou and Hilberg (1965) similarly assume a possible interconversion between the two enzyme systems. Cooper and McIlwain (1967) demonstrated that urea + Na-ATP treatment of the enzyme preparation resulted in a pronounced diminution of the Mg^{++}-dependent ATPase activity while total activity remained essentially unaffected. Identical results were obtained also in our investigations when enzyme preparations were subjected to sonication in the presence of Mg^{++} and EDTA.

In our opinion the dissimilar effects of detergents and those of various other disruptive agents upon ATPase activities detected in the presence of Mg^{++} or $Mg^{++} + Na^+ + K^+$ cannot be fully explained by assuming an interconversion of the two ATPases. This is supported by the observation that detergents not only decreased the Mg^{++}-dependent ATPase activity but were also able to increase at the same time total activity by 40–100 per cent, depending on the experimental conditions. This means, of course, that the increased activity of the $(Na^+ + K^+)$-activated ATPase might be attributed to a large extent to increase in total activity. On the other hand, following application of the detergents at a concentration which completely abolishes the activating effect of $Na^+ + K^+$, approximately 40–60 per cent of the Mg^{++}-dependent activity could still be recovered. Furthermore, digitonin, applied in 2 per cent concentration during preincubation, completely revoked the activating effect of $Na^+ + K^+$, but caused simultaneously an increase of about 100 per cent in the Mg^{++}-dependent ATPase activity. Experimental conditions could be found where reduced Mg^{++}-dependent activity was accomplished by unaltered total ATPase activity. This is, however, no unconditional indication of an interconversion between Mg^{++}-dependent and $(Na^+ + K^+)$-activated ATPase.

Preparation of $(Na^+ + K^+)$-activated ATPase free from Mg^{++}-dependent enzyme is based on the inactivation of Mg^{++}-dependent activity achieved by

different methods (Rendi, Uhr, 1964; Somogyi, 1964; Glynn et al., 1965). A column chromatographic separation of the two enzyme activities in solubilized preparations cannot be carried out satisfactorily (Medzihradsky et al., 1967). A reassuring solution of the problem, whether these two activities could be considered as two convertible forms of the same enzyme, could be achieved only if reversible interconversion of the two separated enzyme activities could be accomplished. Every effort made along these lines, however, was unsuccessful so far.

It has recently been supposed that while $(Na^+ + K^+)$-activated ATPase would play a role in the active ion transport process, the Mg^{++}-dependent enzyme would somehow participate in the passive transport phenomena (Duncan, 1967). Elucidation of the exact nature of differences existing between Mg^{++}-dependent and $(Na^+ + K^+)$-activated ATPases, however, requires further detailed investigations.

References

Ahmed, K., Judah, J. D. (1964) Biochim. Biophys. Acta *93* 603
Albers, R. W. (1967) Ann. Rev. Biochem. *36* 727
Askari, A., Fratantoni, J. C. (1964) Biochim. Biophys. Acta *92* 132
Azzone, G. F., Eeg-Olofsson, O., Ernster, L., Luft, R., Szabolcsi, G. (1961) Exp. Cell Res. *22* 415
Bradford, H. F., Swanson, P. D., Gammack, D. B. (1964) Biochem. J. *92* 247
Cooper, J. R., McIlwain, H. (1967) Biochem. J. *102* 675
Csáky, T. Z. (1965) Ann. Rev. Physiol. *28* 415
Dayan, J., Wilson, I. B. (1964) Biochim. Biophys. Acta *81* 620
Deul, D. H., McIlwain, H. (1961) J. Neurochem. *8* 246
Duncan, C. J., (1967) The Molecular Properties of Excitable Cells, Pergamon Press Oxford.
Dunham, E. T., Glynn, I. M. (1961) J. Physiol. *156* 274
Emmelot, P., Bos, C. J. (1966) Biochim. Biophys. Acta *120* 369
Glynn, I. M. (1963) J. Physiol. *169* 452
Glynn, I. M., Slayman, C. W., Eichberg, J., Dawson, R. M. C. (1965) Biochem. J. *94* 692
Gornall, A. G., Bardawill, C. J., David, M. D. (1949) J. Biol. Chem. *177* 751
Heinz, E. (1967) Ann. Rev. Physiol. *29* 21
Hokin, L. E., Reasa, D. (1964) Biochim. Biophys. Acta *90* 176
Järnefelt, J. (1964) Biochem. Biophys. Res. Commun. *17* 330
Jirgensons, B. (1961) Arch. Biochem. Biophys. *94* 59
Keller, H., Gottwald, P. (1960) Hoppe-Seyler's Z. Physiol. Chem. *318* 201
Kishimoto, U., Adelman, W. J. (1964) J. Gen. Physiol. *47* 975
Landon, E. J., Norris, J. L. (1963) Biochim. Biophys. Acta *71* 266
Lohmann, K., Jendrassik, L. (1926) Biochem. Z. *178* 419
Markus, G., Karush, F. (1956) J. Am. Chem. Soc. *79* 134
Medzihradsky, F., Kline, M. H., Hokin, L. E. (1967) Arch. Biochem. Biophys. *121* 311
Nakao, T., Tashima, Y., Nagano, K., Nakao, M. (1965) Biochem. Biophys. Res. Commun. *19* 755
Pullman, M. E., Racker, E. (1956) Science *123* 1105
Putnam, F. W. (1948) Adv. Protein Chem. *4* 79
Rendi, R., Uhr, M. L. (1964) Biochim. Biophys. Acta *89* 520
Rothstein, A. (1968) Ann. Rev. Physiol. *30* 15
Sacktor, B. (1954) J. Gen. Physiol. *37* 343
Schatzmann, H. J. (1962) Nature *196* 677
Skou, J. C. (1961) in: Membrane Transport and Metabolism, Kleinzeller, A. and Kotyk, A. (eds), Academic Press, New York, p. 228

Skou, J. C. (1962) Biochim. Biophys. Acta *58* 314
Skou, J. C. (1965) Physiol. Rev. *45* 596
Skou, J. C., Hilberg, C. (1965) Biochim. Biophys. Acta *110* 359
Somogyi, J. (1963) Naturwissenschaften *50* 157
Somogyi, J. (1964a) Naturwissenschaften *51* 143
Somogyi, J. (1964b) Biochim. Biophys. Acta *92* 615
Somogyi, J. (1968) Biochim. Biophys. Acta *151* 421
Somogyi, J., Gaál, J. G. (1965) Experientia *21* 573
Somogyi, J., Vincze, S. (1961) Acta Physiol. Acad. Sci. Hung. *20* 325
Somogyi, J., Vincze, I. (1962) Acta Physiol. Acad. Sci. Hung. *21* 29
Swanson, P. D., Bradford, H. F., McIlwain, H. (1964) Biochem. J. *92* 235
Weinbach, E. C., Garbus, J. (1966) J. Biol. Chem. *241* 169
Yoshida, H., Fujisawa, H. (1962) Biochim. Biophys. Acta *60* 443

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (3), pp. 237—249 (1969)

Studies on the Mobilization of Fats in Lower Vertebrates

T. FARKAS

Biological Research Institute, Hungarian Academy of Sciences, Tihany

(Received July 29, 1968)

In vitro the glycerine : fatty acid production of the adipose tissue of fish (Abramis brama L.), amphibia (Rana ridibunda L.) and reptiles (Natrix natrix L.) is approximately 1 : 3. The *in vivo* administration of glucose reduces the free fatty acid level of fish blood plasma. Glucose reduces the free fatty acid production of fish, amphibian and reptilian adipose tissues under *in vitro* conditions.

After the *in vivo* administration of norepinephrine in contrast to mammals, the plasma-free fatty acid level in fish (Cyprinus carpio L., Abramis brama L.) and amphibia (Rana ridibunda L.) decreases and remains unchanged in the turtle (Emys orbicularis L.). The *in vitro* free fatty acid production of fish and amphibian adipose tissue which *in vivo* have been treated with norepinephrine is lower than that of the untreated animals. The free fatty acid production in fish, amphibian and reptilian adipose tissues incubated in the presence of norepinephrine, epinephrine, ACTH and glucagon is lower than that in the controls. Epinephrine has no effect on the glycerol production in the adipose tissue either *in vivo* or *in vitro*. Epinephrine, ACTH and glucagon enhance the phosphorylase activity in fish, amphibian and reptilian adipose tissues. Dichloro-isoproterenol antagonizes the effect of adrenaline on the adipose tissue phosphorylase of frog, as well as the effects of epinephrine, ACTH and glucagon on the production of free fatty acids.

It may be assumed that the adipose tissues of lower vertebrates respond like that of mammals — with an enhanced cAMP synthesis to the lipolytic hormones tested. The effect of hormones on the adipose tissue of lower vertebrates may be explained with the absence of hormone sensitive lipolytic activity and with enhanced re-esterification.

Introduction

It is generally accepted that mammals mobilize the triglycerides stored in their adipose tissue in the form of free fatty acids which reach the various tissues in a form bound to albumin (Fredrickson, Gordon, 1958). The free fatty acid production of mammalian adipose tissues is under metabolic, nervous and endocrine control (Steinberg, 1963). The amount of free fatty acids released by the adipose tissue depends partly on the degree of re-esterification of the produced fatty acids and partly on lipase activity which catalyzes the hydrolysis of triglycerides. Through the first process glucose reduces, and through the second the sympathetic nervous system and the various hormones (epinephrine, ACTH, glucagon, etc.) enhance the production of free fatty acids (Steinberg, 1963).

The lipids are stored in a type of tissue differentiated for this particular purpose, which in the invertebrates may fulfil other functions, too (hepatopancreas).

In the phylogenesis of vertebrates an adipose tissue similar to that of mammals appears first in freshwater fish. In spite of the fact that the processes of lipid mobilization are well studied in mammals, little is known on this process in lower organisms. It appears from the investigations of Goodbridge and Ball (1965), Hoak et al. (1967), and Carlson et al. (1964) that the adipose tissue of birds reacts similarly to that of mammals to certain lipolytic hormones. Our earlier studies on the other hand have proved that triglyceride decomposition is not stimulated by catecholamines and ACTH in fish (Farkas, 1967) and by norepinephrine in green frog (Farkas, 1966). In the work presented here we have investigated the effect of glucose, catecholamines, ACTH and glucagon on the production of free fatty acid in fish, amphibian and reptilian adipose tissue and from the results we attempt to compare the functioning of the adipose tissue of lower and higher vertebrates.

Materials and Methods

The animals used in the experiments were the following: pike perch (Lucioperca lucioperca L.), breams (Abramis brama L.), carps (Cyprinus carpio L.), of 250—300 g weight, male marsh frogs (Rana ridibunda L.) of 80—90 g weight, common snakes (Natrix natrix L.) of 80—100 g weight and turtle (Emys orbicularis L.). The fish were collected from the Lake Balaton, the frogs bought and both types of reptiles collected. The experiments were carried out in the summer months (May to October). The animals were brought into the laboratory one week before the experiment and kept in appropriate aquaria or terraria. No food was given to the animals in captivity.

Norepinephrine dissolved in saline was injected into fish and turtles intramuscularly and into the abdominal lymph sack of frogs. Glucose was introduced intra-abdominally into fish and into the abdominal lymph sack of frogs.

Blood was taken from fish by a scission of the caudal vein and after complete bleeding from frogs by decapitation and from turtles by cardiac puncture. Blood was collected in ice-cooled test tubes containing 0.1 ml of 2 per cent heparine solution.

The quantity of plasma free fatty acids was determined by Dole's method (1965) from 0.5—1.0 ml of blood plasma. For the determination of blood sugar the method of Hyvärinen and Nikkila (1962) was chosen.

For *in vitro* experiments the adipose tissues were collected in frog Ringer solution and 1—2 mm thick slides were prepared. The adipose tissues were incubated in 3 ml of frog Ringer solution at room temperature for 60 minutes. The pH of the incubation medium was adjusted to 7.4. Incubation was carried out in albumin-free media. Under these conditions like in the case of mammals, the produced free fatty acids accumulate in the adipose tissue and the bulk of the produced glycerine in the incubation medium. Up to 90 to 120 minutes of incubation the production of free fatty acids by the adipose tissue was found to be proportional

to the time of incubation (Farkas, 1966, 1967). The active substances used in the *in vitro* experiments (catecholamines, adrenocorticotrophic hormone, glucagon, dichloro-isoproterenol) were dissolved in frog Ringer solution immediately before application.

The free fatty acid content of the adipose tissue was measured at the beginning and end of the experiment; the difference gave the quantity of free fatty acid produced during the experiment. The adipose tissues were homogenized in a Potter-Elvehjem homogenizer in the presence of n-heptane. The homogenates were made up to 5 ml with n-heptane and a 1 ml aliquot was titrated with 0.01 N KOH in the presence of bromothymol blue. In cases when the adipose tissues could not be homogenized at the end of the experiment they were stored in n-heptane at —25 °C.

The quantity of glycerine produced by the adipose tissue was measured from an aliquot part (0.2—0.5 ml) of the incubation medium by the method of Lambert and Neish (1950) and also from an aliquot part (0.3—0.5 ml) of an extract prepared from the homogenized adipose tissue with 3 ml of 0.01 N hydrochloric acid.

For the determination of the adipose tissue glycogen content the adipose tissue was homogenized in 5 per cent trichloroacetic acid and the precipitated proteins and lipids were removed by filtration through filter paper. For the measurement of glycogen van der Vies' method (1954) was used.

The phosphorylase activity of the adipose tissue was assayed by the method of Frerichs and Ball (1962). The adipose tissues (100—150 mg) were homogenized in 0.1 M NaF solution and the bulk of the lipids removed by centrifuging at +4 °C (15 minutes at 900 g). 0.5 ml of the lipid-free homogenate was incubated in the presence of 4.0 mg of glycogen, 31.5 μmoles of glucose-1-phosphate, 1.4 μmoles of 5-adenosine monophosphate and 85 μmoles of NaF at 30 °C for 30 minutes. The pH was adjusted to 6.3, the final volume of the reaction mixture was 1.3 ml. The reaction was stopped with 0.2 ml of 0.1 M perchloric acid and the pH of the system subsequently adjusted to 4 by the addition of 3 ml of 0.2 M acetate buffer solution. Phosphorylase activity was characterized by the liberation of inorganic phosphate in the system. The quantity of inorganic phosphate was determined by the method of Taussky and Shorr (1952).

Results

The effect of glucose on lipid mobilization

After the *in vivo* administration of glucose the free fatty acid level of the plasma of carp decreases. It appears from Fig. 1 that after the administration of glucose the free fatty acid of the plasma and the blood sugar level change in opposite directions. Under *in vitro* conditions the adipose tissue of fish and marsh frogs which have been treated *in vivo* with glucose produces less free fatty acid than the adipose tissue of animals which have been starved for one week. The adipose tissues of starved animals produce less free fatty acid also when glucose is added directly to the incubation medium. While there is a satisfactory agreement

Fig. 1. The effect of glucose on the free fatty acid content of the plasma and on the blood sugar level. Glucose was administered on the 7th, 8th and 9th day of starvation (1 g/animal). Each point corresponds to the average analytical result of blood samples taken by complete bleeding of 5 animals. ●– – –● blood sugar; ○——○ free fatty acid in the plasma

Table 1

The effect of glucose on the in vitro free fatty acid and glycerine production in the adipose tissue of fish, amphibia and reptiles

Experimental animal	No. of experiments	Treatment	Glycerine, µmole/g/h	Free fatty acid, µmole/g/h	
				calculated	measured
Lucioperca lucioperca L.	10	1 week starving	1.16±0.09	3.48	3.47±0.17
	10	+ 10 µmoles glucose *in vitro*	—	—	1.52±0.10
		+ 0.5 g glucose *in vivo*	1.05±0.06	3.15	2.54±0.08
Rana ridibunda L.	15	1 week starving	1.06±0.08	3.18	3.02±0.10
	5	+ 10 µmoles glucose *in vitro*	—	—	0.25±0.05
	5	+ 2×0.5 g glucose *in vivo*	0.83±0.05	2.49	—
Natrix natrix L.	5	1 week starving	0.95±0.09	2.85	2.79±0.12
	5	+ 10 µmoles glucose *in vitro*	—	—	0.50±0.08

Note: Glucose was administered to fish 12 hours, to amphibia 24 and 12 hours before blood sampling. The adipose tissues were incubated for 60 minutes in an albumin-free frog Ringer solution at pH 7.4 and room temperature.

between the experimentally determined free fatty acid production and that determined from the glycerine production of the adipose tissue of starved animals, the adipose tissue of animals treated with glucose produces less free fatty acid than expected from glycerine production (Table 1). Since the *in vitro* glycerine production of the adipose tissues of glucose-treated and starved animals is almost the same, the conclusion may be drawn that the degree of triglyceride decomposition is the same in both cases, but in the adipose tissue of the glucose-treated animals a part of the free fatty acid produced, as in the case of mammals, is re-esterified again to triglyceride. The good agreement between the calculated and measured free fatty acid production of the adipose tissue of starved animals points to the reduced importance of re-esterification in starved animals.

The effect of norepinephrine on lipid mobilization

The quantity of free fatty acids increases in the plasma of mammals after the *in vivo* administration of noradrenaline. Earlier we have found that this hormone reduces the free fatty acid level of the plasma of carp and has no influence

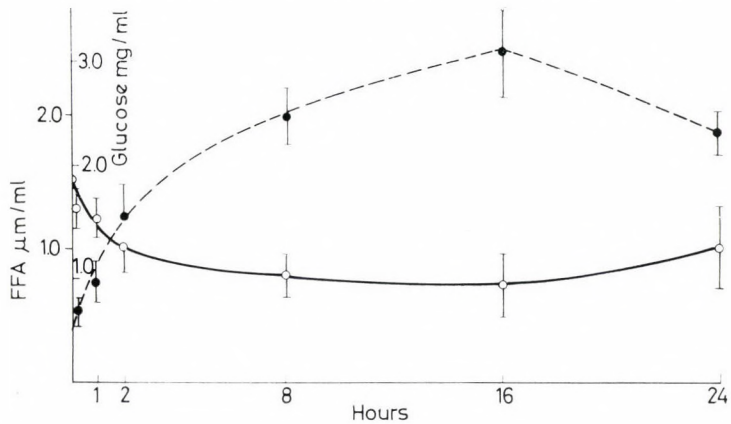


Fig. 2. The effect of norepinephrine on the free fatty acid content of carp plasma and on the blood sugar level of the animal. Norepinephrine was injected intramuscularly into animals fasted 10 days. Each value is the average of the analytical results of blood samples taken by a complete bleeding of 5 animals. ● — — — ● blood sugar; ○ ——— ○ free fatty acid of the plasma

on that of the green frog (Farkas, 1966, 1967). Fig. 2 illustrates the rapid reaction of the carp to the injected hormone. In the 10th minute following the administration of the hormone there is already a significant rise in the blood sugar level and a decrease in the free fatty acid level of the plasma. The blood sugar level and the free fatty acid level of the plasma undergo changes of opposite directions: the reduction of the plasma free fatty acid level will last as long as the blood sugar is higher than the control value.

Fig. 3 shows the effect of norepinephrine on the free fatty acid and glucose contents of the blood of bream, marsh frog and turtle. After the injection of norepinephrine the free fatty acid level of the plasma decreased significantly in

bream and marsh frog ($P > 0.05$) but less markedly in the turtle. In preliminary experiments the plasma glycerine level showed no change in any of the cases compared to the value of the control. The blood sugar level increased significantly ($P > 0.01$) in all three types of animals indicating that the hormone has reached their circulation, and also that the glycogenolytic system of their liver appears to react to norepinephrine in the same way as that of mammals.

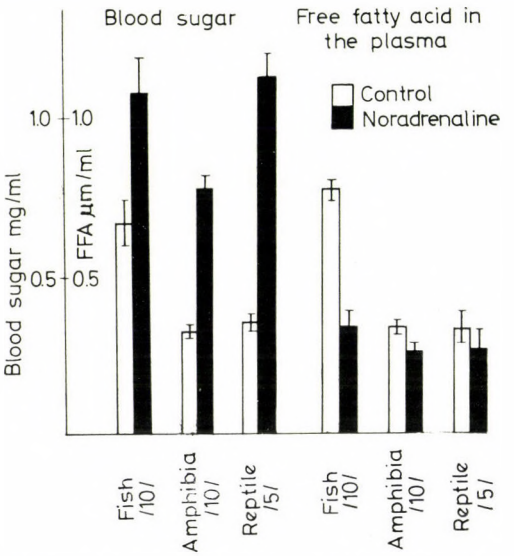


Fig. 3. The effect of norepinephrine on lipid and glycogen mobilization in lower vertebrates. The hormone was injected intramuscularly into fish (Abramis brama, 8 mg/kg), subcutaneously into frog (Rana ridibunda, 6 mg/kg) and turtle (Emys orbicularis, 6 mg/kg) 2 hours prior to taking the blood sample. The figures in brackets are the numbers of animals used in the experiment

The in vitro effect of lypolytic hormones

If norepinephrine or the other lypolytic hormones were to stimulate — as in the case of mammals — the degree of triglyceride decomposition in the adipose tissue, it might be expected that a) under *in vitro* conditions the adipose tissues of animals treated *in vivo* with norepinephrine would produce more free fatty acid than the adipose tissue of untreated animals and b) the hormones would stimulate the hydrolysis of triglycerides under *in vitro* conditions as well.

The data in Table 2 show that contrary to those of mammals (Schotz, Page, 1960) the adipose tissues of fish and green frog treated *in vivo* with norepinephrine produce less free fatty acid than the adipose tissues taken from untreated animals and that the presence of noradrenaline in the incubation medium does not lead to an enhanced free fatty acid production in the adipose tissue.

Fig. 4 illustrates the reduced free fatty acid production of the adipose tissue of fish, frog and snake under the effect of epinephrine, adrenocorticotrophic hormone and glucagon. The glycerine production of adipose tissues incubated

in the presence of epinephrine shows no significant deviation from the glycerine production of the control tissues. The same was observed for marsh frog treated with ACTH and glucagon, but all three hormones were shown to enhance the free fatty acid and glycerine production in the adipose tissue of rat (Vaughan, 1961).

Table 2

The effect of in vivo and in vitro administered norepinephrine on the in vitro free fatty acid production of adipose tissues

Experimental animal	Treatment	Adipose tissue free fatty acid, μmole/g/h	Difference
Abramis brama L. (5)	control	5.02±0.62	—
	NE *in vivo* (8 μg/kg)	3.42±0.24	−1.60
	NE *in vitro* (2 μg/ml)	3.00±0.19	−2.02
Rana esculenta (6)	control	2.42±0.42	—
	NE *in vivo* (6 mg/kg)	1.76±0.11	−0.66
	NE *in vitro* (2 μg/ml)	1.44±0.17	−0.98
Natrix tessalata (4)	control	2.90±0.31	—
	NE *in vitro* (2 μg/ml)	1.45±0.11	−1.55

Note: NE = noradrenaline. The hormone was injected into the animals which had been starved for 6 days 2 hours prior to taking blood sample.

The free fatty acid production of adipose tissues treated with norepinephrine *in vivo* or *in vitro* changed in the same way as of those which were incubated in the presence of glucose.

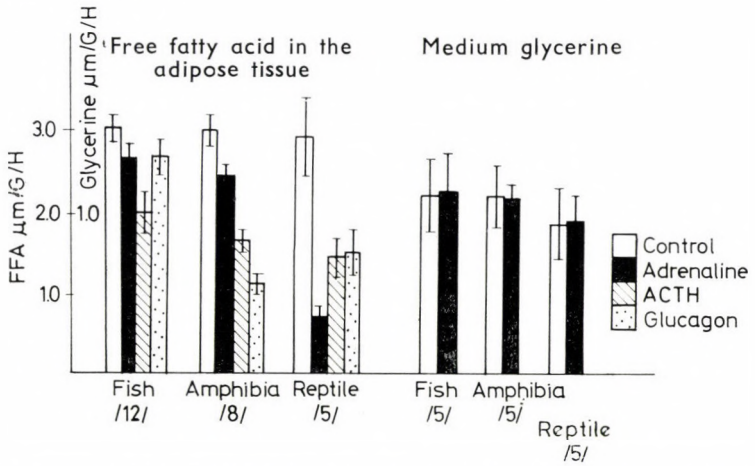


Fig. 4. The effect of lipolytic hormones on the *in vitro* free fatty acid production of adipose tissues. The adipose tissues of fish (Lucioperca lucioperca L.), frog (Rana ridibunda L.) and turtle (Emys orbicularis L.) fasted 6 days were incubated for 60 minutes in an albumin-free frog Ringer solution at room temperature in the presence of 2 μg/ml of epinephrine , 0.3 IU/ml of adrenocorticotrophic hormone (ACTH), and 2 μg/ml of glucagon (Glc). The free fatty acids were determined from the adipose tissue, glycerine from the incubation medium

The effect of lipolytic hormones and dichloro-isoproterenol on the glycogenolytic activity and free fatty acid production of frog adipose tissue

Catecholamines and the other lipolytic hormones stimulate the free fatty acid production of the adipose tissue of mammals by increasing the intracellular level of cyclic 3',5'-adenosine monophosphate (cAMP) (Butcher et al., 1965; Butcher, 1966). We have demonstrated in our earlier work that norepinephrine enhances the *in vitro* phosphorylase activity of frog adipose tissue (Farkas, 1966). The data in Table 3 show a similar effect for epinephrine, ACTH and glucagon.

Table 3

The effect of lipolytic hormones on the phosphorylase activity of the adipose tissues of fish, amphibia and reptiles

Experimental animal	No. of experiments	Adrenaline 2 μg/ml	ACTH 0,3 IU/ml μmol Pi/30'/g	Glucagon 2 μg/ml
Lucioperca lucioperca	3	+2.14±0.15	+2.75±0.22	+0.97±0.10
Rana ridibunda	3	+2.30±0.30	+2.35±0.19	+2.00±0.25
Natrix natrix	3	+2.70±0.70	+0.94±0.45	+0.97±0.35

Note: The adipose tissues were incubated in the presence of hormones for 30 minutes. The lipid-poor homogenates prepared of the adipose tissues were incubated for a further 30 minutes in the presence of 4.0 mg of glycogen, 31.3 μmoles of glucose-1-phosphate, 1.4 μmoles of 5-AMP and 85 μmoles of NaF at 30 °C. Enzyme activity was expressed as the quantity of liberated inorganic phosphate (μmoles/g/30'). The figures represent the differences between the phosphorylase activities of the control and treated adipose tissues.

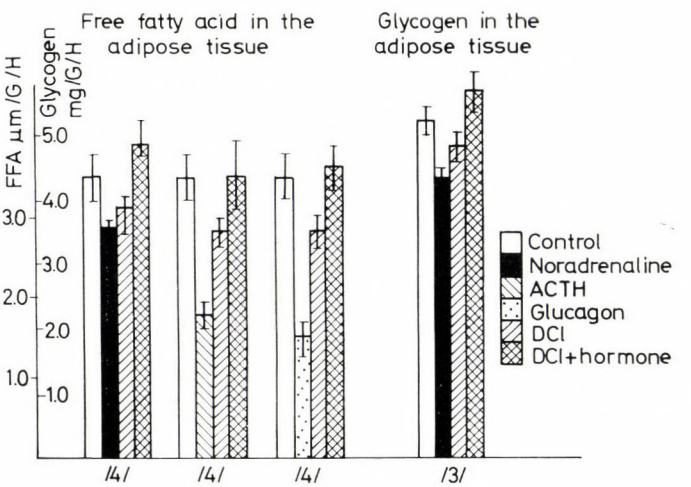


Fig. 5. The effect of lipolytic hormones and dichloro-isoproterenol on glycogen decomposition and on the free fatty acid production of the adipose tissue. The experimental conditions are the same as for Fig. 4. norepinephrine, 2 μg/ml; DCI = dichloro-isoproterenol, 20 μg/ml

The enhanced phosphorylase activity indicates that the adipose tissue of lowe vertebrates similar to that of mammals responds to the lipolytic hormones with an increased cAMP synthesis. If the hormones investigated reduce the accumulation of free fatty acids in the adipose tissues of the experimental animals through the same system, it may be expected that dichloro-isoproterenol (DCI), a potent inhibitor of adenyl cyclase, will antagonize their effect on adipose tissue phosphorylase and on free fatty acid production. Fig. 5 shows that DCI affects glycogen decomposition in the adipose tissue of the marsh frog in the same way as norepinephrine does, but when the two hormones are administered together DCI inhibits the action of norepinephrine. DCI also antagonizes the effect of norepinephrine, ACTH and glucagon on the free fatty acid production of frog adipose tissue This effect of DCI is in agreement with the results obtained with mammals (Zsötér et al., 1966; Love et al., 1963; Mayer et al., 1961).

Discussion

Our earlier work (Farkas, 1966, 1967) and the results of our present investigations show that lower vertebrates mobilize the triglycerides stored in their adipose tissues similarly to mammals in the form of free fatty acids.

These investigations point further to the fact that free fatty acid production of the adipose tissue in lower vertebrates is mainly controlled by the carbohydrate metabolism of the adipose cells. The *in vivo* administration of glucose reduces the free fatty acid level in the blood of fish, amphibia or reptiles (work in progress) and the presence of glucose in the incubation medium has the same effect *in vitro*. From this aspect the lipid depots in lower vertebrates, but even in certain insects (Wlodaver, Baranska, 1965) behave in the same way as the adipose tissues of mammals.

The adipose tissues of fish, amphibia and reptiles differ unequivocally from the adipose tissues of mammals inasmuch as in them catecholamines, ACTH and glucagon fail to enhance the degree of triglyceride breakdown. These hormones, contrary to those of mammals, will induce no increase in the free fatty acid production of the adipose tissue but a decrease.

The fact that the investigated hormones fail to raise triglyceride hydrolysis in lower vertebrates may be explained in the following ways:

1. there is a difference in the structure of peptide hormones between lower vertebrates and mammals,

2. the adipose tissue of lower vertebrates deactivates the investigated hormones,

3. the lipolytic system of the adipose tissue of lower vertebrates differs from that of mammals.

Assumption 1. is supported by the fact that the experiments were carried out with mammal hormone preparations and the structure of the peptide hormones are known to differ even in the different mammal species. Data are, however, indicating that differences in molecular structure have no significant influence on their physiological activity (Geschwind, 1959). There is on the other hand no

direct evidence for any structural difference of hormones between lower vertebrates and mammals. The finding that appropriately prepared extracts of the hypophysis of cartilaginous fish, reptiles and mammals equally stimulated the corticosteroid synthesis in the suprarenal cortex of chicken indicates that the structure of ACTH is essentially the same in all vertebrates (Gist, de Roos, 1966). The situation is probably the same in the case of glucagon since mammal glucagon will bring about a higher blood sugar level in reptiles (Marques, 1967), amphibia (Wright, 1959) and teleost fish (Falkmer, 1965) alike.

In the case of catecholamines no structural problems arise. These compounds are present in various tissues of lower vertebrates (Fänge, 1962), moreover fish blood seems to contain more catecholamines than the blood of mammals (Nakano, Tomilson, 1967).

The second possibility is excluded by the observation that under *in vitro* conditions free fatty acid production in the adipose tissue of fish, amphibia and reptiles decreased in the presence of all three hormones which would not have been the case had their adipose tissue, similarly to the adipose tissue of certain mammals (Rudman et al., 1964), brought about a degradation of the peptide hormones. On the other hand, in the adipose tissue of mammals which were capable of deactivating these hormones there was no reduction in free fatty acid production.

We consider it therefore probable that the lipolytic system of the adipose tissue in lower vertebrates differs from that of mammals. In the case of mammals the catecholamines, ACTH and glucagon enhance the degree of triglyceride hydrolysis by way of raising the intracellular cAMP level of the adipose tissue (Butcher et al., 1965; Butcher, 1966). The hormones act on adenyl cyclase localized in the cell wall and is known to catalyze the synthesis of cAMP. cAMP on the other hand stimulates the so-called "hormone sensitive lipase" which has been described by Rizack for the adipose tissue of rat (Rizack, 1961, 1964). Adenyl cyclase is fairly common in animals (Sutherland et al., 1962) and there can be no doubt that norepinephrine displays its hyperglycaemic effect in the case of fish, amphibia and reptiles through the adenyl cyclase—cAMP—phosphorylase system of the liver. The presence of this system also in other tissues of lower vertebrates is suggested by the observations showing that the physiological effects of catecholamines, mediated by cAMP in mammals, are the same in lower vertebrates and in mammals (Fänge, 1962), or that ACTH stimulates corticosteroid synthesis in the suprarenal cortex of lower vertebrates in the same way as in that of mammals (Macchi, Rizzo, 1962: Macchi, Phyllis, 1966; Carstensen, Burges, 1961).

From our present results it appears probable that the adipose tissue of lower vertebrates responds in the same way with an enhanced cAMP synthesis to the hormones tested as the adipose tissue of mammals, furthermore that the nucleotide mediates the effect of catecholamines, ACTH and glucagon on the free fatty acid production of the adipose tissue. This is indicated by the fact that the catecholamines enhance phosphorylase activity and the degree of glycogen decomposition in frog adipose tissue and also by the cessation of the effect of the above hormones on frog adipose tissue phosphorylase and on the free fatty acid production of the investigated adipose tissues in the presence of DCI.

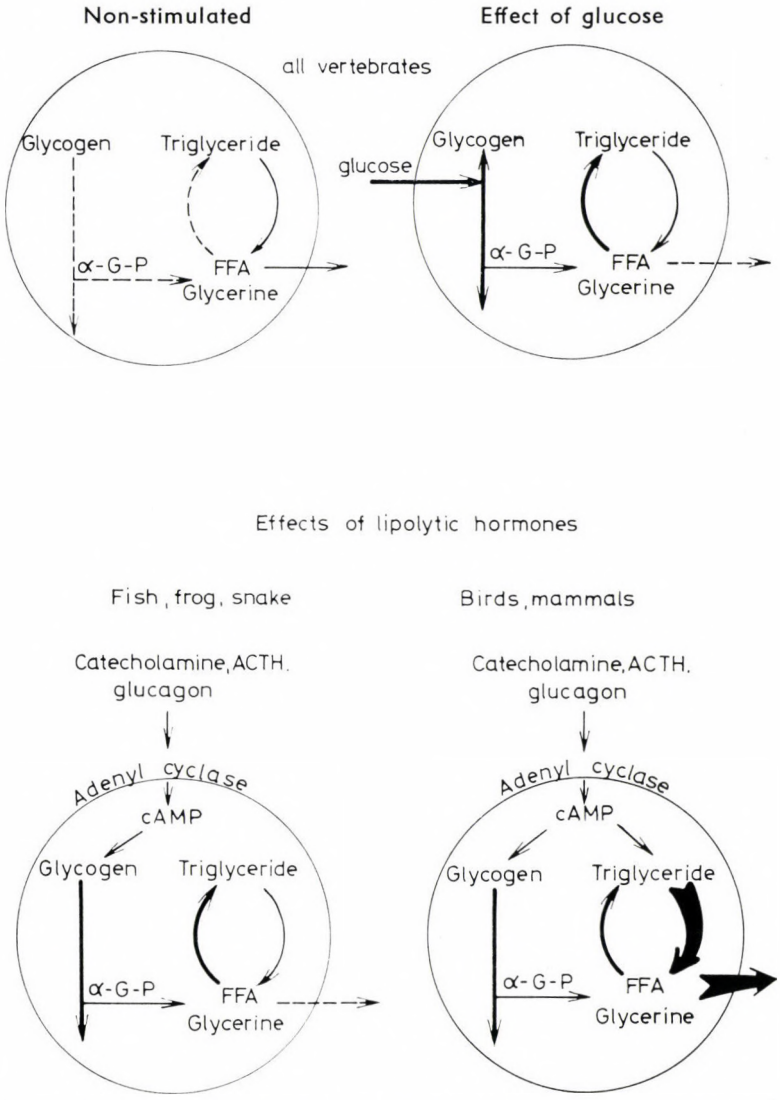


Fig. 6. Schematic diagram illustrating the factors controlling the free fatty acid production of the adipose tissue of vertebrates

To explain the finding that the cAMP formed in the adipose tissue under the action of hormones fails to enhance the degree of triglyceride decomposition we may assume that "hormone sensitive" lipolytic activity is lacking in the adipose tissue of fish, amphibia and reptiles. By accepting this assumption we have attempted to illustrate in Fig. 6 the mechanisms controlling the free fatty acid production in the adipose tissue of vertebrates.

In the adipose tissue of lower vertebrates just as in that of mammals the triglycerides are continuously hydrolyzed into glycerine and free fatty acids.

The further fate of the free fatty acids formed will depend on the availability of α-glycerophosphate for re-esterification. If the amount of α-glycerophosphate is small the fatty acids liberated will leave the adipose tissue, if there is much α-glycerophosphate present the free fatty acid release will decline. Glucose added to the incubation medium is taken up by the adipose tissue and converted partly into α-glycerophosphate.

In the same way as in mammals the lipolytic hormones stimulate adenyl cyclase which responds by an enhanced cAMP synthesis. But while in mammals this nucleotide stimulates besides phosphorylase also a lipase, in fish, amphibia and reptiles it stimulates the phosphorylase only. Higher phosphorylase activity results in an enhanced glycogen decomposition which is finally accompanied by an increase in α-glycerophosphate production. A higher α-glycerophosphate level in the adipose tissue offers a possibility for a higher degree of re-esterification of the fatty acids produced in the adipose tissue, in the same way as when the adipose tissue is incubated in the presence of glucose. It has been demonstrated in mammals that lipolytic hormones enhance the re-esterification of the free fatty acids (Vaughan, 1962), but in mammals this process is less evident, since under the action of hormone-sensitive lipolytic activity more free fatty acids are formed than the adipose tissue would capable to re-esterify. In fish, amphibia and reptiles these re-esterification processes also become more intensive, but their result is far more conspicuous because of the absence of hormone-sensitive lipolytic activity.

References

Butcher, R. W. (1966) Pharmacol. Rev. *18* 237
Butcher, R. W., Ho, R. J., Meng, H. C., Sutherland, E. W. (1965) J. Biol. Chem. *240* 4515
Carlson, L. A., Liljedahl, S. O., Verdy, M., Virsen, C. (1964) Metabolism *13* 227
Cartensen, H., Burges, A. C. J. (1961) Gen. Comp. Endocrin. *1* 37
Dole, V. P. (1956) J. Clin. Invest. *35* 150
Falkmer, S. (1965) Gen. Comp. Endocrin. *5* 674
Farkas, T. (1966) Annal. Biol. Tihany *33* 159
Farkas, T. (1967) Progr. Biochem. Pharmacol. *3* 315
Fänge, R. (1962) Pharmacol. Rev. *14* 281
Fredrichs, H., Ball, E. C. (1962) Biochemistry *1* 501
Fredrickson, D. S., Gordon, R. S. (1958) Physiol. Rev. *38* 585
Geschwind, I. I. (1959) Comparative Endocrinology. Ed.: Gorbman, A. Wiley and Sons, Inc. p. 421
Gist, D. H., DeRoos, (1966) Gen. Comp. Endocrin. *7* 304
Goodbridge, A. G., Ball, E. C. (1965) Comp. Biochem. Physiol. *16* 367
Hoak, J. C., Connor, W. E., Warner, E. D. (1966) Clin. Res. *14* 440
Hyvärinen, A., Nikkila, S. A. (1962) Clin. Chim. Acta *7* 140
Lambert, M., Neish, A. G. (1950) Canad. J. Res. *28* 83
Love, W. C., Carr, L., Ashmore, J. (1963) J. Pharmacol. Exp. Ther. *140* 281
Macchi, J. A., Rizzo, F. (1962) Proc. Soc. Exp. Biol. Med. *140* 433
Macchi, J. A., Phyllips, J. G. (1966) Gen. Comp. Endocrin. *6* 170
Marques, M. (1967) Gen. Comp. Endocrin. *9* 102
Mayer, S., Moran, N. C., Fain, J. (1961) J. Pharmacol. Exp. Therap. *134* 18
Nakano, T., Tomilson, N. L. (1967) J. Fish Res. Bd. Canada *24* 1701
Rizack, M. A. (1961) J. Biol. Chem. *236* 657
Rizack, M. A. (1964) J. Biol. Chem. *239* 392

Rudman, D., Malkin, M. F., Brown, S. J., Garcia, L. A., Abell, L. L. (1964) J. Lipid Res. *5* 38
Schotz, M. C., Page, J. H. (1960) J. Lipid Res. *1* 466
Steinberg, D. (1963) The Control of Lipid Metabolism. (Ed.) Grant, D. K. I. London—New York, p. 111
Sutherland, E. W., Rall, T. W., Menon, T. (1962) J. Biol. Chem. *237* 1220
Taussky, H. H., Shorr, E. (1952) J. Biol. Chem. *202* 675
Van der Vies, J. (1954) Biochem. J. *57* 410
Vaughan, M. (1961) J. Lipid Res. *2* 293
Vaughan, M. (1962) J. Biol. Chem. *237* 3354
Wlodawer, P., Baranksa, J. (1965) Acta Biochim. Pol. *12* 23
Wright, P. A. (1959) Endocrinol. *64* 551
Zsötér, T., Tom, H., Kraml, M., Dvornik, D. (1966) J. Pharmacol. Exp. Ther. *152* 425

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (3), pp. 251—258 (1969)

The Reaction Mechanism of N-acetylglutamate-5-phosphotransferase from Chlamydomonas Reinhardti

ANNA FARAGÓ, G. DÉNES

Institute of Medical Chemistry, University Medical School, Budapest

(Received March 11, 1969)

1. Double reciprocal plots of initial velocity versus acetylglutamate concentration at different levels of ATP result in linear curves, intersecting at a common point on the negative part of the abscissa. A similar kinetic pattern is obtained when the concentration of ATP is varied and that of acetylglutamate kept constant. The result of the initial rate analysis is consistent with a sequential mechanism, in which both substrates must combine with the enzyme before either product is released.

2. Phosphotransferase is inactivated by mushroom tyrosinase. Acetylglutamate increases the inactivation rate, while ATP protects the enzyme against the effect of tyrosinase. If the substrates which accelerate or prevent the tyrosinase induced inactivation, bind to the enzyme at the active site, the addition of substrates to phosphotransferase may occur without an obligate sequence. Consequently, the reaction mechanism of phosphotransferase may be of random nature.

ATP : N-acetylglutamate-5-phosphotransferase catalyses the feed-back sensitive step of the arginine biosynthesis in Chlamydomonas reinhardti. The catalytic activity of phosphotransferase is controlled by the end-product arginine which is an apparent competitive inhibitor with respect to acetylglutamate, and non-competitive with the other substrate, ATP (Faragó, Dénes, 1967).

The substrate saturation curves of phosphotransferase obey in all circumstances the Michaelis-Menten kinetics, but the inhibition caused by arginine exhibits a sigmoidal curve. At the same time, the direct saturation curve of arginine obtained by the method of equilibrium dialysis is a regular rectangular hyperbole (Faragó, Dénes, 1969b). The sigmoid nature of the kinetic pattern of inhibition may originate from interaction between subunits, but it may also be an apparent effect, as discussed in a number of kinetic studies (Keleti, 1968; Ferdinánd, 1966; Sweeny, Fischer, 1968; Henderson, 1968). The evaluation of the complex kinetics of inhibition requires information about the reaction mechanism of phosphotransferase.

Materials and Methods

Chemicals

The inorganic chemicals used were of A. R. grade. Adenosine-5'-diphosphate (ADP) and adenosine-5'-triphosphate (ATP) were obtained from Reanal (Budapest). N-acetylglutamate was prepared chemically (Bergman, Zervas, 1928). L-3-(3,4-dihydroxy-phenyl)-alanine (DOPA) was purchased from Fluka A. G. (Buchs).

Organism and growth conditions

Experimental and stock cultures of minus mating type strain of Chlamydomonas reinhardti were grown as described (Staub, Dénes, 1966). The centrifuged cells were stored at —20 °C for not more than ten days before use.

N-acetyl-L-glutamate-5-phosphotransferase. Preparation and assay

The purification procedure was the same as described previously (Faragó, Dénes, 1967). The enzyme was used for experiments after the second ammonium-sulphate fractionation followed by dialysis against 0.1 M pH 7.5 Tris. HCl buffer changed several times. Dialysis was carried out at 5 °C. The specific activity of the enzyme was about 10—15 units per mg protein. (The unit of the enzyme activity was defined as that amount of enzyme which catalyses the formation of 1.0 μmole of N-acetylglutamate-5-hydroxamate in 60 min. at 37 °C.) The enzyme solution has never been stored for more than ten days before use in our experiments.

The activity of phosphotransferase was determined by measuring the amount of N-acetylglutamate-5-hydroxamate produced, when N-acetylglutamate was incubated with the enzyme in the presence of ATP, Mg^{++} and NH_2OH at pH 7.0. The standard assay mixture contained Tris 200 μmoles, $NH_2OH \cdot HCl$ 200 μmoles acetilglutamate 150 μmoles, ATP 20 μmoles and $MgCl_2$ 20 μmoles in a final volume of 2 ml. The incubation was stopped by the addition of 2 ml $FeCl_3$ reagent consisting of 8 per cent trichloroacetic acid and 3.5 per cent $FeCl_3$ in 2 M HCl. The absorbance of the acetylglutamate-5-hydroxamate-iron complex was determined at 540 mμ.

Tyrosinase

Mushroom tyrosinase (o-diphenol: oxygen oxidoreductase, EC 1.10.3.1) was prepared by the method of Frieden and Ottesen (1959) with the modification of Cory and Frieden (1967). Tyrosinase was used in our experiments after purification by DEAE cellulose chromatography. Its specific activity was about 150 units per mg protein. The unit of tyrosinase activity was defined by Fling et al. (1963).

The assay mixture contained 2.4 mg L-DOPA and 300 μmoles of sodium phosphate (pH 6.0) in a final volume of 3 ml. The formation of dopachrom was followed at 475 mμ spectrophotometrically. The activity of enzyme is completely inhibited by 10^{-4} M KCN. Acetylglutamate, ATP, ADP and arginine in the concentration used for inactivation experiments did not influence the activity of tyrosinase.

Results and Discussion

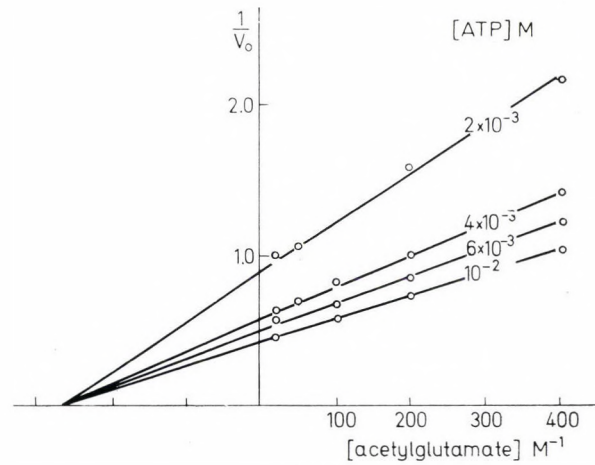
Initial velocity analysis of phosphotransferase

The analysis of initial reaction velocity was carried out according to Cleland (1963). N-acetylglutamate-5-phosphotransferase catalyses the following reaction:

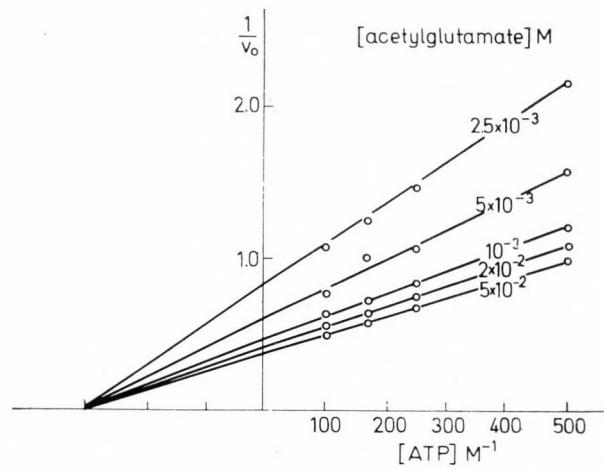
$$\text{N-acetylglutamate} + \text{ATP} \xrightarrow{Mg^{+}} \text{N-acetylglutamate-5-phosphate} + \text{ADP}.$$

In the presence of hydroxylamine N-acetylglutamate-5-hydroxamate is formed according to the equation:

$$\text{N-acetylglutamate-5-phosphate} + NH_2OH \longrightarrow \text{N-acetylglutamate-5-hydroxamate} + P_i.$$



1a



1b

Fig. 1. Double reciprocal plot of initial velocities (v_0) against acetylglutamate concentration at a series of fixed concentrations of ATP (a) and against ATP concentration at a series of fixed acetylglutamate concentration (b). Initial velocity is expressed as μmoles of N-acetylglutamate-5-hydroxamate formed per 60 min. The reaction mixture contains Tris bases 400 μmoles, $NH_2OH \cdot HCl$ 200 μmoles, $MgCl_2$ 20 μmoles, 0.5 mg of protein, acetylglutamate and ATP as indicated, in a total volume of 2 ml

Hydroxylamine is present in the assay mixutre during the incubation, and therefore the concentration of one of the products (acetylglutamate-5-phosphate) is always zero.

The reaction has an absolute requirement for bivalent cations. Mg^{++} and Co^{++} are equally effective, but in our experiments onlyMg^{++} was used. Although the activity of phosphotransferase is about fifty per cent higher at pH 5.5 than at pH 8.0 (Faragó, Dénes, 1967), the initial velocity analysis was carried out at pH 8, because the dissociation constants of Mg-nucleotides are known for this pH only. The Mg^{++} concentration used in these experiments (0.01 M) has no inhibitory effect on the reaction even in the presence of the smallest ATP concentration but it is enough to ensure that the nucleotide is complexed at all times. $MgCl_2$ above 0.02 M, however, causes inhibition regardless of the concentration of ATP.

Figs 1*a* and *b* show the initial velocity pattern obtained with various acetylglutamate concentrations at constant ATP levels, and with ATP as the varied substrate at constant acetylglutamate levels, respectively. The double reciprocal plots of substrates versus initial velocity yield in both cases linear intersecting curves. The intersecting kinetic pattern is consistent only with a sequential reaction mechanism (Cleland, 1963, 1967) according to the equation:

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB}$$

indicating that both substrates must add to the enzyme before either of the products is released. (A and B are the concentration of the two substrates, K_{ia} is the dissociation constant for EA complex, K_a and K_b are the Michaelis constants for A and B, respectively.) Since the cros-sover point of the curves is on the horizontal axis in both cases, the concentration of one of the substrates does not influence the Michaelis constant of the other.

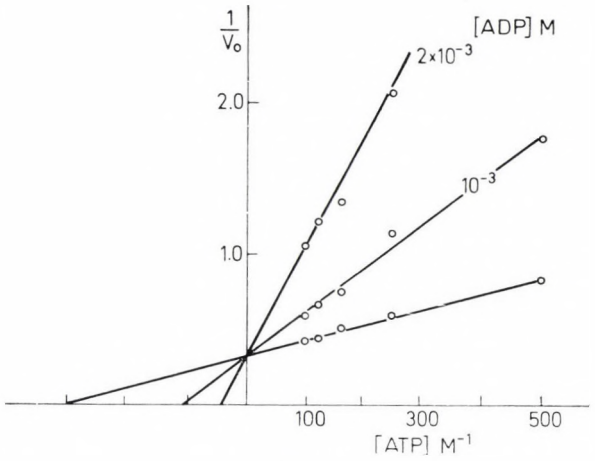
The average Michaelis constant for acetylglutamate is 4×10^{-3} M, and for ATP 3×10^{-3} M, at pH 8.0 and at 37 °C.

The reaction is inhibited by the product ATP. The inhibition caused by ADP is competitive with respect to ATP (in the presence of excess Mg^{++}), and noncompetitive with acetylglutamate (Fig. 2*a*, *b*).

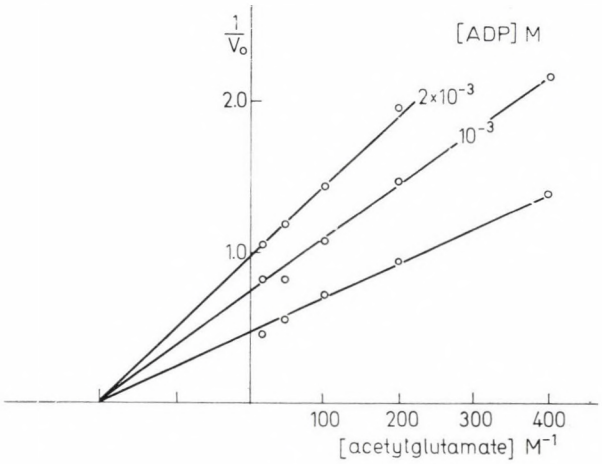
While these results suggest that the reaction mechanism of phosphotransferase is sequential, the data are not sufficient to decide whether the binding of the substrates occurs in ordered or in random sequence. Further data are obtained from inactivation experiments carried out with tyrosinase.

The effect of tyrosinase on N-acetylglutamate-5-phosphotransferase

Mushroom tyrosinase (EC 1.10.3.1) has been used for detecting exposed tyrosines in a protein by several authors, recently by Cory and Frieden (1967). Tyrosinase has proved to be useful in the investigation of the reaction mechanism of phosphotransferase, as the rate of inactivation of this enzyme, caused by tyrosinase, is influenced by both its substrates and also by the allosteric modifier. Fig. 3 shows the inactivation of phosphotransferase by tyrosinase in the absence



2a



2b

Fig. 2. The Lineweaver-Burk plot of ATP (a) and acetylglutamate (b) in the presence of ADP. The reaction mixture contains 30 μmoles of $MgCl_2$, and 75 μmoles of acetylglutamate (a) or 20 μmoles of ATP (b) in a total volume of 2 ml

and in the presence of its substrates. The sensitivity of the residual phosphotransferase activity to the inhibitory effect of arginine remains unaffected. If the incubation mixture contains 10^{-3} M KCN, the activity of phosphotransferase does not change, indicating that the inactivation is due only to the effect of tyrosinase.

Acetylglutamate accelerates the inactivation, while ATP decreases the inactivation rate. The protecting effect of ATP is independent of the presence of Mg^{++}, and Mg^{++} in itself has no effect on the rate of inactivation. In the presence of both ATP and acetylglutamate the protecting effect of ATP dominates over the opposite effect of acetylglutamate. The effective substrate concentrations are in the same

range which are used for the determination of the initial velocity of the enzyme reaction.

The effect of the two substrates on the tyrosinase induced inactivation of phosphotransferase indicates that both substrates can bind to the enzyme independently.

The possibility that the binding of substrates, responsible for this effect, may occur not only at the active site, cannot be ruled out. At this time, however, there

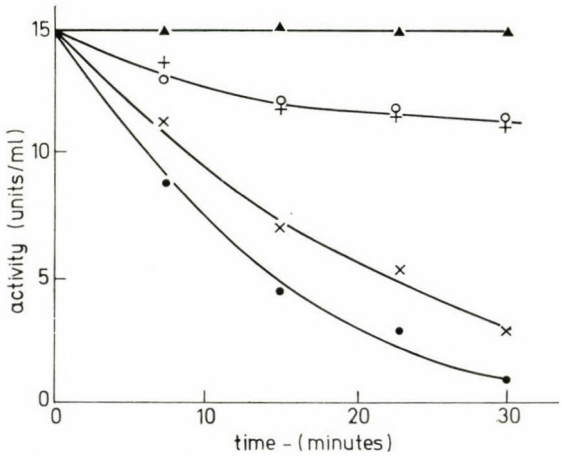


Fig. 3. Inactivation of phosphotransferase by tyrosinase. 30 units of phosphotransferase were incubated with tyrosinase (30 units), in 0.1 M Tris HCl buffer, pH 7.5, at 37 °C. The total volume of the incubation mixture was 2 ml. At times indicated aliquots were taken and the residual activities were determined in a standard reaction mixture containing 10^{-4} M KCN. The curves represent the residual activity in the presence of 10^{-3} M KCN (▲ — ▲), without any addition (× — ×), in the presence of 10^{-2} M ATP (+ — +), 7.5×10^{-2} M acetylglutamate (● — ●) and 7.5×10^{-2} M acetylglutamate + 10^{-2} M ATP (○ — ○)

are no data which would support an aspecific substrate binding. Consequently, the results may suggest that the addition of substrates to phosphotransferase can occur in a random sequence, and the reaction mechanism of phosphotransferase at the investigated pH is in all probability of random nature.

Whether it is a random mechanism without a preferential sequence, or a rapid equilibrium random mechanism, cannot be decided from kinetic data alone. In the latter case the Michaelis constants would be equal to the dissociation constants of EA and EB complexes.

ADP, the product of the reaction catalysed by phosphotransferase can also bind to the enzyme. Mg^{++} is not necessary for the binding of ADP either. It is surprising that ADP bound to the enzyme presumably at the same site as ATP, increases the rate of inactivation caused by tyrosinase (Fig. 4).

The fact that phosphotransferase is inactivated by tyrosinase means only that the intact structure of some tyrosine group(s) is necessary for the catalytic activity of phosphotransferase. The tyrosine group(s) in question may be more or less far from the catalytic centre, having some role only in stabilizing the three-di-

mensional integrity of phosphotransferase. The opposite effect of ATP and ADP on the inactivation rate, however, suggests that this tyrosine group may be situated in the neighbourhood of (if not inside) the active centre. The possibility that the protecting effect of ATP is a direct steric effect and is due to the third phosphate group of ATP seems to be more probable than the assumption that ATP and ADP bring about antagonistic conformational changes far from their binding site. The

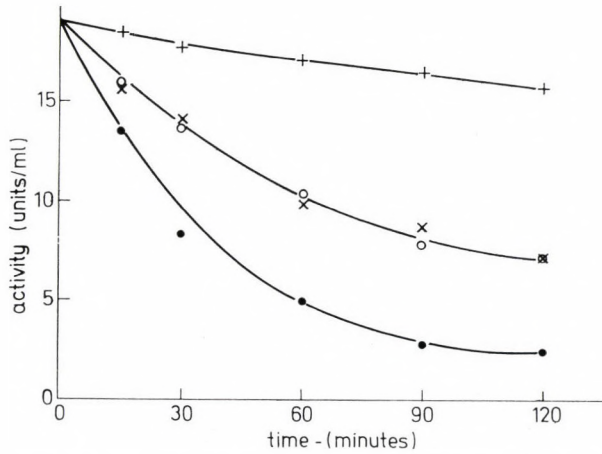


Fig. 4. The opposite effect of ATP and ADP on the tyrosinase induced inactivation of phosphotransferase. 30 units of phosphotransferase were incubated with 8 units of tyrosinase under the same conditions as in Fig. 4 in the presence of 10^{-2} M ATP + 10^{-2} M $MgCl_2$ (+ — +). 5×10^{-3} ADP + 10^{-2} M $MgCl_2$ (●—●), 10^{-2} M $MgCl_2$ (○ — ○) and without any addition (× — ×)

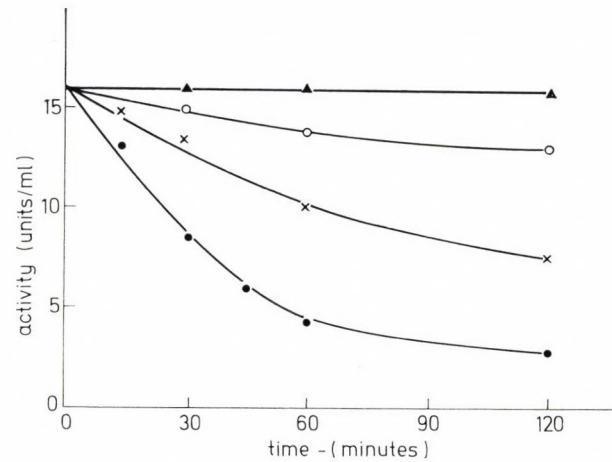


Fig. 5. The protecting effect of arginine against tyrosinase. Curves represent the residual activity in the presence of 10^{-3} M KCN (▲ — ▲), without any addition (× — ×) and in the presence of 10^{-3} M arginine (○ — ○) or 7.5×10^{-2} M acetylglutamate (●—●). The inactivation was carried out by 8 units of tyrosinase

direct steric effect of ATP is also supported by the dominance of the protection by ATP over the accelerated inactivation caused by acetylglutamate.

The allosteric inhibitor, arginine, also protects the enzyme against tyrosinase (Fig. 5). Previous observations (Faragó, Dénes, 1969*a*) have suggested that phosphotransferase has a more compact structure in its allosterically inhibited state, than the free enzyme. It is likely that the protecting effect of arginine against tyrosinase is due to this more compact conformation.

References

Bergman, M., Zervas, L. (1928) Biochem. Z. *203* 280
Cleland, W. W. (1963) Biochim. Biophys. Acta *67* 104
Cleland, W. W. (1967) Ann. Rev. Biochem. *36* 77
Cory, J. G., Frieden, E. (1967) Biochemistry *6* 117
Faragó, A., Dénes, G. (1967) Biochim. Biophys. Acta *136* 6
Faragó, A., Dénes, G. (1969a) Biochim. Biophys. Acta *178* 400
Faragó, A., Dénes, G. (1969b) in preparation
Ferdinand, W. (1966) Biochem. J. *98* 278
Fling, M., Horowitz, N. H., Heinemann, S. F. (1963) J. Biol. Chem. *238* 2045
Frieden, E., Ottesen, M. (1959) *34* 248
Henderson, J. F. (1968) Canad. J. Biochem. *46* 1381
Keleti, T. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 247
Staub, M., Dénes, G. (1966) Biochim. Biophys. Acta *128* 82
Sweeny, J. R., Fisher, J. R. (1968) Biochemistry *7* 561

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (3), pp. 259—264 (1969)

On the Z-Substance of Striated Muscle

VERONIKA HARSÁNYI,* N. GARAMVÖLGYI

Biochemistry Group, Loránd Eötvös University, and Department of Pathology, Postgraduate Medical School, Budapest

(Received April 2, 1969)

Actomyosin and actin were removed from myofibrillar suspensions. As a result of treatment with lipase or trypsin the release of a protein, assumed to be the Z-line substance was observed. These findings are discussed in relation to recent literary data.

The substance of the Z-line has been studied very extensively in the course of the last few years. The Z-substance was found to be very resistant towards treatment with weak acids (Ernst et als, 1958; Guba et al. 1960), or salt solutions of high ionic strength (Hanson, Huxley, 1955; Huxley, Hanson, 1957; Garamvölgyi et al., 1962). It is extremely difficult to bring this substance into solution. By very long extraction with a salt solution of low ionic strength, Corsi and Perry (1958) removed the dense material of the Z-lines and a similar way of extraction was later chosen by Samosudova et al. (1965) and by Stromer et al. (1967).

On the other hand, the Z-substance can be removed very easily from the myofibrils by digestion with trypsin (Ashley et al., 1951), or with lipase (Guba, 1954; Garamvölgyi, 1965, 1968). On the basis of this latter experience we supposed that treatment of the myofibrils with lipase might result in the release of a protein. Since we in fact obtained this protein, it was assumed to originate from the Z-line (Garamvölgyi, Guba, 1967; Garamvölgyi, 1968*a*).

Still earlier a component of the myofibrillar protein, α-actinin, the 6 S component was reported to be localized in the Z-line (Masaki, cited by Ebashi, 1968) on the basis of antibody staining experiments. This assumption has been supported by the team of Mommaerts (Briskey et al., 1967) stating that α-actinin exhibits the property of network formation.

It should be noted that the proportion of the amount of tyrosine and tryptophan in the 6 S component of α-actinin (Ebashi, Nonomura, 1967) was found to be the same as in the protein substance isolated by us as a result of lipase digestion (Garamvölgyi, Guba, 1967). In connection with this, recently Reedy (personal communication) raised the possibility that in our experiments proteolytic contaminations caused the release of the Z-line material, instead of the lipase enzyme itself.

Since the Z-line substance can be removed by tryptic digestion, too, it could have been expected that the Z-protein could be extracted from the myofibril by

* Present address: Central Institute, National Blood Transfusion Service, Budapest

trypsin, provided that it is not highly trypsin sensitive, as e.g. tropomyosin. It is true, however, that in this case one has to consider the release of meromyosins which originate from the splitting of myosin present in the thick myofilaments. Nevertheless, Goll et al. (1969) succeeded in the isolation of an α-actinin -like protein using extremely short tryptic digestion.

At the same time Rash et al. (1968) removed the Z-substance (together with intercalated discs and desmosomes) by treatment with 1.0 M urea from heart muscle and assumed the Z-substance to be tropomyosin, in agreement with an earlier assumption of Huxley (1963).

In the case of extractions performed in the presence of the other myofibrillar proteins the extracting agent may also affect these latter and the product of the extraction can eventually originate from a structure other than the Z-line, even if the removal of the Z-substance can be observed at the same time. Since the Z-substance survives the extraction of myosin as well as that of actin (Huxley, Hanson, 1957) in the present series of experiments we attempted to extract the Z-substance following the complete removal of the main structure proteins. According to Hanson and Huxley (1955) it is only some "S-substance", assumed to be fibrillin by Guba et al. (1968), which may still connect the Z-lines to each other after the subsequent removal of myosin and actin.

Materials and Methods

Myofibrils were obtained from the back and psoas muscle of rabbit by homogenization of the muscle tissue in a standard KCl solution (Huxley, 1963). The myofibrillar suspensions were checked in a phase contrast microscope. The following solutions were used for extraction:

Standard KCl solution: 10^{-1} M KCl, 10^{-3} M $MgCl_2$, 6.7×10^{-3} M phosphate buffer, pH 7.

Weber-Edsall's solution: 6×10^{-1} M KCl, 4×10^{-2} M $NaHCO_3$, 10^{-2} M Na_2CO_3.

KI solution: 6×10^{-1} M KI, 10^{-2} M ME*, 10^{-4} M ATP, 5×10^{-3} M EDTA, 6.7×10^{-3} M phosphate buffer, pH 7.

A. R. chemicals of Reanal Fine Chemicals, Budapest were used. Pancreatic lipase (Reanal) or trypsin (Worthington) were applied for the enzymatic treatment. The reaction was stopped either by trypsin inhibitor (Sigma), or DFP in a final concentration of 10^{-2} M. For centrifugations a Janetzky refrigerated centrifuge was used.

The protein content was estimated by a biuret method (Gornall et al., 1949). UV absorption spectra were taken by a Spektromom 202 spectrophotometer. The tyrosine and tryptophan content of the protein was estimated according to Beaven and Holyday (1952).

Sedimentation experiments were carried out in a MOM ultracentrifuge equipped with a synthetic boundary cell, at a final speed of 50 000 r.p.m.

Polyacrylamid-gel electrophoresis was performed as described by Bálint et al. (1968) based on Davies' (1964) work.

* Abbreviations: DFP, diiospropylfluorophosphate; EDTA, ethylendiamine-tetraacetic acid-disodium salt; ME, mercapto-ethanol.

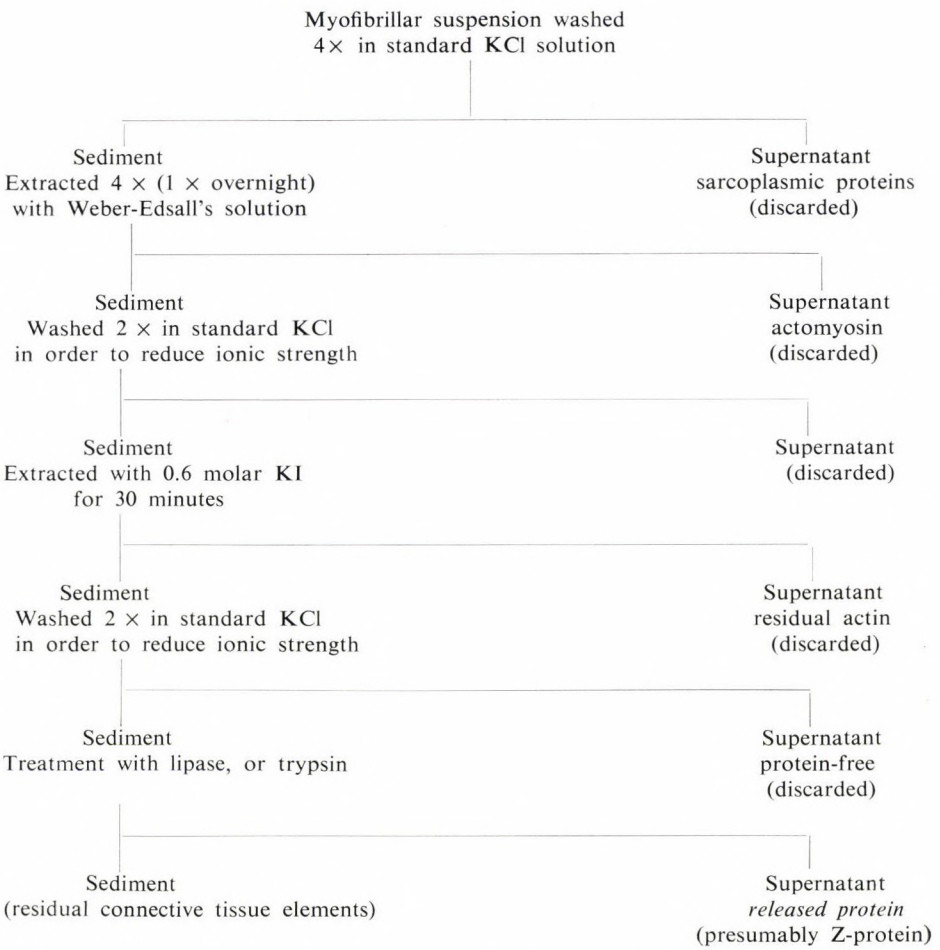
Results

From the myofibrillar suspension first the sarcoplasmic proteins were removed and subsequently the two main myofibrillar proteins were extracted. The details of the preparation are given in Table 1. The repeated treatment of the myofibrils with Weber-Edsall's solution followed by a KI solution resulted in the extraction of about 97 per cent of the total myofibrillar proteins, i.e. most probably no traces of myosin were left behind.

The residue of the extractions was treated either with lipase, or with trypsin in a final concentration of 0.2 mg per ml and 0.005 mg per ml, respectively. Lipase treatment lasted 20 minutes, or longer and trypsin treatment 10 minutes, in the standard KCl solution at 25 °C. In both cases we observed the release of protein as a result of the enzymatic treatment.

Table 1

Flow-sheet of the preparation



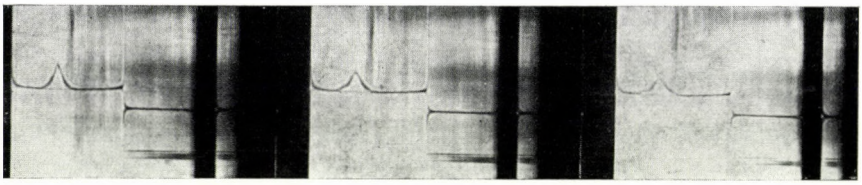


Fig. 1. Ultracentrifuge record of the protein released by lipase. Concentration: 2 mg/ml; t = 0°C; Philpot angle: 20°. The photographs were taken at 4-minute intervals. The first picture was taken 4 minutes after reaching 50 000 r.p.m. $S_{c,\,20} = 3.58 \times 10^{-13}$

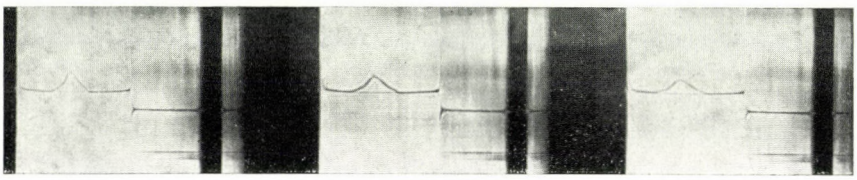


Fig. 2. Ultracentrifuge record of the protein released by trypsin. For data see legend to Fig. 1. $S_{c,\,20} = 2.08 \times 10^{-13}$

The yield of protein varied between 0.9 and 2.0 per cent of the myofibrillar proteins in the case of lipase treatment and between 0.3 and 2.0 per cent in the case of tryptic digestion. A varying part of the released substance was lost when dialysed against standard KCl solution, indicating the presence of low molecular weight substances.

The UV absorption spectra of both protein solutions before, or after dialysis showed a maximum at a wavelength of 276 mμ, at pH 7. $E^{1\%}_{279} = 10.6$ and $E_{276}/E_{260} = 1.14$ were obtained in the case of lipase and $E^{1\%}_{276} = 10.8$ and $E_{276}/E_{260} = 1.35$ in the case of trypsin treated substances. Both proteins were of a high Try content: 13 $gM/10^5$ g. The values for Tyr were 35 $gM/10^5$ g and 30 $gM/10^5$ g in the lipase and trypsin treated substances, respectively. These values were obtained immediately after the solution was adjusted to pH 13, since the protein showed a gradually increased precipitation at this pH. It should be noted that the amount of tryptophan was found to be 16 moles per 10^5 g protein and that of tyrosine 34—35 moles in the protein isolated by Garamvölgyi and Guba (1967) from intact myofibrils. Goll et al. (1969) obtained a value of 33.5 moles for tyrosine.

In most experiments ultracentrifuge runs revealed the presence of a single peak with a sedimentation coefficient of about 3 S. In some cases another peak was also present. Figs 1 and 2 show the sedimentation pattern of the released protein.

Polyacrylamid gel electrophoresis indicated the presence of more components in the protein systems. Fig. 3 shows the disc of the substance released by lipase (a) and by trypsin (b). In both cases some protein remained in the starter gel.

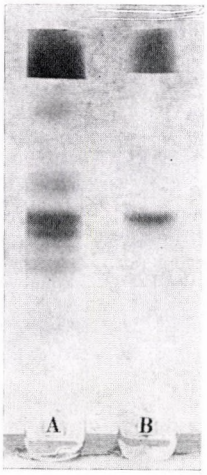


Fig. 3. Gel electrophoresis pattern of the protein released by lipase (a) and trypsin (b)

Discussion

The release of protein in the absence of myosin and actin as a result of lipase digestion clearly indicates that in our earlier experiments the protein released by the effect of this enzyme in fact originated from the Z-line (Garamvölgyi, Guba, 1967). The possibility that the release could be caused by contaminating proteolytic enzymes (Reedy, personal communication) does not influence this latter statement. It is true, however, that as a result of the treatment with lipase we perhaps obtained protein molecules which suffered some alterations. Notwithstanding, the high tryptophan content indicated that it was a protein other than tropomyosin (see also Stromer et al., 1967).

The same can be said from the product of the tryptic digestion. Since tropomyosin is rapidly split by trypsin, the isolated protein is very probably not tropomyosin. It is possible, however, that it is just the splitting of tropomyosin which brought about the release of a second protein. It is also possible that the small sedimentation coefficients in our case are the result of the extraction time being much longer than that used by Goll et al. (1969). The repeated extraction procedures could also have influenced the properties of the released protein. On the other hand, we have avoided the danger of a release of meromyosins, or their subfragments.

Our present observation is in agreement with that of Goll et al. (1969) since it shows that the protein extracted by the latter authors is in fact localized in the Z-line. The release of protein upon trypsin digestion after the extraction of the two main myofibrillar proteins, i.e. when virtually the Z-lines alone survive (Huxley, Hanson, 1957) seems an unambiguous evidence in favour of the work of Goll et al. (1969), too.

The authors thank Drs P. Závodszky and Gy. Fekete for their help in the sedimentation and gel electrophoretic experiments. They are also indebted to Prof. N. A. Biró and Dr P. Elődi for helpful discussions.

References

Ashley, C. A., Porter, K. R., Philpott, D. E., Hass, G. M. (1951) J. Exptl. Med. *94* 9
Bálint, M., Szilágyi, L., Fekete, Gy., Blazsó, M., Biró, N. A. (1968) J. Mol. Biol. *37* 317
Beaven, G. H., Holyday, E. R. (1952) Adv. Prot. Chem. *7* 319
Briskey, E. J., Mommaerts, W. F. H. M., Seraydarian, K. (1967) Biochim. Biophys. Acta *133* 424
Corsi, A., Perry, S. V. (1958) Biochem. J. *68* 12
Davies, B. J. (1964) Ann. N. Y. Acad. Sci. *121* 404
Ebashi, S. (1968) Symp. Muscle, Budapest 1966. Eds. Ernst, E., Straub. F. B. Symp. Biol. Hung. Vol. 8. p. 77. Publ. House Hung. Acad. Sci. Budapest
Ebashi, S., Nonomura, Y. (1967) Abstr. Brit. Biophys. Soc. Muscle Meeting, p. 11, London
Ernst, E., Garamvölgyi, N., Guba, F. (1958) Acta Physiol. Hung. *14* Suppl. 40
Garamvölgyi, N. (1965) J. Ultrastr. Res. *13* 425
Garamvölgyi, N. (1968) Acta Biochim. Biophys. Hung. *3* 77
Garamvölgyi, N. (1968a) Symp. Muscle, Budapest 1966. Eds. Ernst, E., Straub, F. B. Symp. Biol. Hung. Vol. 8, p. 114. Publ. House Hung. Acad. Sci., Budapest
Garamvölgyi, N., Guba, F. (1967) Acta Biochim. Biophys. Hung. *2* 417
Garamvölgyi, N., Metzger-Török, G., Tigyi-Sebes, A. (1962) Acta Physiol. Hung. *22* 223
Goll, D. E., Mommaerts, W. F. H. M., Reedy, M. K., Seraydarian, K. (1969) Biochim. Biophys. Acta *175* 174
Gornall, A., Bardawill, C. J., David, N. (1949) J. Biol. Chem. *117* 751
Guba, F. (1954) Thesis of Cand. Biol. Sci. Budapest
Guba, F., Garamvölgyi, N., Ernst, E. (1960) 4th Internatl. Congr. Electr. Micr. Berlin, 1958. Vol. 2, p. 324. Springer Verl., Berlin.
Guba, F., Harsányi, V., Vajda, E. (1968) Acta Biochim. Biophys. Hung. *3* 433
Hanson, J., Huxley, H. E. (1955) Symp. Soc. Exptl. Biol. *9* 228
Huxley, H. E. (1963) J. Mol. Biol. *7* 281
Huxley, H. E., Hanson, J. (1957) Biochim. Biophys. Acta *23* 229
Rash, J. E., Shay, J. W., Biesele, J. J. (1968) J. Ultrastruct. Res. *24* 181
Samosudova, N. V., Kalamkarova, M. V., Ogievetskaya, M. M. (1965) Biofizika *10* 268
Stromer, M. H., Hartshorne, D. J., Rice, R. V. (1967) J. Cell Biol. *35* c23

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (3), pp. 265—272 (1969)

Inhibition of Rabbit Skeletal Muscle Aldolase by an Alkylating Agent

B. SZAJÁNI, P. FRIEDRICH, GERTRUDE SZABOLCSI

Institute of Biochemistry, Hungarian Academy of Sciences, Budapest

(Received April 10, 1969)

1. Three out of seven cysteinyl residues per subunit have been selectively carboxymethylated in rabbit muscle aldolase with bromoacetate. The modified enzyme is practically inactive.

2. The three cysteinyl residues display different reactivities towards the reagent and in the order of decreasing reactivity they have been designated as Cys-I, Cys-II and Cys-III.

3. Kinetic analysis of the process shows that inactivation is due to the blocking of Cys-II.

Several observations indicate that certain cysteinyl residues of rabbit muscle aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate liase, E. C. 4.1.2.13) play some role in the catalysis (Swenson, Boyer, 1957; Szabolcsi, Biszku, 1961; Kowal et al., 1965). Though 10 SH-groups per mole of enzyme can be blocked with p-mercuribenzoate without loss of activity, blocking of 5 more sluggish reacting residues leads to 50 per cent inactivation (Swenson, Boyer, 1957). The formation of mercaptide bonds is always accompanied by the alteration of the conformation of aldolase. However, if FDP* is added to aldolase containing 10 mercaptide bonds, the conformation of the enzyme will be similar to that of native aldolase (Szabolcsi, Biszku, 1961). Kowal et al., (1965) have shown that dinitrophenylation or treatment with N-ethylmaleimide or β,β'-carboxymethyl disulphide of 10—12 SH-groups per mole of aldolase inactivate the enzyme. Substrates and to some extent phosphate ion protect 4 to 6 SH-groups from dinitrophenylation. Kowal et al. (1965) conclude that 4 to 6 SH-groups are located at the substrate binding site of the enzyme and more recent experiments with o-phenantroline seem to support this idea (Kobashi, Horecker, 1967).

In the present paper the carboxymethylation of different cysteinyl residues of aldolase will be described and kinetic evidence will be presented that alkylation of one cysteinyl residue per subunit leads to the inactivation of the enzyme.

* Abbreviations: FDP = fructose-1,6-diphosphate; TCA = trichloroacetic acid; CM = carboxymethyl group.

Materials and Methods

Aldolase was prepared from rabbit skeletal muscle and recrystallized three times according to Taylor et al. (1949). Enzyme concentration was determined spectrophotometrically by using the extinction coefficient: $E_{280}^{0.1\%} = 0.74$ (Biszku et al., 1964). This value does not change between pH 7.5 and 13. All calculations were based on a molecular weight of 160 000 (Kawahara, Tanford, 1966; Závodszky, Biszku, 1967). Specific activity of the preparations varied between 100 and 130, as determined by the dinitrophenyl hydrazine test (cf. *Enzyme assay).*

Fructose-1,6-diphosphate was a commercial (Reanal) preparation. The monobarium salt was freed from barium with sodium sulphate. The solution of the sodium salt was purified by repeated charcoal treatment.

^{14}C-monobromoacetic acid was purchased from Reanal. Specific activity was 1.4 to 1.6 mCi/mmole, as determined by Sajgó's (1969) method.

Tris(hydroxymethyl)aminomethane was recrystallized twice from 70 per cent ethanol.

All other chemicals were commercial preparations of reagent grade.

Enzyme assay was performed by the dinitrophenylhydrazine test at 30 °C and pH 7.5 as described by Swenson and Boyer (1957). The assay mixture (final volume 2.0 ml) contained: 0.5 ml of 0.5 M hydrazine sulphate, 1.0 ml of 0.04 M FDP and 0.5 ml enzyme solution in 0.1 M Tris buffer, pH 7.5. If the reaction was allowed to proceed for 1 min, about 100 μg of native aldolase was used. Care was taken that saturation with substrate be maintained in all assays. Specific enzyme activity was calculated according to Swenson and Boyer (1957) from the relationship: S. A. = $= \Delta E_{520}^{10\text{min}}$ divided by the absorption of the enzyme at 280 mμ in the test system.

Carboxymethylation. Aldolase crystals were dissolved in 0.1 M Tris buffer, pH 7.5, and gel-filtered on a Sephadex G-25 column equilibrated with the same buffer. Alkylation was carried out at 37 °C in 0.35 M Tris buffer, pH 7.5 and 8.5. Protein concentration varied between 2.5 to 10×10^{-5} M, bromoacetate concentration was in the range of 1 to 2.5×10^{-2} M. The molar ratio of reagent/mole of cysteine reacted was 20 : 1 or higher and this allowed the application of a kinetic treatment for an apparent first order reaction. Bromoacetate is decomposed at 37 °C with a half life of 22 hours at pH 7.5, and 6 hours at pH 8.5, respectively. In the experiments from which rate constants were calculated care was taken to maintain a constant concentration of bromoacetate.

Inactivation during carboxymethylation was determined in the following way. At time intervals samples were withdrawn and diluted 20- to 200-fold with 0.1 M Tris buffer, pH 7.5. Dilution practically arrested the carboxymethylation reaction. Within 30 seconds 0.5 ml of these solutions was added to the assay mixture and the activity was determined as described in *Enzyme assay.* Increasing amounts of enzyme were used as inactivation proceeded. Controls, run at each pH in the absence of bromoacetate, showed negligible inactivation.

Determination of ^{14}C-CM content. Samples containing about 2 mg of protein were withdrawn from the alkylation mixture precipitated with 7 per cent TCA and centrifuged. The excess of reagent was removed by washing 3 times with 3 per cent and 3 times with 1 per cent TCA. The precipitate was dissolved in 0.02 N

NaOH. The radioactivity of solutions was measured in a Tricarb liquid scintillation counter. Protein content was determined spectrophotometrically by using the extinction coefficient $E_{280}^{0.1\%} = 0.74$.

DEAE-Sephadex chromatography. For chromatographic analysis carboxymethylation was carried out on a large batch of enzyme and the reaction was stopped by transferring the mixture from 37 °C to 0 °C and immediately gel-filtering on a Sephadex G-25 column equilibrated with 0.05 M Tris, pH 7.5. The protein solution was freeze-dried and stored in the cold. Freeze-drying and subsequent storage up to several months did not affect the residual enzymatic activity of the samples.

For each chromatographic run modified aldolase was freshly dissolved in 0.02 M Tris buffer, pH 7.5, containing 0.02 M NaCl and 10^{-3} M EDTA, and gel-filtered on a Sephadex G-25 column equilibrated with the same buffer. About 20 mg of enzyme was applied to a DEAE-Sephadex A 50 column, 0.9×40 cm in size, equilibrated with 0.02 M Tris buffer, pH 7.5, containing 0.02 M NaCl and 10^{-3} M EDTA. All manipulations were carried out at 5 °C. The enzyme was eluted by a linear concentration gradient of NaCl increasing from 0.02 to 0.16 M, in 0.02 M Tris-buffer, pH 7.5, containing 10^{-3} M EDTA. The flow rate was about 8 ml per hour. Fractions of 4 ml were collected and tested for enzyme activity (see above), protein (E_{280}), and radioactivity. The presence of 5×10^{-3} M 2-mercaptoethanol in the eluting buffers did not affect the chromatographic pattern.

Results and Discussion

Kinetics of the inactivation of aldolase during carboxymethylation

The reaction of the cysteinyl residues of aldolase with monobromoacetate is very slow at 25 °C even above pH 8.5. At 37 °C, however, alkylation proceeds at a well measurable rate and there is a simultaneous loss of enzyme activity. Fig. 1A shows the time course of inactivation at pH 7.5 and 8.5. At these pH values inactivation apparently follows first order kinetics. As inactivation has never exceeded 95 per cent even after prolonged incubation, the theoretical curves in Fig. 1A have been calculated for this end point. The apparent first order rate constants of inactivation at both pH 7.5 and 8.5 are linear functions of bromoacetate concentration (Fig. 2 curves A and A'), as expected.

Kinetics of the carboxymethylation of aldolase

Fig. 1B shows the time course of carboxymethylation at pH 7.5 and 8.5. Since we have shown elsewhere (Szabolcsi et al., 1969; Sajgó et al., 1969) that under these experimental conditions the CM-groups bound to aldolase are all present in the form of CM-cysteine contained in only 3 tryptic peptides, Fig. 1B indicates that at pH 8.5 up to 3 cysteinyl residues per subunit react with bromoacetate. Our considerations are based on the assumptions that aldolase is an oligomer consisting of 4 subunits each comprising an identical set of cysteinyl residues and that the subunits are catalytically independent units.

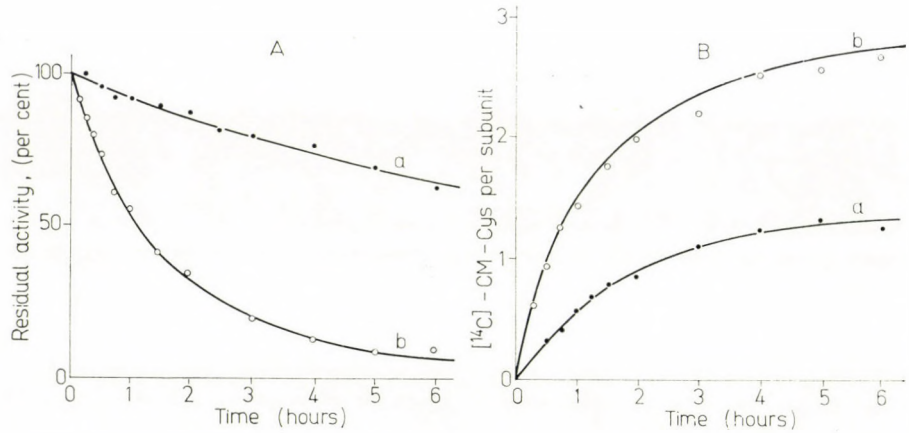


Fig. 1. Progress curves for the inactivation and alkylation of aldolase with bromoacetate at 37 °C, pH 7.5 and 8.5. 0.35 M Tris buffer was used in each case. Protein concentration: 6×10^{-5} M, bromoacetate concentration: 1×10^{-2} M. A: progress of inactivation; *a* at pH 7.5 and *b* at pH 8.5. The solid lines are theoretical curves for a single first order process, with apparent first order rate constants of 8×10^{-2} and 6×10^{-1} hr^{-1}, respectively. B: progress of alkylation in the same experiments

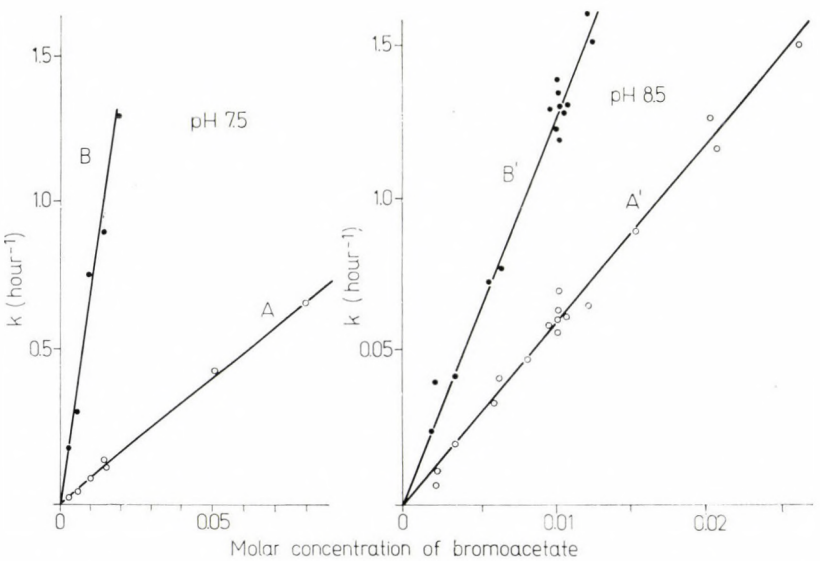


Fig. 2. Dependence of the apparent first order rate constants of inactivation and alkylation on bromoacetate concentration at pH 7.5 and 8.5. A and A': rate constants of inactivation which are also equal to the rate constants of alkylation of Cys-II (see text); B and B': rate constants of alkylation of Cys-I

Plotting inactivation *vs.* CM-groups bound, obtained in several experiments, we get the relationships presented in Fig. 3. The S-shape of the curves indicates that by blocking the most reactive cysteinyl residues, or the one that reacts first in a consecutive reaction (Cys-I), enzyme activity is not affected. As inactivation reaches an end-point at about 2.5 CM bound per subunit, it must be a consequence of the blocking of the "second" cysteinyl residue (Cys-II). Accordingly inactivation may be considered as an index of carboxymethylation of Cys-II and, since this process follows apparently first order kinetics (cf. Fig. 1A and Fig. 2), a sequential reaction of Cys-I and Cys-II seems to be excluded. The data suggest parallel alkylating reactions involving two SH-groups, of which the less reactive one is somehow connected with enzymatic activity. This is supported by the finding that: 1. In the early phase of alkylation the radioautograms of the peptide maps only show high, but different labelling of two cysteinyl peptides. 2. The difference in the labelling of these peptides decreases with time and 3. The inactive enzyme which contains about 2.5 CM per subunit, only contains one additional labelled peptide (Szabolcsi et al., 1969; Sajgó et al., 1969).

To check these ideas we have attempted to calculate the second order rate constant for the alkylation of Cys-I in the following way. We have assumed as discussed above, that the rate constant of alkylation of Cys-II is identical with the rate constant of inactivation, i.e. $k_{Cys-II} = 60$ and 8 $M^{-1}hr^{-1}$ at pH 8.5 and 7.5, respectively (cf. Fig. 2, curves A and A'). The apparent first order rate constant for Cys-I has been calculated by subtracting the amount of CM-Cys-II from the total amount of CM-cysteine (Fig. 4). Data from the early phase of alkylation have been considered for which the reaction of Cys-III is still negligible. In this way apparently first order reactions have been found for Cys-I. As seen in Fig. 2 (curves B and B'), the rate constants for Cys-I are also linear functions of bromoacetate concentration at both pH 7.5 and 8.5. From this the second order rate constants were 130 and 70 $M^{-1}hr^{-1}$ at pH 8.5 and 7.5, respectively.

Little can be said about Cys-III. It might react sequentially after Cys-I or Cys-II due to a conformational change. At any rate it does not seem to have any effect on enzymic activity.

Since the reactivity of Cys-I is markedly higher than that of Cys-II we have compared it to the reactivity of 2-mercaptoethanol towards bromoacetate at pH 7.5 and under the conditions of carboxymethylation described in this paper. The obtained second order rate constant is about 200 $M^{-1}hr^{-1}$, that is, three times higher than that of Cys-I. An even lower reactivity of Cys-II is by no means difficult to account for, whether or not it is located in the area of the active centre.

Characterization of carboxymethylated aldolase

The K_M of FDP and fructose-1-phosphate was tested with samples of aldolase which contained about 2.5 CM-Cys per subunit and the specific activity of which was reduced to 10 per cent of the native enzyme. No change in the K_M values was found.

To test the homogeneity of the modified enzyme and to account for its residual activity, aldolase containing about 2.5 CM-groups per subunit was subjected

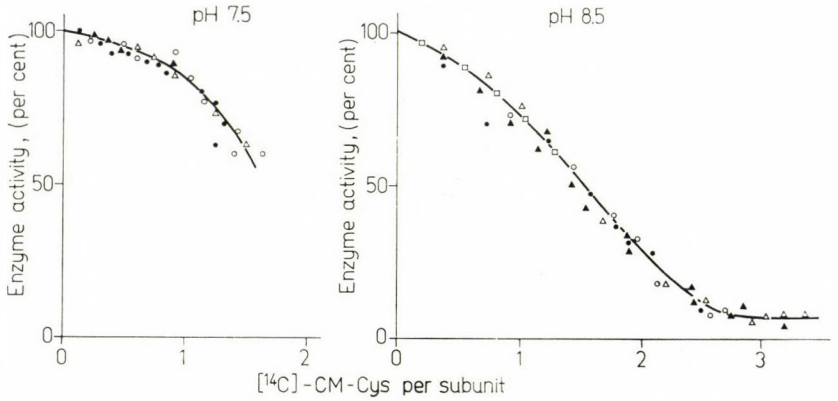


Fig. 3. Activity of aldolase as a function of carboxymethyl groups bound to the enzyme in experiments performed with different concentrations of bromoacetate. Concentration of the reagent in the alkylating mixture at pH 7.5: ▲ = 0.005 M; ● = 0.010 M; ○, △ = 0.015 M. At pH 8.5: □ = 0.0035 M; ○, ● = 0.01 M; △, ▲ = 0.015 M

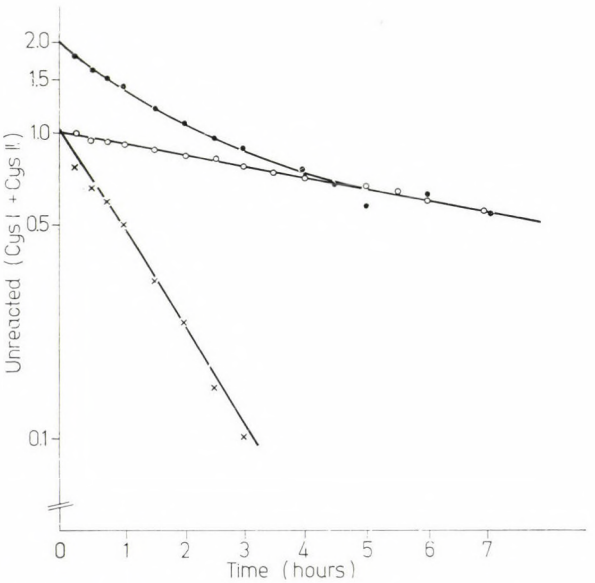


Fig. 4. Resolution of the carboxymethylation of Cys-I and Cys-II at pH 7.5. Protein concentration: 5×10^{-5} M, bromoacetate concentration: 1×10^{-2} M. ● — ●, overall process. The solid line is a theoretical curve of two parallel apparently first order reactions with the rate constants given below. ○ — ○, alkylation of Cys-II, calculated from data on the loss of enzyme activity in the same experiment. $k_{Cys\text{-}II} = 8.5\times10^{-2}$ hr^{-1}. × -- ×, alkylation of Cys-I. $k_{Cys\text{-}I} = 7.2\times10^{-1}$ hr^{-1}. For calculations see text

to DEAE-Sephadex chromatography. The elution profile shown in Fig. 5 indicates that the modified product is not homogeneous. The main bulk of the protein displays constant specific radioactivity. The peak which represents enzyme activity is shifted somewhat in advance of the protein peak. No better resolution could be achieved by varying the chromatographic conditions such as pH or ionic composition or when DEAE-cellulose was used instead of DEAE-Sephadex. Under the experimental conditions of Fig. 5 native aldolase did not bind to the column whether tested separately or mixed with the modified enzyme.

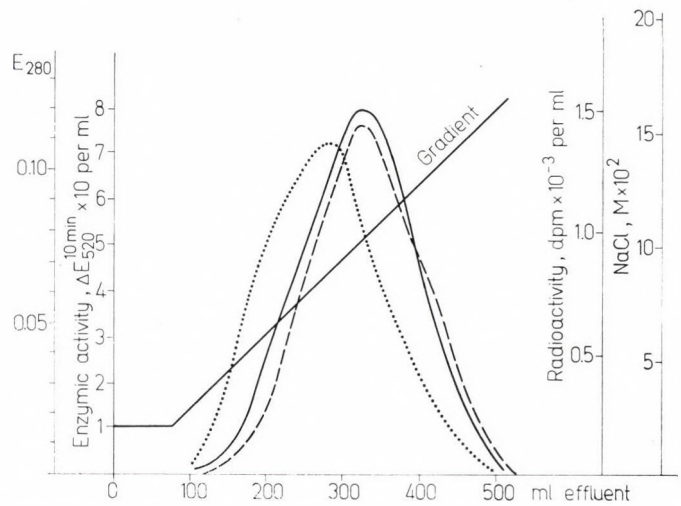


Fig. 5. DEAE-Sephadex chromatography of modified aldolase. 22 mg of aldolase containing 2.5 CM per subunit was put on the column. The specific enzyme activity of the sample was 10. —— = E_{280}; · · · = enzyme activity; — — — = radioactivity. About 95 per cent of the total protein, enzyme-activity and radioactivity have been recovered

It follows from the above findings that the residual activity of modified aldolase cannot be due to the presence of small amount of the native enzyme. Carboxymethylation results in enzyme molecules which contain a varying number of CM-groups, and/or oligomeric molecules in which the CM-groups are not equally distributed. Since enzymic activity could be detected even in fractions with a high CM-content, it is possible that blocking of Cys-II with bromaocetate does not fully inactivate the enzyme, but rather results in enzyme molecules (active centres) which still display a few per cent of their original catalytic power.

The possible role of Cys-II in the catalytic activity of aldolase, and the characterization of the tryptic peptides containing Cys-I, Cys-II and Cys-III, their location in the polypeptide chain will be described in other papers (Sajgó et al. 1969; Sajgó, 1969*a*).

References

Biszku, E., Boross, L., Szabolcsi, G. (1964) Acta Physiol. Acad. Sci. Hung. *25* 161
Kawahara, K., Tanford, C. (1966) Biochemistry *5* 1578
Kobashi, K., Horecker, B. L. (1967) Arch. Biochem. Biophys. *121* 178
Kowal, J., Cremona, T., Horecker, B. L. (1965) J. Biol. Chem. *240* 2485
Sajgó, M. (1969) in preparation
Sajgó, M. (1969*a*) in preparation
Sajgó, M., Szajáni, B., Biszku, E., Szabolcsi, G. (1969) in preparation
Swenson, A. D., Boyer, P. D. (1957) J. Am. Chem. Soc. *79* 2174
Szabolcsi, G., Biszku, E. (1961) Biochim. Biophys. Acta *48* 355
Szabolcsi, G., Sajgó, M., Szajáni, B., Biszku, E., Závodszky, P., Abaturov, L. (1969) Proceedings *5th* FEBS Meeting, Praha 1968; Symposium I, in press.
Taylor, J. F., Green, A. A., Cori, G. T. (1949) J. Biol. Chem. *173* 591
Závodszky, P., Biszku, E. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 109

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (3), pp. 273—278 (1969)

Thermal Investigations on Structural Glycosaminoglycans and Proteins

1. The Influence of Age on the Thermal Decomposition of Aortic Intima

JUDIT SIMON, MAGDOLNA BIHARI-VARGA, L. ERDEY, S. GERŐ

3rd Department of Medicine, University of Budapest and Institute for General and Analytical Chemistry, Technical University, Budapest

(Received May 19, 1969)

Results obtained by the use of thermoanalytical methods and infrared spectrophotometry have shown that thermal decomposition of glycosaminoglycans in aortic intima takes place at maximum rate in the temperature interval 240—270 °C.

Structural proteins were found to be decomposed at maximum rate around 290—320 °C.

Variations in the percentage weight losses of aorta intima with age at these characteristic temperatures were studied.

As a continuation of investigations previously reported (Simon et al., 1968; Bihari-Varga et al. 1968) in our present experiments the thermal decomposition of structural glycosaminoglycans and proteins was studied in aortic intimas of different age.

Materials and Methods

Apparently lesion-free human aortas were obtained from patients at autopsy within 24 hours after death. After washing the endothelial surface by gentle rubbing under running tap water, the intima was stripped and dried for 3 hours with 50 per cent saturated air at constant flow rate. Delipidation of the samples was performed in 25 per cent ether in ethanol for 2 days, followed by 50 per cent chloroform in methanol for 2 days, both at room temperature.

Reference materials used in the experiments were: elastin (ligamentum nuchae) and collagen (Achilles tendon) both generous gifts of Dr. Ilona Banga, Budapest, and a chondroitin sulfate preparation of Light, Colnbrook.

Thermal analysis was carried out using a F. Paulik—J. Paulik—L. Erdey MOM derivatograph (Paulik et al., 1958). The heating rate was 10 °C/min up to 900 °C. The weight of the samples was about 100 mg. The instrument measured and recorded simultaneously the weight change (TG curve), rate of weight change (DTG curve), enthalpy change (DTA curve) and the temperature (T).

Cleavage of chemical bonds was identified by their infrared spectra, using a Zeiss UR-10 spectrophotometer.

Results and Discussion

Fig. 1 shows the derivatograms of a human aorta intima. Decomposition of glycosaminoglycans reaches its maximum rate at 240 °C, according to DTG curve (as described previously, Simon et al., 1968). The peak at 240 °C on the DTG curve of aortic intima indicates the decomposition of the chemical bonds of glycosaminoglycans, present in the tissue.

This statement was proved also by infrared spectrophotometry.

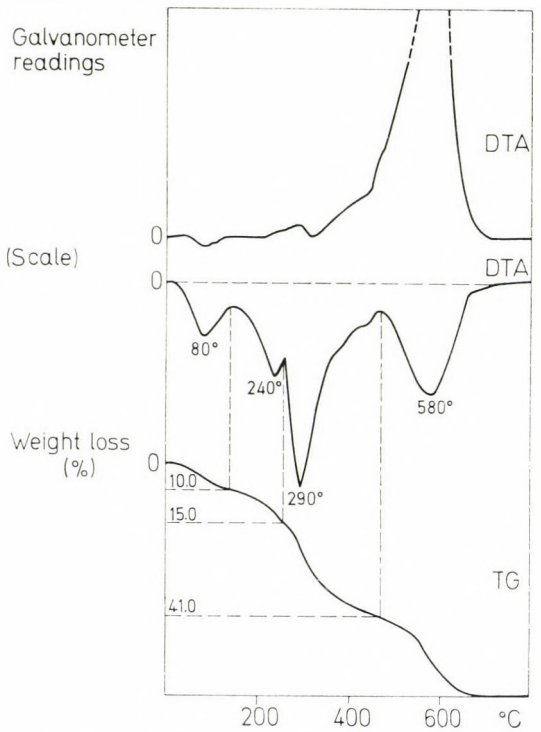


Fig. 1. Thermal decomposition curves for human aorta intima

Infrared spectra of chondroitin-sulfate and other glycosaminoglycans were in good agreement with literary data (Bihari-Varga, Gerő, 1966). The spectrum of the residue obtained by heating chondroitin-sulfate up to 250 °C at a rate of 10 °C per min revealed the disappearance of the peaks characteristic of glycosaminoglycans, and the appearance of sodium sulfate and an intermediate oxidation product which showed an absorbance characteristic of oxo groups at 1720 cm^{-1} wave number. Thermal decomposition of chondroitin sulfate at 600 °C resulted in a product which contained mainly sodium sulfate.

The same investigations were performed with aortic intimal tissues. In the spectrum of native aortic samples the same glycosaminoglycan absorbance maxima

could be detected as in chondroitin-sulfate. In addition, between 1500—1700 cm^{-1} wave numbers a series of peaks appeared which were characteristic of peptid linkages of structural proteins of the connective tissue.

When aortic intimal tissue was heated to 250 °C as described above, in the infrared spectrum the intensity of glycosaminoglycan peaks diminished, the absorbance maxima of the intermediate product appeared, while those characteristic of peptid-linkages remained unchanged.

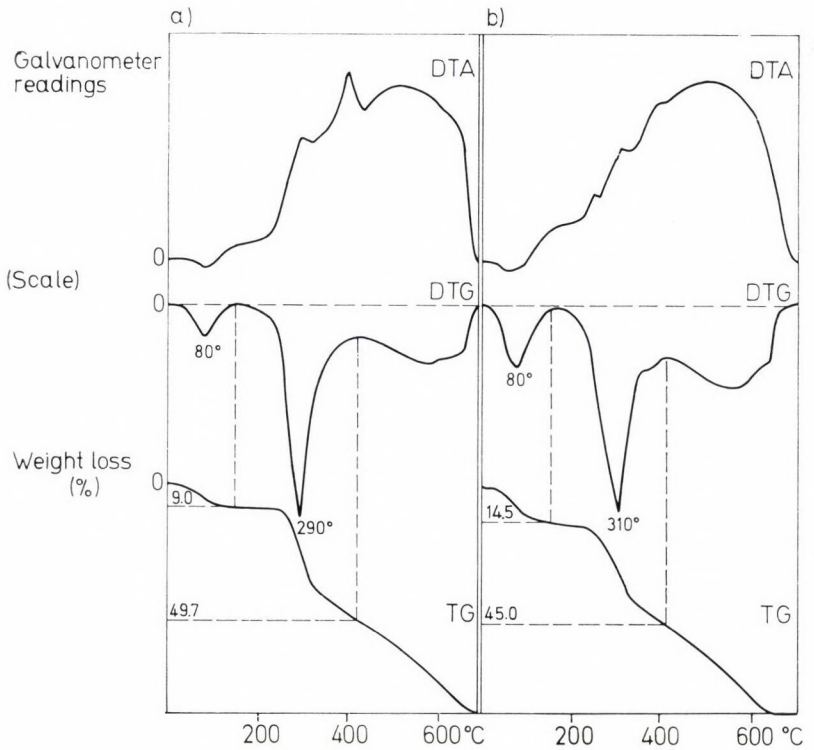


Fig. 2. Thermal decomposition curves for a) elastin; b) collagen

The results obtained by infrared spectrophotometry thus confirmed the conclusions based on thermal analysis, namely, that in the 240—270 °C temperature interval the chemical bonds of glycosaminoglycan components of human aorta are decomposed at a maximum rate.

In Fig. 1 the DTG curve of aorta shows that after the removal of adsorbed moisture decomposition of the sample starts at 150 °C and reaches a maximum at 240 °C. The DTG curve does not return to the base line, a second decomposition process starts at 260 °C. This takes place at a maximum rate at 290 °C and is completed at 470 °C.

Next, an attempt was made to identify the compounds which are decomposed at the highest rate at 290—320 °C.

As some proteins are known to break down in this temperature interval (Lóránt, 1965a, b) we investigated individually the thermal decomposition of elastin and collagen, because these fibrillar proteins are the main components of connective tissue.

Fig. 2 represents the obtained derivatograms. In elastin (Fig. 2a) the 9 per cent adsorbed water gets lost first, the decomposition of the protein starts at about 200 °C and according to the DTG curve reaches its maximum rate at 290 °C. This

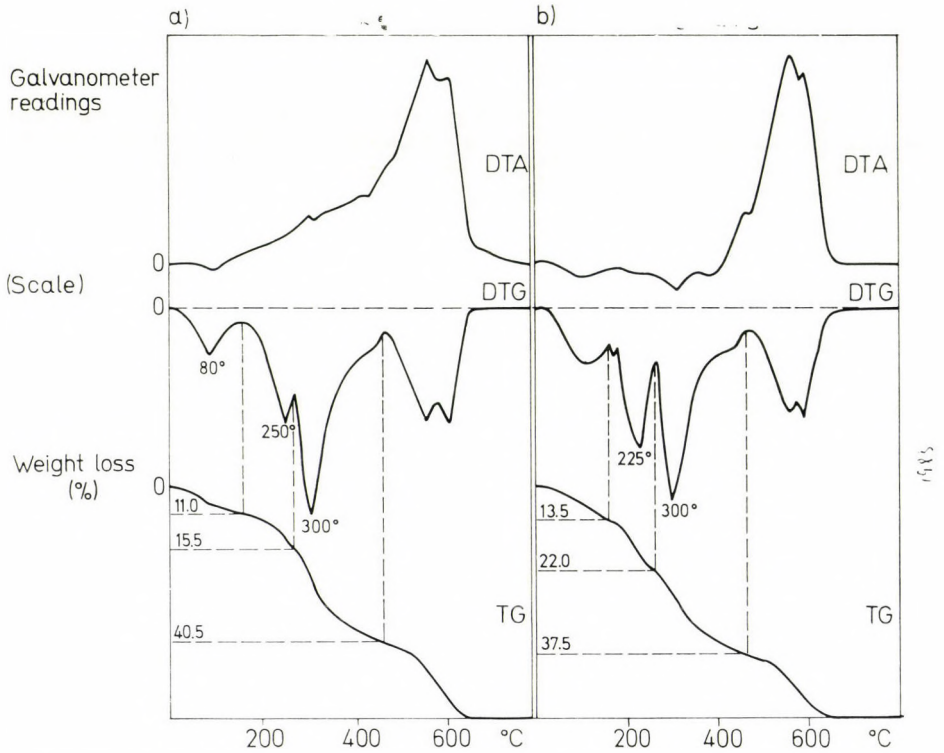


Fig. 3. Thermal decomposition curves for defatted human aorta intimas. a) Age: 14 years; b) Age: 26 years

is accompanied by a weight loss of 49.7 per cent (54.4 per cent calculated for dry tissue).

The thermal decomposition process of collagen (Fig. 2b) is very similar to that of elastin, the maximum rate is at 310 °C, with a weight loss of the same order (45.0 and 52.6 per cent, respectively).

Infrared spectra of elastin and of aortic tissue were found to be very similar concerning the absorbance maxima characteristic of protein linkages.

Samples of both types were heated to 290 °C, 320 °C and 350 °C and infrared analysis of the residues was performed.

Depending on the completion of decomposition, the amid (III)-band disappeared first at 1250 cm^{-1} wave number. This was followed by the disappearance of the intensive amid (I)- (1680 cm^{-1}) and amid (II)-bands (1550 cm^{-1}), confirming the presumption based on the results of thermal analysis.

The percentage weight loss, connected with the 240—270 °C DTG peak, probably indicating the cleavage of chemical bonds characteristic of glycosaminoglycans could be quantitatively estimated in delipidated samples. (Extraction with

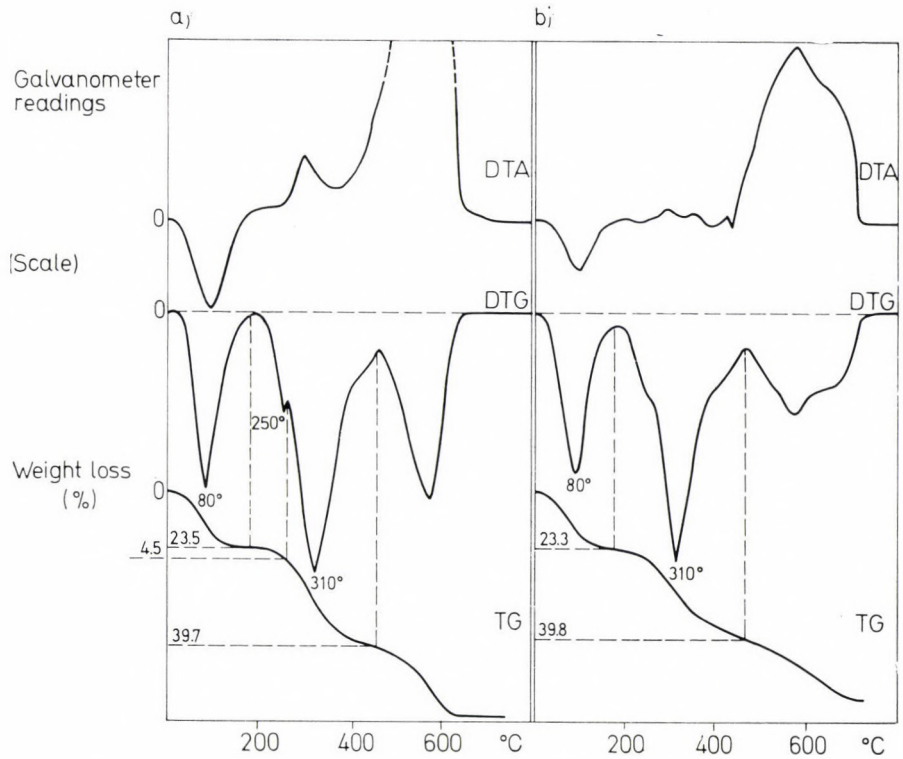


Fig. 4. Thermal decomposition curves for defatted human aorta intimas. a) Age: 45 years; b) Age: 90 years

organic solvents used for delipidation did not influence the shape of decomposition curves of the reference glycosaminoglycans and proteins.)

Experiments were performed to study the variation in the weight loss of aorta intima taking place with maximal rate in the 240—270°C and 290—320°C temperature intervals, with age.

Fig. 3 and Fig. 4 represent some typical thermoanalytical curves.

In Fig. 3 derivatograms of aorta intima samples obtained from a 14 (Fig. 3a) and a 26 (Fig. 3b) years old subject are shown. The DTG curves demonstrate the increase in weight loss at 250 °C with maximum rate as a function of age, that was

found 17.4 and 25.4 per cent of dry weight, respectively. At the same time the values of weight loss concerning the 300 °C peak were 45.5 and 43.3 per cent calculated for dry tissue.

Fig. 4 shows thermal decomposition curves for the aorta of a 45 (Fig. 4a) and a 90 (Fig. 4b) years old person. In the first case the weight loss belonging to the 250 °C maximum was found to be 6.0 per cent (calculated for dry weight), while in the sample from the 90 years old person the process diminished to an inflexion in this temperature interval. The weight loss at 310 °C was in both cases 52.3 per cent of dry weight.

Chemical analysis carried out by Kumar et al. (1967) pointed to an increase in the amount of intimal glycosaminoglycans with age up to about 20—30 years and a decrease in their concentration in older tissue. In our experiments the variation in the percentage weight loss caused by the decomposition of structural glycosaminoglycans (240—270 °C) was studied in a series of aortic intimas of different ages. The weight loss in this temperature interval showed a rising trend to about the third decade, followed by a subsequent decline with age. Thus it may be concluded that the amount of glycosaminoglycans in the intima and the percentage weight loss brought about by the cleavage of chemical bonds characteristic of these compounds, change parallel with increasing age.

The weight loss in the 290—320° temperature interval, as a result of the cleavage of protein bonds showed a decrease with age, a minimum concentration being attained around the ages of maturity. In the subsequent age group the TG values rise. The same inverse tendency has been described for changes in the concentration of glycosaminoglycan and of protein components in aortic tissue with age, based on the results of chemical analysis (Kaplan, Meyer, 1960; Smith, 1965).

Authors are indebted to Dr Klára Erős, Institute for General and Analytical Chemistry Technical University, Budapest for infrared spectrum analysis.

References

Bihari-Varga, M., Gerő, S. (1966) Acta Physiol. Acad. Sci. Hung. *29* 273
Bihari-Varga, M., Simon, J., Gerő, S. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 375
Kaplan, D., Meyer, K. (1960) Proc. Soc. Exp. Biol. Med. *105* 78
Kumar, V., Berenson, G. S., Ruiz, H., Dalferes, E. R., Strong, P. (1967) J. Atheroscler. Res. *7* 573
Lóránt, B. (1965a) Nahrung *9* 33
Lóránt, B. (1965b) Nahrung *9* 573
Paulik, F., Paulik, J., Erdey, L. (1958) Z. Anal. Chem. *160* 241
Simon, J., Bihari-Varga, M., Erdey, L., Gerő, S. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 365
Smith, E. B. (1965) J. Atheroscler. Res. *5* 241

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (3), pp. 279—285 (1969)

Thermal Investigations on Structural Glycosaminoglycans and Proteins

2. The Influence of Atherosclerosis on the Thermal Decomposition of Aortic Intima

MAGDOLNA BIHARI-VARGA, JUDIT SIMON, J. FEHÉR, S. GERŐ

3rd Department of Medicine, University of Budapest and Institute for General and Analytical Chemistry, Technical University, Budapest

(Received May 19, 1969)

Thermal decomposition of structural glycosaminoglycans and proteins in human aortic intima at different stages of atherosclerosis was studied.

1. The percentage weight loss brought about by the thermal cleavage of chemical bonds in glycosaminoglycan constituents of the tissue was highest in early lesions and decreased with the severity of the disease. The amount of saline-extractable beta-lipoprotein, probably bound to glycosaminoglycans, exhibited a parallel change.

2. The relative amount of protein and that of lipids probably bound to structural scleroproteins increased with the development of atherogenic process.

In previous communications (Simon et al., 1968; Bihari-Varga et al., 1968; Simon et al., 1969) we have reported on the application of a thermoanalytical method for the identification and assay of glycosaminoglycans and proteins in native biological materials and on the relationship found between age and the thermal decomposition of these compounds in human arterial tissue.

In our present investigations the thermal behaviour of glycosaminoglycans and proteins in aortic intima at different stages of atherosclerosis was studied. The concentration of saline-extractable beta-lipoprotein in the same tissue was also determined.

Materials and Methods

Atherosclerotic aortas were obtained from patients at necropsy within 24 hours after death. Some aortas were subdivided according to the type of lesions. Four types of tissue were selected for analysis: a) apparently lesion free intimal areas; b) oedematic thickening; c) fatty streaks and nodules; d) calcified plaques. After washing the endothelial surface, the intima was stripped and dried for 3 hours with 50 per cent saturated air at constant flow-rate.

Delipidation of aortic samples was performed by extraction in 25 per cent ether in ethanol for 2 days, followed by 50 per cent chloroform in methanol for 2 days, both at room temperature.

For thermal analysis a F. Paulik—J. Paulik—L. Erdey MOM derivatograph was used (Paulik et al., 1958). The derivatograph measured and recorded simultaneously the weight change (TG curve), rate of weight change (DTG curve),

enthalpy change (DTA curve) and the temperature (T) of the same sample. The heating rate was 10 °C/min up to 900 °C.

Extraction and assay of beta-lipoprotein from aortic intima was carried out by the immunological method of Slater and Smith (1967). The amount of extracted beta-lipoprotein was expressed as mg beta-cholesterol/g dry tissue, calculated from the cholesterol content of standard beta-lipoprotein.

Results and Discussion

In Fig. 1 typical thermal decomposition curves of an average sample of an individual atherosclerotic aorta intima are presented. In Fig. la the TG curve of the dried tissue indicates 10.8 per cent moisture content which disappears before

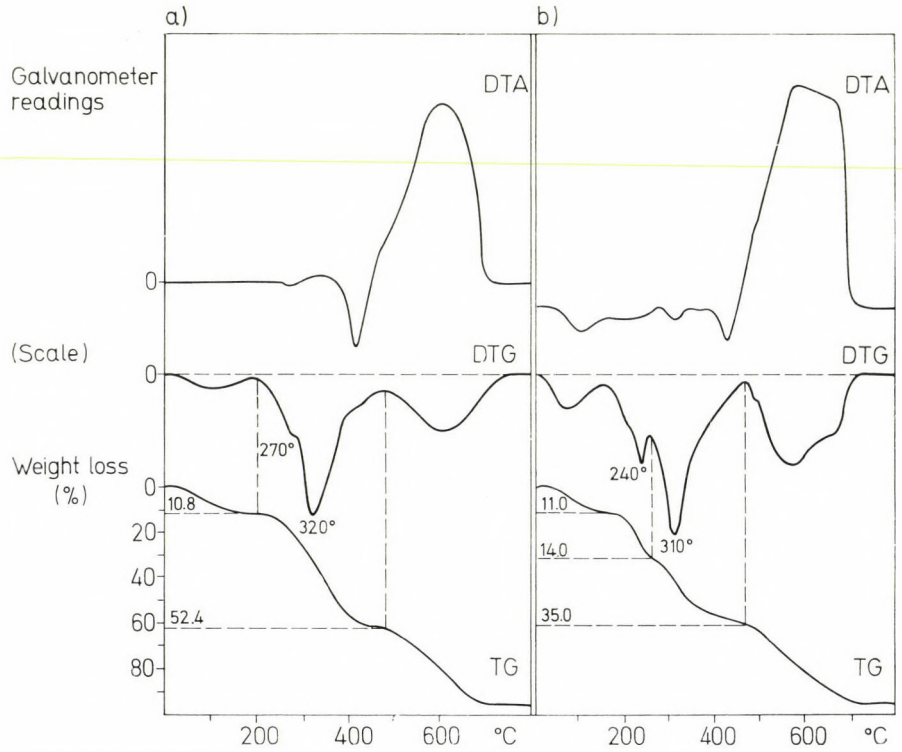


Fig. 1. Thermal decomposition curves for atherosclerotic human aorta intima. a) air-dried; b) delipidated

200 °C is reached. At about 200 °C decomposition starts. At 270 °C there is an inflexion on the DTG curve, the decomposition takes place at a maximum rate at 320 °C and is completed at 480 °C. The weight loss in this temperature interval is 52.4 per cent (58.5 per cent calculated for dry weight). This is caused by the cleavage of chemical bonds involving glycosaminoglycan-lipoprotein complexes, structural proteins and some other, lipid containing compounds, as previously described

(Simon et al., 1968; Bihari-Varga et al., 1968; Simon et al., 1969). The sample became thermostable at 800 °C.

Fig. 1b shows the derivatogram of the same aortic sample after lipid extraction. In this case the weight loss up to 150 °C was 11.0 per cent. At 240 °C a maximum appeared on the DTG curve accompanied by a weight loss of 14.0 per cent (15.7 per cent calculated for dry tissue), while the 310 °C DTG peak indicated a decomposition resulting in 35.0 per cent weight loss (39.3 per cent of dry weight).

A comparison of the two derivatograms presented in Fig. 1 indicates that as a result of delipidation the thermostability of some tissue components (previously identified as glycosaminoglycans) decreased and the decomposition ranging from 200 °C to 480 °C was separated into two individual processes.

There was a difference between dried and delipidated samples in the total weight loss measured up to 480 °C: according to the TG curves the losses amounted to 58.5 and 55.0 per cent of dry weight, respectively. This difference may be due probably to the extraction of various lipoproteins; these compounds were found to be decomposed in the same temperature interval (Bihari-Varga et al., 1968).

Next, the thermal behaviour of dried and delipidated samples of aortas, subdivided according to the type of atherosclerotic lesions, was studied.

In Fig. 2 typical derivatograms of air-dried intimal tissues are presented.

In Fig. 2a after the departure of water, decomposition starts at 200 C°, there is an inflexion on the DTG curve at 240 °C and a maximum at 310 °C. The process is completed at 480 °C and the weight loss is 64.8 per cent calculated for dry tissue.

In Fig. 2b there is still an inflexion at 240 °C and the amount of the material decomposed up to 480 °C is 66.4 per cent of dry weight.

In Fig. 2c there is no visible inflexion at 240 °C and the weight loss in the above temperature interval is 66.4 per cent.

In Fig. 2d the 240 °C DTG inflexion disappears and the main decomposition maximum about 300 °C is disproportionated into two separate processes, showing maximum rates at 300 °C and 340 °C. The weight loss up to 480 °C is 68.4 per cent of dry tissue weight.

These results indicate that there is a slight increase with the severity of atherosclerotic lesion in the total amount of chemical bonds characteristic of glycosaminoglycans, proteins and lipids which decompose in the 200—480 °C temperature interval.

In Fig. 3 thermal decomposition curves of the same aorta sections as in Fig. 2, after a previous delipidation procedure, are presented.

There is a difference in the shape of the corresponding curves; delipidation causes a decrease in the thermostability of glycosaminoglycan-type compounds, thus leading to the formation of a DTG peak about 240 °C.

The total amount of material decomposed up to 480 °C is 63.5, 63.3, 62.0 and 50.8 per cent of dry weight for lesion free parts, oedematic thickening, lipid containing lesions and calcified plaques, respectively. A comparison of these data with the results obtained with dried tissues shows an increase in lipid content with the severity of the lesions. This is indicated by the differences in the percentage weight losses between the corresponding parallel samples.

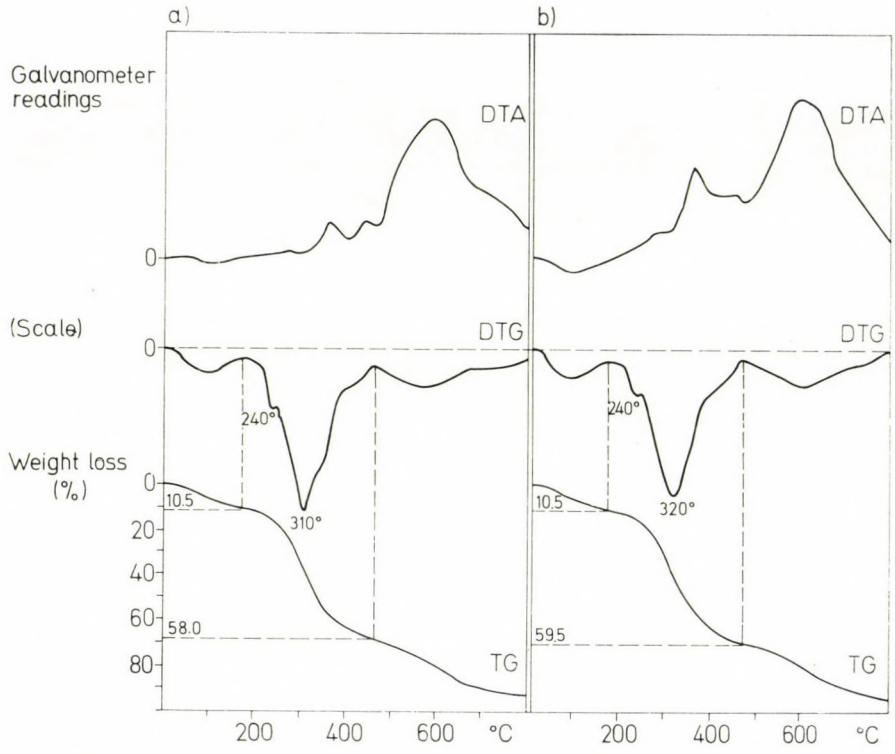


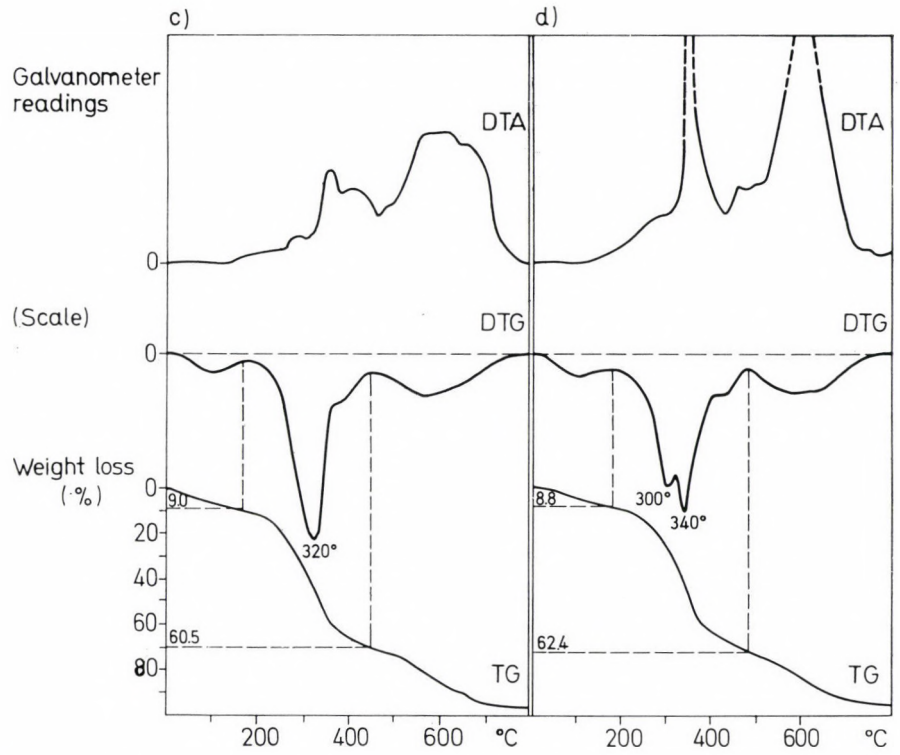
Fig. 2. Thermal decomposition curves for different parts of air-dried atherosclerotic aorta

The same tendency is observed in a series of individual atherosclerotic aortas, with slightly different numerical values.

These findings are in good agreement with the results of Smith (1965a) who described an increase in lipid concentration of the aorta related to increasing atherosclerosis.

On the basis of histochemical, chemical and biochemical studies it has been suggested that the glycosaminoglycans of the ground substance may in some way render lipids (lipoproteins) of the blood insoluble, and thereby set the stage for the development of the lesions of atherosclerosis.

In our experiments the changes in the amount of glycosaminogylcans in different types of lesions could be followed, because the concentration of these compounds was proportional to the percentage weight loss of delipidated sample in the 240 °C region. Data calculated from the TG curves (see e.g. Fig. 3) seem to confirm those obtained by chemical analysis (Bertelsen, 1960; Buddecke, 1962; Böttcher, Klynstra, 1962; Berenson et al., 1963; Smith, 1965b; Klynstra et al., 1967; Manley, Mullinger, 1967). Highest values were found in the early lesions and there was a decrease with increasing severity of the disease.



intima. a) lesion-free parts; b) oedematic thickening; c) fatty steaks and nodules; d) calcified plaques

The beta-lipoprotein content of saline extracts of the same involved areas (Table 1) changes parallel with the amount of glycosaminoglycans. This is further evidence for the role of glycosaminoglycans in the fixation of lipoproteins.

Table 1

Amount of saline-extractable beta-lipoprotein in different parts of atherosclerotic aortas

Type of lesion	mg beta-lipoprotein cholesterol/g dry tissue
Lesion free areas	26.4
Oedematic thickening	22.5
Fatty streaks + nodules	10.8
Calcified plaques	7.0

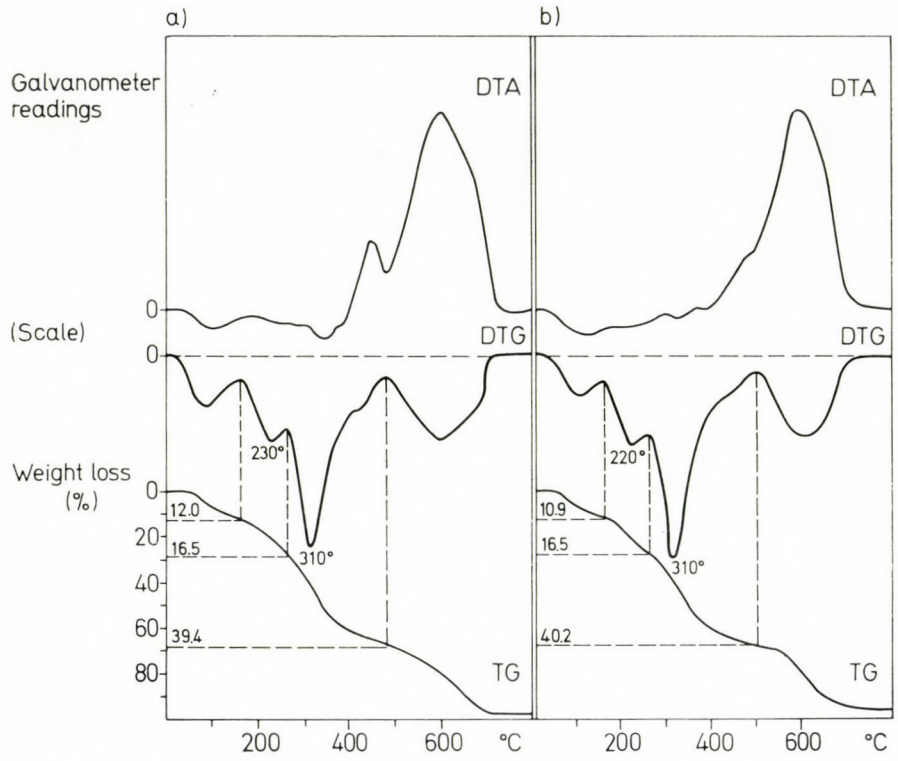
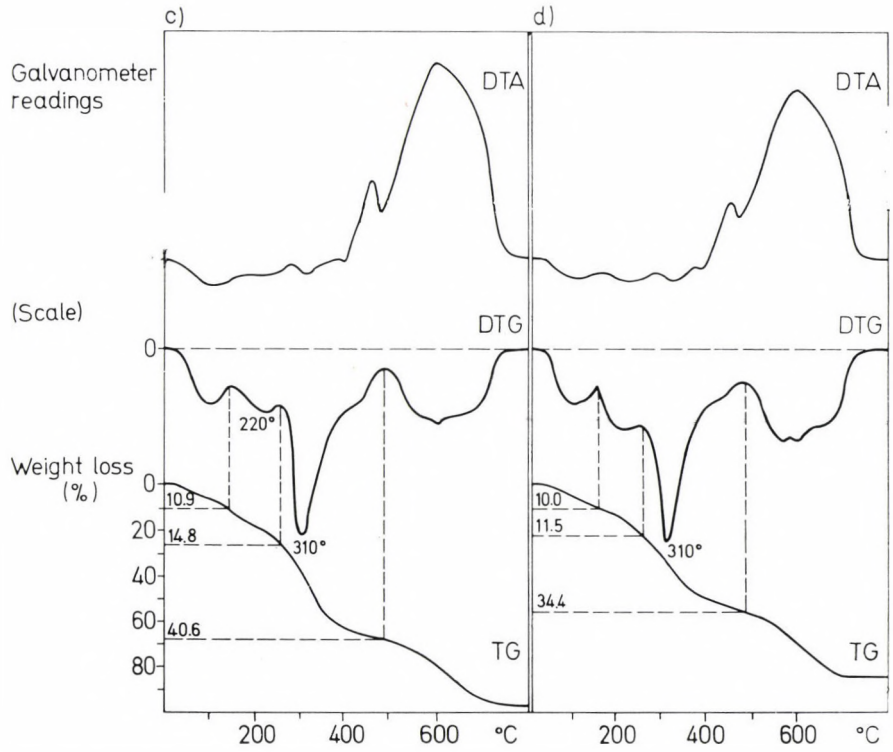


Fig. 3. Thermal decomposition curves for different parts of delipidated atherosclerotic aorta

Some part of the lipids which accumulate in the aorta during atherogenesis are known to be attached to the scleroproteins. The decomposition of the structural proteins in the defatted samples was characterized by the 320 °C DTG maximum. It is of interest, that while in the first three types of defatted tissues weight losses of the same order were observed (Fig. 3a, b, c) in calcified plaques (Fig. 3d) there was a decrease in the amount of compounds decomposed at about 320 °C. This phenomenon points to the possibility that some lipids are bound to the structural proteins, the amount of which increases with the severity of the lesions. The presence of lipid complexes seems to increase the thermostability of the proteins as well. This may explain the appearance of a second protein peak at 340°C in the DTG curves of dried calcified plaques (Fig. 2d). In our view these compounds may consist of complex macromolecules containing fibrous proteins, calcium and lipid components. The identification and assay of these complexes will be the object of our further studies.

Nevertheless, it has to be emphasized that other various processes (e.g. local alterations in enzymatic activities taking part in lipid synthesis etc.) have also a great influence on the formation of the atheromatous plaques.



intima. a) lesion-free parts; b) oedematic thickening; c) fatty steaks and nodules; d) calcified plaques

References

Berenson, G. S., Dalferes, E. R., Robin, R., Strong, J. P. (1963) Mucopolysaccharides and Atherosclerosis. In: Evolution of the Atherosclerotic Plaque. Ed. Jones, R. J. Univ. Chicago Press, p. 139

Bertelsen, S. (1960) Acta Pharmacol. *16* 20

Bihari-Varga, M., Simon, J., Gerő, S. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 375

Böttcher, C. J. F., Klynstra, F. B. (1962) J. Atheroscler. Res. *2* 263

Buddecke, E. (1962) J. Atheroscler. Res. *2* 32

Klynstra, F. B., Böttcher, C. J. F., Van Melsen, J. A., Van der Laan, E. J. (1967) J. Atheroscler. Res. *7* 301

Manley, G., Mullinger, R. N. (1967) Brit. J. Exptl. Path. *58* 529

Paulik, F., Paulik, J., Erdey, L. (1958) Z. Anal. Chem. *160* 241

Simon, J., Bihari-Varga, M., Erdey, L., Gerő, S. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 365

Simon, J., Bihari-Varga, M., Erdey, L., Gerő, S. (1969) Acta Biochim. Biophys. Acad. Sci. Hung. in the press

Slater, R. S., Smith, E. B. (1967) Biochem. J. *104* 11P

Smith, E. B. (1965a) J. Atheroscler. Res. *5* 224

Smith, E. B. (1965b) J. Atheroscler. Res. *5* 241

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (3), pp. 287—295 (1969)

New Biochemical Micro-method for the Determination of Prolactin in Rat Adenohypophysis

M. KURCZ, I. NAGY, P. BARANYAI

Department of Biology and Department of Biochemistry, National Institute of Health, and Central Laboratory of "Heim Pál" Children's Hospital, Budapest

(Received September 30, 1968)

Polyacrylamide gel electrophoresis was used for the determination of prolactin in the adenohypophysis of rat. By this procedure prolactin could well be separated from other proteins of the hypophysis. The standard relationship between the prolactin activity of the disc and the amount of dye bound by the disc, measured by the absorbancy of the alkaline-methanolic extract, made quantitative determinations possible.

About 80 per cent of prolactin activity of the sample was detected in the prolactin-disc after separation. From other parts of the gel prolactin activity could be demonstrated in traces. This slight diffuse spreading of the material can be explained on the basis of model experiments by interactions between proteins of the sample. Once the location of the prolactin disc is known, the method can be applied with other species as well. The new method surpasses the pigeon crop-sac micro-method in several respects.

Introduction

Starch- and polyacrylamide gel electrophoresis has been used by several authors to detect changes in the prolactin and somatotropin content of rat adenohypophysis; the intensity of staining was found to be proportional to the amount of hormones present (Baker et al., 1963; Lewis et al., 1965a, 1965b; Jones et al., 1965; Catt, Moffat, 1965; Baker, Zanotti, 1966; Kragt, Meites, 1966; Kurcz et al., 1968, 1969).

According to our earlier assumption (Kurcz et al., 1969), if the relationship between absorbancy and biological activity, as well as the location of tropic hormones in the gel are known, the method may also be suitable for quantitative measurements. In the present paper we describe the quantitative determination of prolactin from rat adenohypophysis by polyacrylamide gel electrophoresis.

Materials and Methods

Electrophoresis. Stock solutions and reagents for the electrophoresis were prepared according to Ornstein (1964) and Davis (1964).

Into glass tubes, 90 mm long and 6 mm in inner diameter, 2 ml small-pore gel solution was polymerized. On the top 0.2 ml spacer- and sample-gel solutions

were layered, respectively. When preparing the sample-gel, we diluted the homogenate of known volume and concentration with large-pore gel solution so that 0.2 ml should contain the desired quantity to be examined; the solution thus obtained was centrifuged, layered on the top, polymerized and then subjected to electrophoresis.

In our apparatus 12 samples could be analyzed simultaneously. The duration of an electrophoretic run in the case of 12 samples, 300 V, and 46—48 mA initial current intensity was always 120 minutes. At the end of separation the gels were removed from the tubes and stained for 60 minutes in 1 per cent Naphtalene Black 10B (C. I. 20470, Reanal, Budapest), dissolved in 7 per cent acetic acid. The excess dye could be washed out from the gel completely with several changes of 7 per cent acetic acid in 3—4 days. This manipulation can be carried out conveniently in a compartmented and perforated plastic box, where the gels can be treated simultaneously.

Colorimetric determination. After differentiation discs to be examined were cut our from the gel with a blade and homogenized in 0.5 ml of a 1 : 1 mixture of NaOH and methanol. This mechanical disintegration accelerated the removal of dye. After 15 minutes standing the gel homogenate was spun in a Beckman Spinco ultramicro analytical centrifuge M-152 for 5 minutes, then the absorbancy of the supernatant was read in a Beckman Spinco ultramicro spectrocolorimeter (M-151) at the absorption maximum of the dye, 610 mμ. (Light path 6.4 mm, volume of cell 100 μl).

Preparation of hypophyses. The hypophyses of rats were removed immediately after decapitation carried out in deep ether narcosis. The adenohypophysis was separated from neurohypophysis, weighed with 0.2 mg accuracy, then homogenized with a manual glass homogenizer in 0.02 M phosphate buffer, pH 7.0 at 0° C. The homogenate suitably contained 70—80 mg wet tissue per ml. The homogenate could be stored in the homogenizer at 0° C for 48 hours without any detectable change.

For the calibration curve homogenates were prepared from 50—60 mg adenohypophysis in each case, and these homogenates were used for the determination of biological activity and absorbancy.

Assay of prolactin activity. Prolactin activity was assayed by a modification (Kurcz, 1966) of the pigeon crop-sac micromethod of Grosvenor and Turner (1958). The acrylamide gel columns were cut up according to their stained parallels and the pieces thus obtained were homogenized in an appropriate volume of distilled water, extracted at 4° C for 24 hours and centrifuged in the cold. Samples from the supernatants were injected intracutaneously over the sensitive region of the pigeon crop.

Other materials. In model experiments prolactin preparations of known activity (Panlitar, Armour, NIH, Bethesda, Md., USA, 15 I.U. per mg and Prolactin, Ferring, Malmö, Sweden, 1000 I.U. per flacon), somatotropin (Hormone Somatotrope, Choay, Paris), purified bovine albumin (Sigma Chem. Co., St. Louis, USA), and sheep and human sera were used.

Results and Discussion

Analysis of the electrophoretogram of rat adenohypophysis extract. Fig. 1 shows the electrophoretograms of extracts prepared from 1, 2, 3 and 4 mg of rat adenohypophysis, respectively. The electrophoretic pattern is characterized by three fractions of different mobility. According to literary data (Jones et al., 1965; Kragt, Meites, 1966) fraction "S" is somatotropin and this was confirmed by the identical mobility of rat STH prepared by us according to the method of Reisfeld et al. (1964a) and Lewis et al. (1965a). Fraction "A" of intermediate mobility

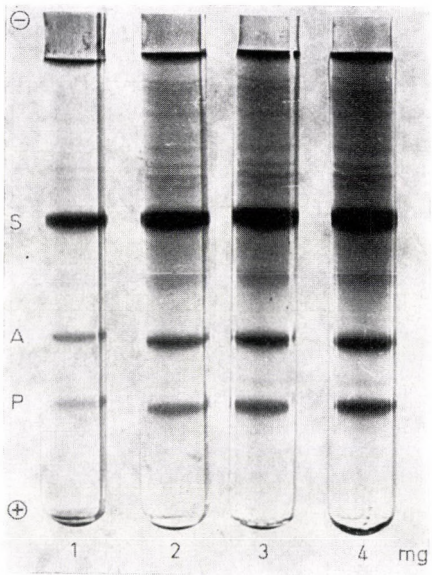


Fig. 1. Electrophoretograms made with 1, 2, 3, and 4 mg of rat adenohypophysis. P = prolactin, A = albumin, S = somatotropin

and intensive staining comprises albumin and its location in the electrophoretogram agrees with that of the albumin fraction of rat serum.

Prolactin is the fraction with the greatest mobility. This observation of ours is in agreement with that of Lewis et al. (1965b), Jones et al. (1965), and Kragt and Meites (1966). Some authors, however, could detect prolactin activity in other parts of the gel as well, though their results are contradictory (Baker et al., 1963; Kragt, Meites, 1966; Robboy, Kahn, 1966). Therefore, we examined the quantitative distribution of prolactin in the gel. When 3 mg of adenohypophysis was used for electrophoresis, crop-stimulating effect could be elicited only with gel-portion "P", while using 4 mg of adenohypophysis we found prolactin activity also in the sample-spacer gel. From the other parts of the gel prolactin could be demonstrated only after combining the extracting solutions. Fig. 2 shows the results in detail. It can be calculated from the activity values per mg of adenohypophysis that if 3—4 mg of adenohypophysis are subjected to electrophoresis, the prolactin content

of only fraction "P" and the sample-spacer gel reaches the sensitivity-threshold of the pigeon crop-sac test (0.02—0.05 I.U.). This would explain the negative results for a single gel-portion. The contradictory distributions known from the literature (Baker et al., 1963; Jones et al., 1965; Catt, Moffat, 1965; Robboy, Kahn, 1966), which in fact differ from our results, might be due to the lack of

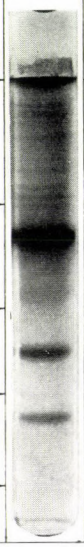
Length of the gel mm	Fraction		Number of determinations + Reaction	−	Activity, IU/mg adenohypophysis	Distribution of prolactin activity per cent
0	Sample and spacer gel		8	0	0.0092 ± 0.003^{x}	14
17			2	2	≈ 0.0055	≈ 9
33	S		4	4	≈ 0.0028	≈ 4
45	A		2	2	≈ 0.0017	≈ 3
60	P		8	0	0.0450 ± 0.001^{x}	70
66			0	4	0.0000	0

Fig. 2. Distribution of prolactin activity in the gel column. The sites of cuttings are marked by arrows. X = standard error; P = prolactin, A = albumin, S = somatotropin

detailed analyses. As can be seen in Fig. 2 the majority of prolactin activity (about 70 per cent) appeared in a single band, fraction "P". The remaining 30 per cent were smeared in a gel segment four times larger than the gel segment contraining fraction "P" and could be detected only indirectly.

The above data are confirmed by another experiment. A sample containing 3 mg of adenohypophysis of a homogenate (0.096 I.U. prolactin per mg) was analyzed by electrophoresis. In disc "P" 0.076 I.U. per mg activity was found (12 determinations), i.e. about 80 per cent of the introduced prolactin activity. In this case no other parts of the gel were analyzed. We checked, however, the loss of activity in sample-gel after centrifugation. This proved to be 0.009 I.U. per mg (9 determinations).

Because of the activity losses it is advisable to base the calibration curve on the activities found in the extracting liquids of discs "P". The prolactin content of the segment can be completely extracted with 2 × 1 ml of distilled water, as shown by a vast number of experiments. When 50 μg of prolactin preparation of known specific activity was run, about 75—80 per cent of the original activity could be

recovered from the gel-portion containing the hormone even in the first extraction step. The gel itself does not adsorb prolactin. Pieces of gel of the same size, not containing protein, were homogenized in a prolactin solution of known activity. The activity was quantitatively recovered from the supernatant solution. The absorbancy values with the corresponding activities are given in Table I, while the

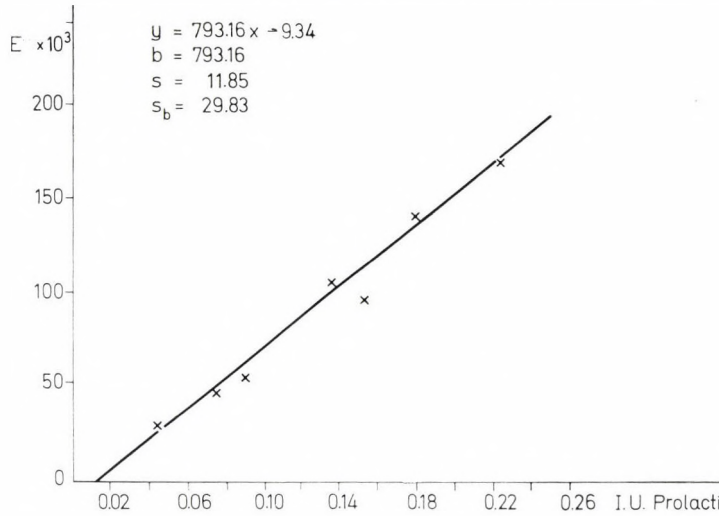


Fig. 3. Relationship between the biological activity and Naphtalene Black-binding capacity of rat prolactin. s = scattering within the group, b = regression coefficient, sb = scattering of the regression coefficient, Y = equation of the regression straight line

calibration curve is shown in Fig. 3. The results of mathematical analyses are also summarized in Fig. 3.

The linear relationship between absorbancy and activity was proved in model experiments as well. Purified sheep prolactin preparations are electro-

Table 1

Experimental data of the calibration curve. Relationship between biological activity and absorbancy

Activity of prolactin disc after electrophoresis I.U.	Number* of measurements	Absorbancy $\times 10^3$ at 610 mμ
0.0450	5	29.40± 7.64**
0.0760	8	45.25± 3.54
0.0900	5	55.20± 2.77
0.1350	4	104.25±11.95
0.1520	8	97.25± 8.35
0.1800	4	138.25± 3.50
0.2280	8	170.25± 8.11

* The number of measurements gives the number of gels used for activity and absorbancy determinations.
** Standard deviation

phoretically inhomogeneous (Fig. 4b). The bulk of activity appears in the slowest, intensively stained fraction (Reisfeld et al., 1964b). Electrophoretic runs were made with the following amounts of preparation; 1.2, 1.5, 2.3, 3.0, 4.7, 6.2, 9.3, 12.5, 18.7, 25.0, 37.5, 50.0 and 75.0 μg). The absorbancy of the principal fraction increased proportionally with the weight of the sample.

Distribution of prolactin activity

When 1—4 mg of adenohypophysis was separated by electrophoresis, the resolving capacity of the gel was perfect, regular discs were formed (Fig. 1). When more than 4 mg of adenohypophysis was used, the sides of the prominent discs

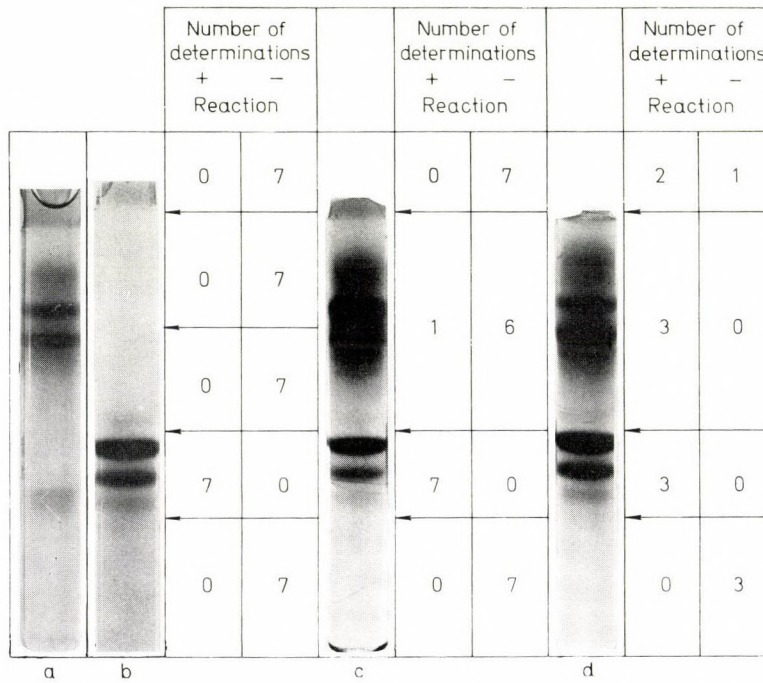


Fig. 4. Distribution of prolactin activity after the electrophoresis of mixtures of prolactin and somatotropin. Electrophoretograms of a) 400 μg of somatotropin, b) 100 μg of prolactin, c) a mixture of 800 μg of somatotropin and 50 μg of prolactin, d) a mixture of 800 μg of somatotropin and 100 μg of prolactin

were blurred and large diffuse areas developed between them. The formation of smaller fractions could not be observed. The analyses are therefore suitably made with 1—4 mg of sample. In our experimental set-up this is the optimal amount to be separated.

In spite of the good resolving capacity we attempted to clarify the cause of the above distribution pattern of prolactin activity by model experiments. We

loaded gels with 50, 100, and 200 μg of sheep prolactin, i.e. with relatively large amounts of protein. From these gels the total amount of activity could be recovered after electrophoresis from the portions indicated by staining (13 determinations). 800 μg of somatotropin has no stimulating effect on the pigeon crop. When this amount was mixed with 50—100 μg of prolactin and run in the gel (10 determinations), the extract from the gel-portion containing somatotropin exhibited prolactin activity (Fig. 4).

From a mixture of sheep serum and sheep prolactin the various components were well separated by electrophoresis, however, some activity was left in the macroglobulin and diffuse gammaglobulin regions. From a mixture of human serum and sheep prolactin, activity was left in traces in the starting zone (Fig. 5).

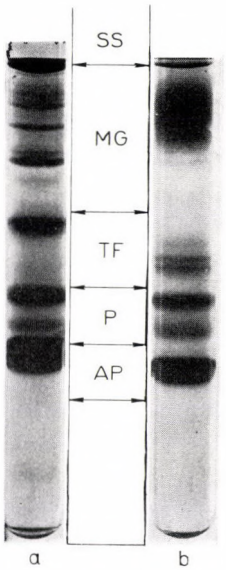


Fig. 5. Distribution of prolactin activity after the electrophoresis of mixtures of prolactin and serum. Electrophoretograms of a) a mixture of 5 μl of human serum and 50 μg of prolactin, b) a mixture of 5 μl of sheep serum and 50 μg of prolactin. SS = sample and spacer gels, MG = macroglobulins, TF = transferrin, P = prolactin, AP = albumin and prolactin

The mixture of purified bovine albumin and prolactin showed activity only in the fraction corresponding to prolactin. It seems possible that in protein mixtures, such as an adenohypophysis homogenate, if a critical amount of the components is present, complexes exhibiting biological activity are formed. These complexes may not be broken up even by forces that act during separation. This phenomenon cannot be observed on the stained gel with the naked eye, since perfect discs are obtained when adenohypophyses, preparations, sera, or their mixtures are being analyzed.

The above data do not exclude the possibility of the existence in the hypophysis of other proteins (which might be called "iso-prolactin") that exert a stimulating effect on the pigeon crop. The assumption put forward by Kragt and Meites

(1966) according to which active polymers of prolactin may be formed during electrophoresis, cannot be disporved either. It has also been shown that rat prolactin prepared by disc electrophoresis (Jones et al., 1965) and the principal fraction of sheep prolactin prepared chromatographically (Reisfeld et al., 1964b) remain homogeneous on electrophoresis. The formation and break-down of polymers could so far be brought about only enzymatically (Lewis, 1962, 1963, 1964) or as a result of drastic chemical effects (Reisfeld et al., 1964b).

Evaluation and scope of application of the method

On the basis of the above experimental results polyacrylamide gel electrophoresis seems to be suitable for the determination of prolactin content in rat adenohypophysis. If 1—4 mg of wet tissue is used for the analysis the method is more sensitive than the generally applied pigeon-crop micro-method (Grosvenor, Turner, 1958; Kurcz, 1966). As a biochemical procedure it eliminates all sources of error of biological tests: individual and species differences in sensitivity, age of animals, seasonal differences, aspecific effects, etc. Its specificity resides in the fact that prolactin can be separated from other proteins of the adenohypophysis, and the biological activity as well as the absorbancy of the bound dye can be determined. The possible error due to incomplete recoveries can be reduced if two or three samples of different weights are run from the same material. This circumstance was taken into consideration when we made the calibration curve. The activity *vs.* absorbancy plot is linear.

It contributes to the precision of the method that its sensitivity allows the analysis of several samples from a single hypophysis. A further advantage of the method is that within a short time a great number of well reproducable determinations can be performed. Furthermore, it is cheaper and less laborious than any other method mentioned above.

Among the drawbacks it should be mentioned that the method is less sensitive than the radioimmunoassay (Kwa, Verhofstad, 1967a, 1967b; Kwa et al., 1967; Arai, Lee, 1967) and is unsuitable for the determination of prolactin in blood.

We have developed the method for the determination of prolactin in rat adenohypophysis. The criteria of application to other species are the following: a) knowledge of the distribution of prolactin; b) location of prolactin disc in the gel; c) establishment of the activity *vs.* absorbancy plot.

Our earlier investigations indicate (Nagy et al., 1969) that the method described in the present paper can be used for the determination of prolactin content in the adenohypophysis of other species as well.

Extension of the method to the determination of somatotropin in adenohypophysis

According to our experiments and literary data (Lewis et al., 1965b; Jones et al., 1965; Kragt, Meites, 1966) STH can also be separated by disc electrophoresis. In our opinion the criteria for quantitative determination would also hold in

the case of STH. Thus prolactin and STH could be simultaneously determined from the same sample. Results concerning this problem will be presented in our next paper.

The authors express their thanks to Mrs G. Csukás, Chief of the Department of Statistics, Natl. Institute of Health, for the mathematical analysis of data, to Mrs I. Káldi, Miss I. Szarka, Mrs L. Hejtejer, and Miss I. Mayer for their competent assistance in the experimental work. Thanks are due to the National Institute of Health, Bethesda, Md., USA for providing us with the prolactin preparation used in the present work.

References

Arai, Y., Lee, T. H. (1967) Endocrinology *81* 1041
Baker, B. L., Clark, R. H., Hunter, R. L. (1963) Proc. Soc. Exptl. Biol. (N. Y.) *114* 251
Baker, B. L., Zanotti, D. B. (1966) Endocrinology *78* 1037
Catt, K., Moffat, B. (1965) Endocrinology *76* 678
Davis, B. J. (1964) Ann. N. Y. Acad. Sci. *121* 404
Grosvenor, C. E., Turner, C. W. (1958) Endocrinology *63* 530
Jones, A. E., Fisher, J. N., Lewis, U. J., Vanderlaan, W. P. (1965) Endocrinology *76* 578
Kragt, C. L., Meites, J. (1966) Proc. Soc. Exptl. Biol. *121* 805
Kurcz, M. (1966) Ann. Univ. Sci. Budapest de Rolando Eötvös, Sect. Biol. *8* 149
Kurcz, M., Nagy, I., Gerhardt, V. J., Baranyai, P. (1968) Acta Biol. Acad. Sci. Hung. *19* 123
Kurcz, M., Nagy, I., Baranyai, P., Gerhardt, V. J. (1969) Acta Biol, Acad. Sci. Hung. *20* 163
Kwa, H. G., Verhofstad, F. (1967a) Biochim. Biophys. Acta (Amst.) *133* 186
Kwa, H. G., Verhofstad, F. (1967b) J. Endocrin. *38* 81
Kwa, H. G., Verhofstad, F., van der Bent, E. M. (1967) Acta Physiol. Pharmacol. Neerl. *14* 514
Lewis, U. J. (1962) J. Biol. Chem. *237* 3141
Lewis, U. J. (1963) J. Biol. Chem. *238* 3330
Lewis, U. J. (1964) J. Biol. Chem. *240* 247
Lewis, U. J., Cheever, E. V., Vanderlaan, W. P. (1965a) Endocrinology *76* 210
Lewis, U. J., Cheever, E. V., Vanderlaan, W. P. (1965b) Endocrinology *76* 362
Nagy, I., Kurcz, M., Baranyai, P. (1969) Acta Biochim. Biophys. Acad. Sci. Hung. *4* 27
Ornstein, L. (1964) Ann. N. A. Acad. Sci. *121* 321
Reisfeld, R. A., Muccilli, A. S., Williams, D. E., Steelman, S. L. (1964a) Nature *201* 821
Reisfeld, R. A., Williams, D. E., Cirillo, V. J., Tong, G. L., Brink, N. G. (1964b) J. Biol. Chem. *239* 1777
Robboy, S. J., Kahn, R. H. (1966) Endocrinology *78* 440

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (3), pp. 297—299 (1969)

Modified Single-column Procedure for the Automatic Analysis of Amino Acids

(Short Communication)

T. DÉVÉNYI

Institute of Biochemistry, Hungarian Academy of Sciences, Budapest

(Received July 15, 1969)

In a previous paper we described a single-column procedure for the automatic analysis of amino acids (Dévényi, 1968). By using only two buffers, an analysis can be completed within 4 hours. By the use of an additional buffer (1 N sodium citrate, pH 5.28) the time necessary for an analysis is reduced to $3^1/_2$ hours, however, in this case there is a sharp increase of the baseline indicating the buffer-change. A similar effect was also observed by Dus et al. (1966), and this phenomenon is described in most amino acid analyzer manuals. Increased height of the baseline was accompanied by a slight decrease in the sensitivity of the ninhydrine reaction.

In the present paper a modification of the previous procedure is described which allows the completion of an amino acid analysis without any considerable change in the baseline, in about 3 hours.

Materials

Apparatus

The analyses were carried out with Beckman Unichrom amino acid analyzers (No 61001 and 61036).

Resin

A recently developed spherical resin (CHROMEX—U8 Reanal, Budapest) was applied. A standard long column was used with a filling height of 55 cm. The flow-rate was 100 ml buffer per hour and the back-pressure was 26—32 atm. at 55° C.

Buffers

a) 0.2 N sodium citrate, pH 3.28. This buffer was identical with that described by Spackman et al. (1958).

b) 0.2 N sodium citrate, pH 4.25, containing 0.6 M sodium chloride. This buffer was similar to that described by Hamilton (1963).

c) 0.1 N sodium citrate, pH 6.0, containing 1.5 M sodium chloride.

The buffers were prepared from analytical grade reagents using deionized water obtained from a mixed-bed resin.

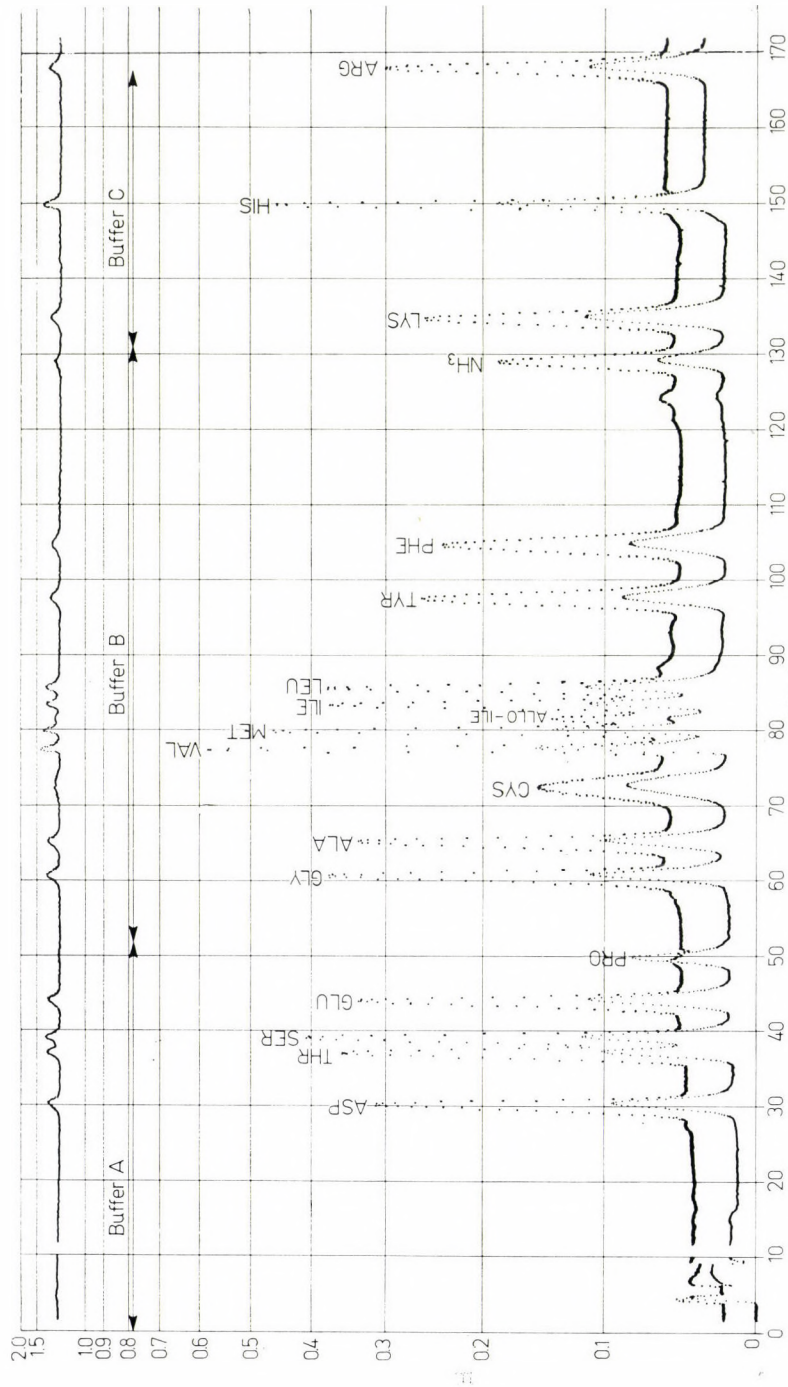


Fig. 1. Fractionation of a calibration mixture on a single column with the three-buffer system

Results and Discussion

Figure 1 shows a typical analysis of a calibration mixture. The programming of the analysis is as follows:

a) The elution is started with buffer A.

b) The first buffer-change (from A to B) is set to 65 minutes.

c) The second buffer-change (from B to C) is set to 145 minutes. This is carried out with the "regeneration system" built into our analyzers by Beckman Instruments GMBH Munich, by courtesy.

d) Run control is set to 190 minutes.

e) Recyclization with buffer A is finished in 20 minutes. Due to the high molarity and pH of buffer C, regeneration with sodium hydroxide is unnecessary.

With buffer C arginine elutes shortly after histidine, so the analysis can be completed in 3 hours, without any considerable change in the baseline.

The sharp increase of the baseline observed by the previous procedures is due to the relatively low capacity of the acetate buffer used for the preparation of the ninhydrine solution. In the reaction mixture formed in the mixing manifold (a mixture of two parts of effluent and one part of ninhydrine solution) the final concentration of the acetate buffer is 0.33 M. If the molarity of the effluent is higher than that, and the pH is higher than 5, the color of the ninhydrine solution turns yellow which will be indicated by a sharp increase of the baseline. Since during chromatography the molarity of sodium ions is critical, the concentration of sodium citrate is maintained at 0.2 N and the molar concentration of sodium ions is brought to a final concentration of 0.8 N in the case of buffer B and to 1.6 in the case of buffer C with the addition of NaCl. Under such conditions the capacity of acetate buffer present in the reaction mixture will be sufficiently high to maintain the pH of the mixture in the vicinity of 5 and the baseline remains stable until the analysis is completed.

Using an automatic sample injector six analyses can be completed within 24 hours with this revised single column procedure.

The technical assistance of Mrs Judit Báti is gratefully acknowledged.

References

Dévényi, T. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 429
Dus, K., Lindroth, S., Pabst, R., Smith, R. M. (1966) Anal. Biochem. *14* 41
Hamilton, P. B. (1963) Anal. Chem. *35* 2055
Spackman, D. H., Stein, W. H., Moore, S. (1958) Anal. Chem. *30* 1190

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (3), pp. 301—304 (1969)

Characterization of the SH-Groups of D-Glyceraldehyde-3-phosphate Dehydrogenase

(Preliminary Communication)

L. Boross, Emilia Cseke, Mária Vas

Institute of Biochemistry, Hungarian Academy of Sciences, Budapest

(Received August 11, 1969)

One of the 4 SH-groups per subunit of pig muscle D-glyceraldehyde-3-phosphate dehydrogenase (GAPD), namely Cys-149, is the nucleophilic or "active" SH-group of the enzyme, which forms a thiol ester with the oxidized substrate (Harris, Perham, 1968; Velick, Furfine, 1963). The thiol groups of GAPD display differential reactivities towards p-mercuribenzoate (PMB): Cys-149 reacts instantaneously (SH_I), whereas the other three thiol groups react only after prolonged incubation. In the order of decreasing reactivity they have been called SH_{II}, SH_{III}, and SH_{IV} (Szabolcsi et al., 1968; Friedrich, Szabolcsi, 1967).

Our recent experiments provide a subtler quantitative characterization of the different thiol groups of GAPD and, furthermore, contribute to a more accurate delineation of the active center of the enzyme.

Characterization of SH_I

1. SH_I reacts with iodocatete faster than aliphatic mercaptans, including cysteine (Racker, Krimsky, 1958; Boross, Cseke, 1969). In the presence of NAD the rate constant of carboxymethylation is unusually high in dilute tris or acetate buffer and decreases when sulfate, phosphate or pyrophosphate ions are added (Table 1). In the presence of these anions the pH dependences of rate constants are also different and agree well with those of the ultraviolet absorption (Racker-band) of the GAPD-NAD complex (Boross, Cseke, 1967; Trentham, 1968).

The carboxymethylation of SH_I proceeds much slower in the absence of NAD or in the presence of NADH, although the rate constants in neutral solution are still higher than for cysteine. We assume that a functional group of the enzyme activates SH_I and that NAD further accelerates carboxymethylation by promoting the oriented binding of iodoacetate close to SH_I. The activating group may exhibit either acidic or basic dissociation and only its deprotonized form is able to activate SH_I. The effect of anions is due to their binding in the vicinity of the activating group, thereby changing its acidic dissociation as well as weakening its interaction with SH_I.

Table 1

The second order rate constants of the alkylation of SH_I

Experiments were carried out at 5 °C in tris/HCl buffer of 0.05 ionic strength, containing different anions as indicated. The reactions were followed in three different ways, indicated in the last column: 1) determination of the absorption at 365 mμ (Boross, Cseke, 1967); 2) determination of the unreacted $SH_I + SH_{II}$ groups with dithio-bis nitrobenzoate in the presence of 1.5 M sodium chloride at pH 7 (Boross, 1969); 3) determination of unreacted SH_I by addition of $CuSO_4$ in the presence of 5 per cent ammonium sulfate and 0.5 M phosphate buffer, pH 7

Reagent	Presence of coenzyme, 4 mole equ.	Anions added 0.1 M	pH	k liter / mol×minute	Apparent pK of SH_I	Method
Iodoacetate	NAD	–	7.4 or 8.5	4800	<5	1
	NAD	chloride	7.4	2600	<5	1
	NAD	sulfate	7.4	700	5.4	1
	NAD	phosphate	7.4	1100	*	1
	NAD	pyrophosphate	7.4	1050	6.8	1
	NADH	–	7.4	180		2
	–	–	8.5	250	≥7	3
Iodoacetamide	NAD	–	7.4	140	>8	3
	NAD	–	8.5	350		3
	–	–	7.4	540	>8	3
	–	–	8.5	3700		3
	–	chloride	8.5	3330		3
	–	phosphate	8.5	640		3
	NADH	–	7.4	35		2

* According to Trentham (1968) the apparent pK is 6.86 in phosphate + pyrophosphate buffer.

This anion-binding center of GAPD might be a substrate-binding group since not only phosphate but also chloride and sulfate ions inhibit the enzymatic reaction competitively with the substrate.

In contrast to iodoacetate, iodoacetamide reacts faster with SH_I in the absence of NAD. The very high rate constant in this case also indicates a stereospecific binding of the reagent in the vicinity of SH_I. Both NAD and NADH decrease the rate of the reaction, presumably by occupying the binding site for the reagent.

2. SH_I reacts instantaneously with heavy metal ions, such as Hg^{++} and Ag^+ ions, and with PMB. Whereas the binding of PMB abolishes the Racker-band (Velick, Furfine, 1963), the binding of Ag^+ ions only changes the absorption spectrum of GAPD—NAD complex (Boross, 1965). We have found that SH_I binds cupric ion, too, which increases about 3 times the absorption at 365 mμ. However, this increased absorption is due to the formation of Cu—GAPD binary complex rather than to a ternary Cu—GAPD—NAD complex, since the absorption increases also in the absence of NAD. The spectrum of Cu—GAPD resembles

that of bovine serum albumin-Cu complex (Klotz et al., 1955), which supports the hypothesis that there are similar chelating groups around these thiol groups in both proteins.

The alkylation of SH_I prevents the formation of the specific Cu-complex. In fact, this phenomenon served as a tool in some of our experiments for the spectrophotometric determination of the rate of alkylation.

Characterization of SH_{II}, SH_{III} and SH_{IV}

The reactivity of SH_{II} toward PMB depends on several factors (Table 2). Mercaptide formation follows first order kinetics in all cases, indicating that SH_{II} is not a free thiol group and some rate limiting structural changes should occur prior to the reaction with PMB. Carboxymethylation of SH_I does not influence

Table 2

The apparent first order kinetic constants of the reaction of SH_{II},SH_{III} and SH_{IV} groups with PMB

The experiments were carried out at 5°C with 175 μg/ml NAD-free enzyme in tris/HCl buffer of 0.05 ionic strength, containing additional components as indicated. The reactions were followed by determination of the changes in absorption at 255 mμ

pH	Additional components	k(minute^{-1})	
		SH_{II}^{*}	SH_{III} and SH_{IV}^{**}
7.0	—	0.52	0.050
	NAD (4 mole equ.)	0.30	0.050
	NADP (4 mole equ.)	0.52	
	NADH (4 mole equ.)	0.52	
	GAP (40 mole equ.)	0.52	0.050
	0.5 M sulfate	0.075	0.006
	0.1 M phosphate	0.045	0.023
	1.0 M chloride	1.90	0.035
8.5	—	0.76	0.087
	NAD (4 mole equ.)	0.55	0.085
	0.5 M sulfate	0.21	0.021
	0·1 M phosphate	0.15	0.025
	1.0 M chloride	2.20	0.036

* PMB concentration: 10^{-5} M
** PMB concentration: 2×10^{-5} M

the reaction of SH_{II} with PMB. Mercaptide formation is completed within a few minutes at pH 8.5, 5°C, consequently SH_{II} seems to be on or near the surface of the enzyme, presumably involved in a complex bond. This hypothesis is supported by the fact that under the same conditions SH_{II} does not react with iodoacetate.

The rate of mercaptide formation for SH_{III} and SH_{IV} are indistinguishable and the average rate constant is much smaller than that of SH_{II}. The first order

rate constant is independent of the presence of coenzyme, but depends on the presence of various anions (Table 2). The reaction of SH_{III} and SH_{IV} with dithio-bis-nitrobenzoate is also an apparently first order reaction. The rate of this reaction is likewise influenced by anions; the rate constants at 5 °C in 0.05 ionic strength tris-buffer and in 1 M ammonium sulfate solution, PH 7, are 0.009 and 0.0014 min^{-1}, respectively.

The effect of various anions on the rate of modification of these buried SH_{II} and SH_{IV} groups (Friedrich, Szabolcsi, 1967) seems to be due to their influence on the rate of structural changes of the protein, as a result of which these groups become accessible to the reagent.

These results allow us to divide the SH-groups of GAPD into three classes 1. SH_I, the unusually reactive thiol group, which is activated by a side-chain interaction influenced by bound anions, 2. SH_{II}, which is less reeactive than aliphatic mercaptanes and whose reactivity is further decreased by polyvalent anions and increased by chloride ion, 3. buried thiol groups, SH_{III} and SH_{IV}, whose reactivity is influenced by anions through their effect on the structure of whole protein molecule.

References

Boross L. (1965) Biochim, Biophys. Acta *96* 52
Boross, L. (1969) Acta Biochim. Biophys. Acad. Sci. Hung. *4* 57
Boross, L., Cseke, E. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 47
Friedrich, P., Szabolcsi, G. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 59
Harris, J. I., Perham, R. N. (1968) Nature *219* 1025
Klotz, I. M., Urquhart, J. M., Klotz, T. A., Ayers, J. (1955) J. Am. Chem. Soc. *77* 1919
Racker, E., Krimsky, I. (1958) Fed. Proc. *17* 1135
Szabolcsi, G., Biszku, E., Sajgó, M. (1960) Acta Physiol. Acad. Sci. Hung. *17* 183
Trentham, D. R. (968) Biochem. J. *109* 603
Velick, S. F., Furfine, C. (1963) in The Enzymes, vol. 7, p. 243 Ed. by Boyer, P. D., Lardy, H. and Myrbäck, K., New York, Academic Press Inc.

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (3), pp. 305—312 (1969)

On Step-like Plaque-growth*

J. HEMELA, GY. RONTÓ, I. TARJÁN

Biophysical Institute, Medical University, Budapest

(Received November 26, 1968)

The authors have developed a method for the examination of plaque-growth which is more accurate than that employed before. It has been demonstrated that the growth curve of T7 phage plaques in the second phase is a step-like one. The length of the steps (τ) as well as their height (h) are only slightly diminished by increasing the concentration of bacterial microcolonies ($5 \times 10^6 - 5 \times 10^8$ cm^{-3}). None of these quantities depend significantly either on the age of the plaque or on the concentration of the soft agar. τ approximately equals to the minimal latent period and h falls within the order of magnitude of the average distance between the bacterial microcolonies. In order to interpret the empirical results the authors suggest that the bacterial-viral interaction is effective even at a distance several times greater than the size of bacterial colonies.

Introduction

One of the oldest methods used for the demonstration and identification of phages is the examination of the plaques caused by them. It is a generally accepted fact that plaque development and growth are rather complex phenomena including several physical and chemical processes. There is no doubt about the fact either that the size of the plaque and its morphology are determined, above all, by the characteristics of the phage, of the host cell and of the phage–host cell complex (virulency, burst size, etc.) but, in a given case, the actual experimental conditions (incubation time, temperature, the viscosity of the medium) also play a remarkable role (Adams, 1959; Herčik, 1959; Mayr-Harting, 1958). In spite of the many observations, only a few data at our disposal are suitable for quantitative evaluation of the exact mechanism of the development and growth of the plaque, and of the factors playing an important part in the process.

The growth of a plaque can be divided macroscopically into two phases: the first (phase I) includes the period from the primary infection to the set-in of the constant growth-rate, the second (phase II) can be characterized just by the constant growth-rate. The latter is interpreted by Koch (1964), considering the increase of the virus concentration during the process, as a diffusion of a virus wave. But the calculated plaque sizes do not agree in each case with the values obtained experimentally by the author. According to the model of Schwöbel and his collaborators (1966) diffusion does not play a part in the increase of

* Delivered at the 5th Congress of the Hungarian Biophysical Society.

plaque-diameter, but the growth rate of the plaque is determined by the virus--production (presuming a constant latent period).

In the present paper we report our observations concerning phase II which, in our opinion, give new informations on the expanding mechanism of the lysis-front.

Material and Method

We have examined the plaques of T7 phages in our experiments. We used E. coli B bacteria as host cells which were synchronized by a method elaborated for non-auxotroph strains (Maaloe, 1962; Cummings, 1965; Cutler, Evans, 1966).

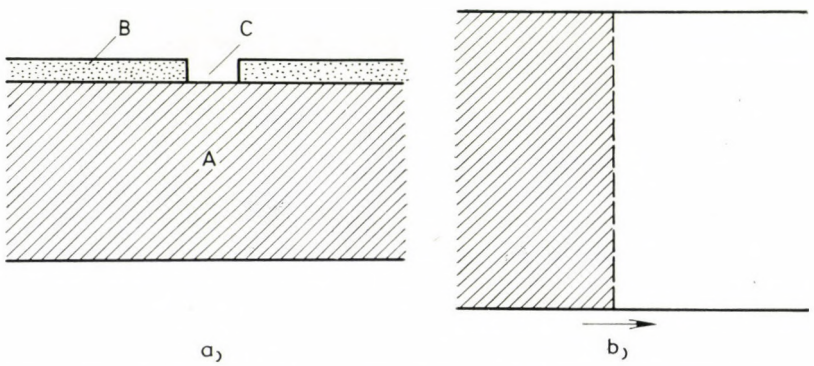


Fig. 1a. A scheme for the so-called one-dimensional technique (side-view). *A* — basal agar layer, *B* — soft-agar of 0.35 mm thickness with the host-cells, *C* — a groove of 40×5 mm in the soft-agar; this is where the phage-suspension is dropped. b. A schematic representation of the plaque (top-view) in the case of the one-dimensional technique; the dotted line indicates the lysis-front, the shaded part — the range of the lysed bacteria and the arrow — the direction of the shift of the lysis-front

In one part of our experiments the plaques were produced with the soft agar-layer technique of Gratia (if not stated otherwise: bouillon agar of 1 per cent). The size of the plaques was determined by means of a measuring microscope with a magnification of 18× provided with a crosswebbed ocular. We measured the plaque diameter with an error smaller than ± 0.01 mm, which means an accuracy of about one order of magnitude greater than that in previous measurements of similar kind (Mayr-Harting, 1958). The Petri-dish was placed in a closed transparent holder which was connected to the outer water circuit of an ultrathermostat ensuring thus continuous reading during the incubation at 37 °C.

In other experiments we employed a special technique, the essence of which is shown in Fig. 1. In this arrangement the lysis-front — viewed from above — appears to be a straight line moving away continuously from the edge of the groove. The progress of a 3 mm long section of the lysis-front was followed under the microscope. The advantage of this method is that the progress of the lysis-front can be treated as a one-dimensional diffusion problem. This method is called further on briefly *one-dimensional technique*.

Results

The second phase of the plaque growth is usually characterized by a constant growth rate. In our experiments with 5×10^7 cm^{-3} bacterial concentration, 1 per cent soft agar concentration and 37 °C incubation temperature we found a growth rate of 0.0065 mm/min. This value means an average growth rate for a longer period of time (more than 10 minutes). On the basis of our more accurate determinations *plaque growth is not continuous but periodically interrupted* (step-like). The same phenomenon can be observed also in the measurements performed with our one-dimensional technique, which is shown in Fig. 2. The "points" in the figure indicate the position of the lysis-front in every minute.

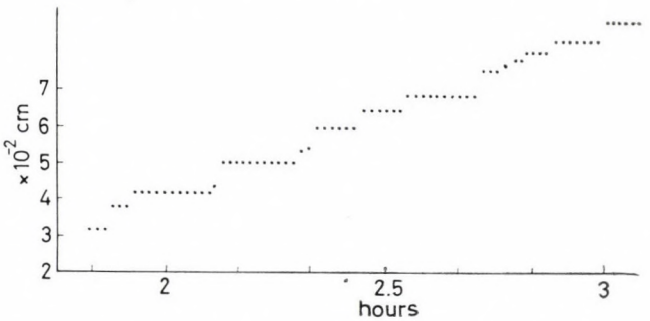


Fig. 2. Step-like growth curve taken at a temperature of 37 °C in the case of 5×10^6 cm^{-3} bacterial colony concentration. The abscissa shows the incubation time and the ordinate the position of the lysis-front

The length of the plateaus illustrates the period during which the position of the lysis-front does not change and the distance between the plateaus (height of the steps) gives the size of the lysis-front-shifts ("steps"). The distance between the end of a plateau and the beginning of the next one represents the time necessary for one "step".

In the following we are going to analyse the average values of the data characterizing the steps. Let τ denote the average time between the beginning (resp. the end) of two neighbouring plateaus (briefly: step-length); h the average height of the steps and τ' the average time necessary for one "step". We examined in our one-dimensional system how the τ', h and τ quantities were influenced by

a) the concentration of the bacteria seeded into the agar-layer

b) the age of the plaque*

c) the concentration of the soft-agar.

We have summarized in Table 1 the average values of our experimental results got from about 30 data in each case. The bacterial concentration (C_b) means the concentration of bacteria seeded into soft-agar and having presumably a statistical distribution. The average distance between the bacteria is character-

* Previously we called plaques only the round holes; for simplicity we are going to speak further on about plaques also in the case of our one-dimensional technique.

ized by $d = 1/\sqrt[3]{C_b}$. The bacteria multiply during the observation lasting for several hours and the progeny of the originally single bacteria bring about micro-colonies. Their concentration is also C_b. We characterize the average distance between the colonies also by the d, defined above, which clearly corresponds to the average distance between the centres of colonies. The value of C_b was changed in our experiments by two orders of magnitude.

Table 1

		0—4 hours		0—2 hours		2—3 hours		3—4 hours	
C_b cm^{-3}	$d\times10^3$ cm	τ min	$h\times10^3$ cm	τ min	$h\times10^3$ cm	τ min	$h\times10^3$ cm	τ min	$h\times10^3$ cm
5×10^8	1.3	6.7	4.3	6.5	4.2	7.1	4.5	6.6	3,9
5×10^7	2.7	8.4	5.3	7.6	5.5	8.5	5.6	9.7	4,4
5×10^6	5.8	8.9	7.0	8.3	6.7	9.4	7.6	9.2	6.7

Values of τ' are not listed in the Table, since τ' does not depend significantly either on the concentration of colonies or on the age of the plaque. According to our measurements: $\tau' \approx 2$ min.

Table 2 contains the values of τ and h given on the basis of about 20 data for different soft-agar concentrations each (37 °C, $C_b = 5\times10^6$ cm^{-3}).

Table 2

Concentration of agar per cent	τ min	$h\times10^3$ cm
0.75	8.4	6.3
1.0	8.9	7.0
1.5	8.1	6.1
2.0	8.3	6.5

We should like to make the following comments on our results:

a) τ approximately corresponds to the minimal latent period of phages ($T \approx 8$ min), and h lies in the range of d.

According to the variance-analysis of the data the values τ as well as h belonging to the different bacterial concentrations are significantly different on a level of 0.1 per cent.

b) The age of plaques does not significantly influence the height of the steps within the observed period. The quotient h/τ shows a decreasing tendency with age, which may be due rather more to the increase of step-length than to the variation of step-height.

c) Variance analysis shows that the concentration of soft-agar influences neither the length nor the height of the steps significantly.

Discussion

1. From our experimental results the following conclusions can be drawn:

a) As the lysis is always preceded by infection one can speak also of an infection-front similarly to the lysis-front. Let us establish the correlation between the lysis-front and infection-front. Our conception is schematically illustrated in Fig. 3. At a given time the lysis-front reaches the position taken T time earlier by the infection-front. (T is the average period of the phage developmental cycle.) Thus the position of the infection-front is obtained by shifting the curve illustrating the lysis-front to the left by a value of T. As the progress of the lysis-front results in the release of new viral-progeny, the appearence of new phages causes the infection-front to shift as a consequence. For this reason the infection-front

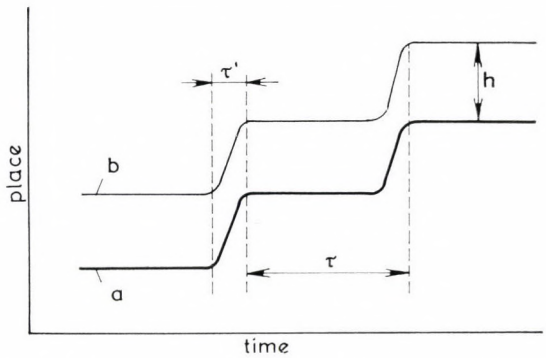


Fig. 3. The position of the lysis-front (a) and the infection-front (b) in function of time

moves forward at the same time as the lysis-front. In this case τ is equal to T and through this statement the length of the steps is interpreted. Besides, our experimental values for τ are in good agreement with our data on the minimal latent period measured in synchronous cultures. The above results imply that the *infectionfront also moves step-like always at a distance h before the lysis-front.*

b) Based on the periodicity in infection we can characterize the distribution of phage concentration in time and space. At the moment of lysis the phage concentration in the region behind the lysis-front must be high enough to infect in a period τ' all bacterial colonies within a distance h. At the same time the concentration should decrease very abruptly at the new lysis-front in order to prevent a considerable infection before the moment of lysis in the newly infected colonies.

2. The hypotheses concerning plaque-growth are so far based on the assumption that phages diffuse freely in the space between bacterial colonies, and interaction between phages and bacteria only arises when they get close to each other on a molecular scale. This condition means in other words that the cross-section of bacteria (bacterial colonies) for phage-capture equals to their mean geometrical cross-section. Let us compare our results with the foregoing assumption.

a) According to the deduction treated above in section 1b the bacteria in a zone of width h must be infected during a time τ'. Supposing that the phages ap-

proach the bacteria by a random movement we tried to estimate the required minimal concentration of phages to ensure infection in a time τ' by at least one phage.

Let r denote the average distance which a phage reaches by diffusion from a given point O during τ' time. It is known that

$$r = \sqrt{2D\,\tau'} \tag{1}$$

(D is the diffusion-constant of the phages). If the phage can get with equal probability to any point of a spherical surface of centre O and radius r, the probability that it reaches a given bacterium is $q/4\,r^2\pi$ (q being the mean bacterial cross-section). Let us denote the concentration of phages in the environment of the bacteria by ρ. It is easy to see that the number of phages equals to $4r^2\,\pi\,\rho\,dr$ in the spherical shell of thickness dr placed at a distance r from the bacterium. As each phage reaches the bacterium with the above mentioned probability, the number of phages getting to the bacterium from the spherical shell during τ' time is $\rho\,q\,dr$. And the number of phages reaching the bacterium during a time τ' from an imaginary sphere of radius r is $\rho\,q\,r$, that is $\rho\,q\sqrt{2D\,\tau'}$. The bacterium is infected within a time τ' if at least one phage gets to the bacterium, i.e.:

$$\rho\,q\sqrt{2D\,\tau'} \geqq 1. \tag{2}$$

Accepting that $q \approx 2\times10^{-8}$ cm^2, $\tau' \approx 2$ min and $D \approx 2\times10^{-7}$ cm^2 s^{-1} (Tamás et al., 1966), (2) is valid if

$$\rho \geq 0.6\times10^{10}\ \text{cm}^{-3}. \tag{3}$$

Hence, in order that the bacteria be infected through random meeting with the phages within $\tau' \approx 2$ min, a phage-concentration of at least 0.6×10^{10} cm^{-3} is needed. In our experiments when $C_b = 5\times10^6$ cm^{-3}, a colony consists of 20 bacteria as an average and the burst size is 150/bacterium, the average phage density is 1.5×10^{10} cm^{-3} in the environment of the lysed colonies. Naturally, a higher concentration of colonies and larger colony size result in a higher phage density. Thus it is possible that the concentration of phages near the lysis-front is high enough to satisfy condition (3). This calculation shows that the first requirement of section 1b) can be satisfied even if we consider the encounter of phages and bacteria to be simply the result of a random-like diffusion process.

b) Let us examine whether the second requirement of section 1b) is satisfied, accepting that the phages *diffuse freely* in the space between bacteria and *interactions only arise within molecular distances*. Let us look at Fig. 4 which indicates the relative concentration of phages (c/c_0) obtained from Fick's law in dependence of the distance measured from the lysis-front. We presume a homogeneous phage distribution in the range of the lysed bacteria; the concentration is c_0. Curve a shows the situation developed in $\tau' = 2$ min, and curve b in $\tau = 8$ min. It is evident that the phage concentration is high enough, even at distances exceeding $h = 4.3\times10^{-3}$ cm, for the infection of even more remote bacteria during the time τ. Thus according to Fig. 4 the lysis-front should move continuously and not step-

like. Consequently the step-like plaque growth cannot be satisfactorily explained by the above idea.

3. We propose the following working hypothesis for the interpretation of our empirical results. Let us assume that in the given medium an *interaction exists* between bacteria and phages *even at distances exceeding molecular distances*, i.e., the phage-capturing cross-section of bacteria is greater than their average geometrical cross-section. The interaction may result from Coulomb-like or electric dipole forces created e.g. by ion-adsorption of micro-organisms from their environment (Mackal, Kozloff, 1954; Christensen, 1965). The phage-capturing

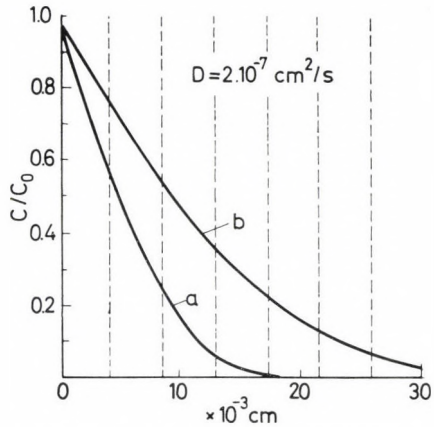


Fig. 4. Relative concentration of phages in function of the distance measured from the lysis-front; a — 2 min after the lysis, b — 8 min after the lysis

cross-section of bacteria (bacterial colonies) is so great that e.g. in the case of the lowest bacterial concentration the phages released from the lysed bacteria can reach only the neighbouring colonies ($h \approx d$), but they cannot cause infection beyond them. The phages surround the neighbouring colonies *in the form of a cloud;* the value of h is determined by the size of the cloud. Within this distance h the concentration of phages is relatively high, but it is negligibly low at distances greater than h until the release of a new generation of phages. Since in the case of the lowest bacterial concentration applied in our experiments $h \approx d \approx 6 \times 10^{-3}$ cm, the cross-section of a bacterial colony is at least $d^2 \approx 36 \times 10^{-6}$ cm^2, i.e. it is at least a hundred times greater than the geometrical cross-section of a colony of medium size. The experience that the phages are able to infect two, even three rows of bacteria in one step in the case of a higher C_b seems to be contradictory to the above hypothesis ($h \approx 2d$, resp. $h \approx 3d$; see Table 1). This fact can be explained in the following way. The cross-section of a cloud of phages developing around a bacterial colony obviously cannot be smaller than a certain value which depends not only on the magnitude of the interacting forces but also on the Brownian movement of phages. We can give an upper limit for the diameter of the cloud (at a temperature of 37 °C) based on the smallest height of steps observed in our experiments; this limit is about 4×10^{-3} cm.

Our working hypothesis is in agreement with our other empirical results. In our opinion the size of h is mainly determined by the size of the phageclouds developed around the colonies, which is influenced by the concentration of colonies only insofar as at the decrease of concentration, i.e. at the increase of d the cloud slightly spreads out. With the growth of the plaque the diameter of the colonies increases and the burst size decreases. The former factor leads to a slight increase of the cloud diameter and the latter one to its decrease. The decrease is presumably greater than the increase and this may explain the decrease in the values of h shown with the increasing age of the plaque (see Table 1). The diffusion constant of phages is decreased approximately to its half value by the increase of agar-concentration from 0.75 to 2 per cent. As diffusion plays a secondary role in the progress of lysis, neither τ nor h changes with the concentration of agar (see Table 2).

According to our informative measurements both τ and h vary sensitively with temperature. If the temperature decreases, τ increases and h diminishes (at 25° C $\tau \approx 12.6$ min, $h \approx 3 \times 10^{-3}$ cm). These results are also in accordance with expectations. Namely the value of τ is determined mainly by the duration of the phage developmental cycle which evidently grows with the decrease of temperature for its value is related to complicated chemical processes. The rapid decrease of h exhibited at the decrease of temperature is probably caused by several factors, e.g. the decrease of burst size (Pollard, Woodyatt, 1964) as well as that of the Brownian movement, etc.

Hereby we thank Mr. M. Herczeg and Mr. Gy. Szabó, our students, for their participation in the experiments.

References

Adams, H. M. (1959) Bacteriophages. Interscience Publ., New York
Christensen, J. R. (1965) Virology *26* 727
Cummings, D. J. (1965) Biochim. Biophys. Acta *85* 341
Cutler, R. G., Evans, J. E. (1966) J. Bact. *91* 469
Herčik, F. (1959) Biophysik der Bakteriophagen. VEB Verl. d. Wiss. Berlin.
Knorre, W. A. (1968a) Biochem. Biophys. Res. Comm. *31* 812
Knorre, W. A. (1968b) Biophysik *5* 24
Koch, A. S. (1964) J. Theoret. Biol. *6* 413
Maaloe, (1962) Synchronous Growth. In: Gunsalus, Stanier: The Bacteria. Vol. 6. Acad. Press, New York
Mackal, R. P., Kozloff, L. M. (1954) J. Biol. Chem. *209* 83
Mayr-Harting, A. (1958) Zbl. Bakt. *171* 380
Pollard, E. C., Woodyatt, S. (1964) Biophys. J. *4* 367
Schwöbel, W. (1965) Arch. f. ges. Virusforsch. *17* 73
Schwöbel, W., Geidel, H., Lorenz, R. J. (1966) Zschr. Naturforsch. *21b* 953
Tamás, Gy., Rontó, Gy., Pozsonyi, T. (1966) Acta Biochim. Biophys. Hung. Acad. Sci. *1* 179

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (3), pp. 313—318 (1969)

Effects of Veratrine and Procaine on Nerve Excitation

G. Biró, K. Gábor

Biophysical Institute, Medical University, Pécs

(Received January 14, 1969)

Experiments were performed on frog's sciatic-gastrocnemius preparation to investigate the effect of veratrine and procaine on nerve excitation. The experimental results demonstrate that, though these two compounds when applied separately give rise to a block of conduction of nerve excitation and to an increase of the threshold, procaine can suspend the effects of veratrine. These results are significant from the point of view of the electron process of the excitation.

Introduction

A nerve treated with veratrine or procaine in suitable concentration loses its ability to conduct excitation, and both substances give rise to an increase of the threshold (Bennett, Chinburg, 1946; Lorente de Nó, 1947; Shanes, 1950, 1951, 1958; Fleckenstein, 1951; Rud, 1961). Nevertheless, according to Shanes' results, these commonly used compounds prevent the repetitive activity brought about by veratrine, and Fleckenstein has shown by means of recording the muscle contraction that a common solution of these compounds suspends the block previously produced by veratrine. To explain the mechanism of action of these two compounds, Ernst (1968) has suggested the possibility of an electron donor–acceptor process.

In the present study the changes of threshold and the conduction of excitation in nerves treated with veratrine and procaine have been investigated from the point of view of the electron process mentioned above.

Methods

The experiments were performed with isolated sciatic-gastrocnemius preparations of the frog (Rana esculenta). The preparations were sustained in a moist chamber containing a small vessel used for soaking the muscle in Ringer solution.

The nerve was connected with three pairs of platinium electrodes of 1 mm diameter. Two pairs of them (I and II) were used for stimulation and one pair (III) was used for recording the action potentials (Fig. 1). An about 1 cm part of the nerve was treated with Ringer solution containing 0.025 per cent of veratrine or 0.25 per cent of procaine. This part of the nerve was between electrodes I and II

in the case of investigation of the blocking effects, and at electrodes I in investigations into changes of the threshold, respectively.

The action potentials recorded on the upper beam of a double-beam oscilloscope were elicited by square-wave stimuli of 2 V and 0.1 ms. The time markers were recorded by extinguishing the lower beam of the oscilloscope. A normal record of the action potentials of the nerve stimulated at electrodes I as well as

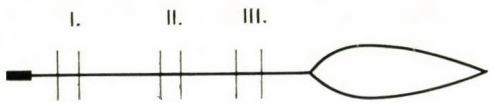


Fig. 1. Sketch of the experimental arrangement. I and II: stimulating electrodes, III: recording electrodes

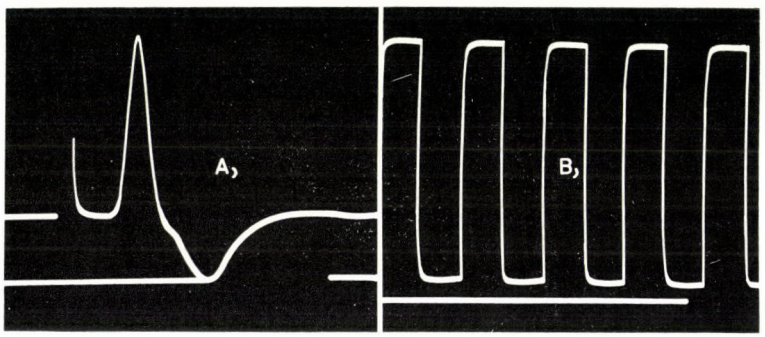


Fig. 2. Action potential of the nerve (A) and calibration impulses of 10 mV and 1000 Hz (B)

calibration impulses of 10 mV and 1000 Hz are shown in Fig. 2. In the experiments examining the blocking effect, the amplification of the oscilloscope was gradually increased up to 16 times higher than that in Fig. 2.

When investigating the changes of threshold, the threshold was defined as the amplitude of a stimulus required to evoke an action potential having an amplitude of 0.05 mV at least. This stimulus was produced by a device which divided the square-wave pulse of 2 V into 520 parts.

About 100 preparations were used in the course of the investigations. The experiments were carried out at room temperature (20 to 25 °C).

Results

Soaking the part of a nerve between electrodes I and II in veratrine-Ringer caused the amplitude of the action potentials evoked at electrodes I gradually to decrease below 0.1 mV. Finally a block of conduction developed under the effect of veratrine. The action potentials evoked at electrodes II did not change substantially (Fig. 3A). Similar results were obtained in experiments performed with soaking solution containing procaine (Fig. 3B). Substituting after development of the blocking effect the veratrine-Ringer for procaine-Ringer led to the reappearance of action potentials of increasing amplitude at electrodes I (Fig. 4).

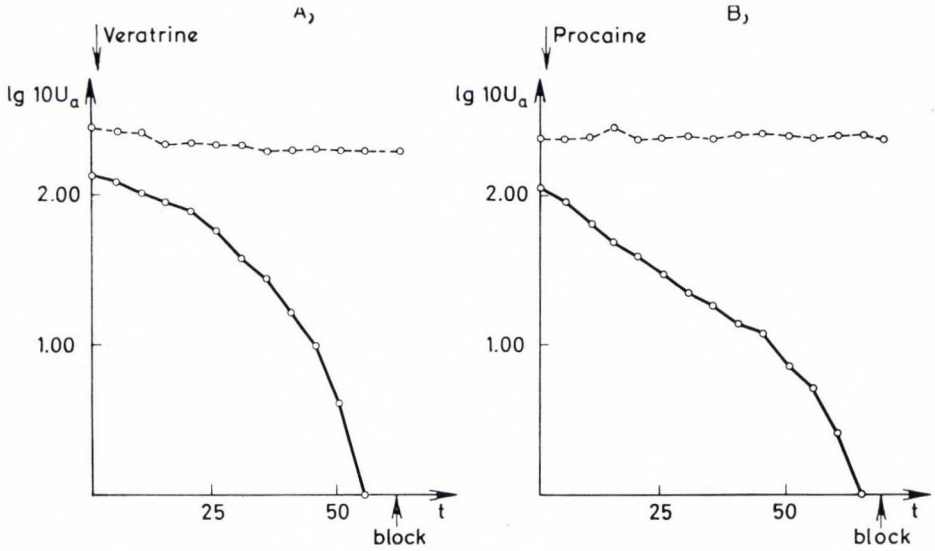


Fig. 3. The change of the amplitudes of the action potentials when the nerve was treated with veratrine (A) and procaine (B) separately. U_a: the pick to pick amplitude of the action potentials in mV; t: time in minute. Full and dotted lines: amplitude of the action potentials evoked at electrodes I and II, respectively

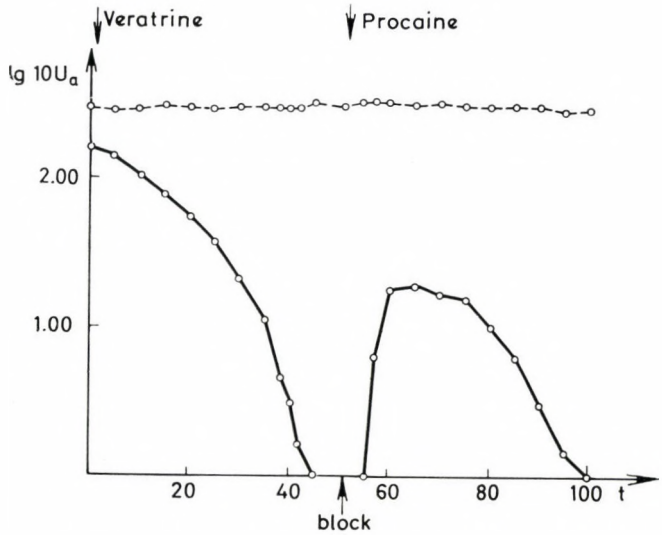


Fig. 4. The change of the amplitudes of the action potentials when the nerve was treated with veratrine and procaine after each other. U_a: the pick to pick amplitude of the action potentials in mV; t: time in minute. Full and dotted lines: amplitude of the action potentials evoked at electrodes I and II, respectively

Another type of the experiments in which the soaked part of the nerve was at electrodes I demonstrated that both veratrine and procaine increased the threshold (Fig. 5A and B) and a threshold of 2 V brought about by veratrine was followed by a decrease of the threshold after substituting the veratrine-Ringer for procaine-Ringer (Fig. 6).

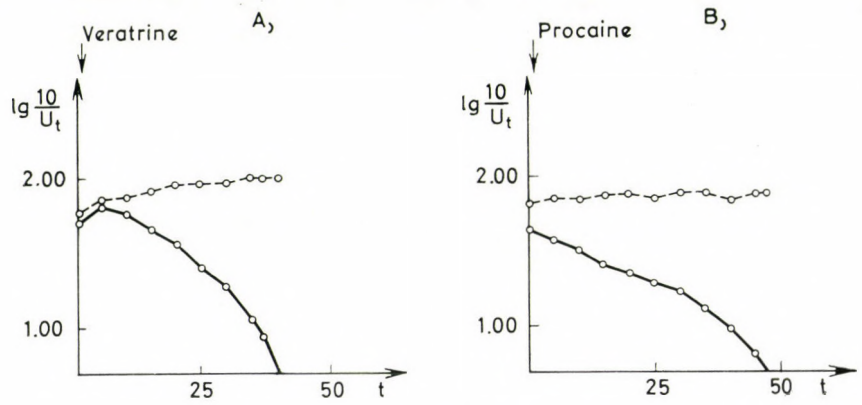


Fig. 5. The change of the threshold of the nerve treated with veratrine (A) and procaine (B) separately. U_t: threshold in V; t: time in minute. Full and dotted lines: threshold at electrodes I and II, respectvely

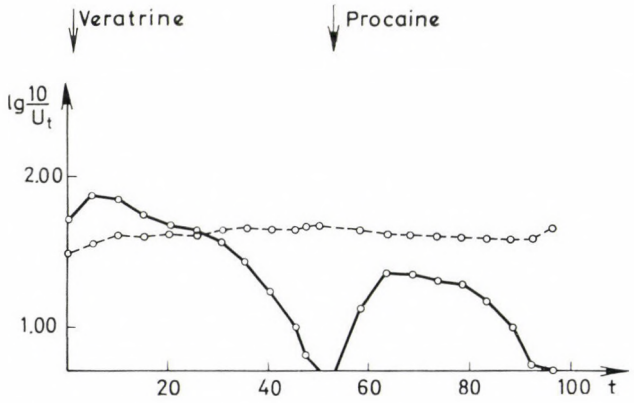


Fig. 6. The change of the threshold of the nerve treated with veratrine and procaine after aech other. U_t: threshold in V; t: time in minute. Full and dotted lines: threshold at electrodes I and II, respectively

In the different experiments the procaine action counteracting the veratrine effects appeared in 4 $\pm$ 2 minutes after the exchange of the solutions.

Control experiments in which the veratrine solution was exchanged for normal Ringer showed that Ringer solution without procaine did not abolish the block and did not decrease the threshold. The time periods during which the nerves were treated with different solutions in different phases of the experiments are shown in the Table 1.

Table 1

Durations of treating the nerves with different solutions

Veratrine in the first phase	61±20 min.
Procaine in the first phase	64±24 min.
Procaine in the second phase	53±18 min.
Ringer in the second phase	49±19 min.

The experiments, in which the procaine solution was used in the first phase of the experiment and then exchanged for veratrine solution, gave results similar to those presented above. However, these results need further examinations because the Ringer solution also suspended the effects of procaine.

Figs 4 and 6 also show that the amplitude of the action potentials and the threshold did not reach their original values after the exchange of the solutions, and that the block as well as the threshold of 2 V developed again. The quantitative aspects of the suspending effect variable in various experiments also deserve further investigations.

Discussion

Although both veratrine and procaine have inhibitory effects on the activity of the nerves, our experimental results shown in Figs 4 and 6 demonstrate that procaine can suspend the effects of veratrine which latter drug causes a block of conduction and an increase of the threshold. Supposing these inhibitory effects exerted on the excitation to be produced by a connection between some substance of the nerve and the easily mobile electrons belonging to these compounds, the suspending effects would be the result of an electron donor–acceptor process taking place between the molecules of veratrine and procaine (Ernst, 1968).

Veratrine is also known for its ability to induce in the nerve (Achenson, Rosenblueth, 1941; Pórszász, 1959) and muscle (Dittler, Tichomirow, 1908; Kuffler, 1945) repetitive responses and to raise the frequency of the miniature end-plate potentials (Hofmann et al., 1966). The antagonism between veratrine and procaine has been demonstrated by experiments in which procaine abolished the repetitive activity produced by veratrine (Shanes, 1950, 1951).

The repetitive response is connected with the electron process of the excitation (Ernst, 1963). Therefore, the examinations of the effects of veratrine and procaine, referring to a donor–acceptor role in the productions of the repetitive response, or in other words, in the transformation of a stimulus into frequent excitation (Ernst et al., 1967; Biró, 1968), are important also from the point of view of electron biology and biocybernetics.

The authors are indebted to Professor E. Ernst for rising the problem and for his encouragement during the work.

References

Achenson, G. H., Rosenblueth, A. (1941) Amer. J. Physiol. *133* 736
Bennett, A. L., Chinburg, K. G. (1946) J. Pharmacol. Exp. Ther. *88* 72
Biró, G. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 107
Dittler, R., Tichomirow, N. P. (1908) Pflügers Arch. *125* 111
Ernst, E. (1963) Biophysics of the Striated Muscle. Publishing House of the Hung. Acad. Sci., Budapest, p. 218
Ernst, E., Gábor, K., Varga-Mányi, P. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 179
Ernst, E. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 115
Fleckenstein, A. (1951) Arch. exp. Path. Pharmacol. *212* 416
Hofmann, W. W., Parsons, R. L., Feigen, G. A. (1966) Amer. J. Physiol. *211* 135
Kuffler, S. W. (1945) J. Neurophysiol. *8* 113
Lorente de Nó, R. (1947) A Study of Nerve Physiology. The Rockefeller Institute for Medical Research *131*, Part I, p, 142
Rud, J. (1961) Acta Physiol. Scand. *51* Suppl. *178*
Pórszász, J. (1959) Acta Physiol. Acad. Sci. Hung. *15* 291
Shanes, A. M. (1950) J. gen. Physiol. *33* 57
Shanes, A. M. (1951) J. Cell Comp. Physiol. *38* 17
Shanes, A. M. (1958) Pharmacol. Rev. *10* 165

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (3), pp. 319—326 (1969)

The Role of K and Ca in the Stimulation Process of Muscle

K. GÁBOR, P. VARGA-MÁNYI

Biophysical Institute, Medical University, Pécs

(Received October 14, 1968, rewritten January 16, 1969)

We have examined how the stimulus-threshold value and the K- and Ca-content, resp., of the frog gastrocnemius muscle change in the case of direct stimulation, depending on the composition of isotonic Ringer's solution perfused through the preparation. During an 8-day perfusion with K-free Ringer's solution the stimulus-threshold value of the muscle increased from 1 V to 14 V, its K-content decreased by 65 per cent and its Ca-content increased. During a 2- to 3-day perfusion with K- and Ca-free Ringer's solution the stimulus-threshold value of the muscle increased from 1 V to 26 V, 50 per cent of the K was washed out, while the Ca-content did not change. During a 2- to 3-day perfusion with Ca-free Ringer's solution the stimulus-threshold value increased from 1 V to 31 V. In our experiments we were looking for a connection between the change of K- and Ca-content resp., and the excitability.

Introduction

Several authors have dealt with the role of K and Ca in connection with the excitability of the muscle. Former experiments show muscle-K to have an important role in the stimulation process of the muscle (literature e.g. Ernst, 1963); recently, a great number of research workers considered calcium to be of great importance (literature e.g. Ernst, 1963; Ernst, Straub, 1968). We examined the role of K- and Ca-lack, resp., in connection with the excitability of the muscle.

Methods

K- and Ca-free isotonic "Ringer's solutions", resp., were perfused through Läwen–Trendelenburg's frog preparations (Rana esculenta). The left common iliac artery of the frog was ligated in each case in order to obtain control data. (The control left gastrocnemius muscle was protected from desiccation by covering it with cotton-wool imbibed with normal Ringer's solution.) Stimulation was made with a special stimulator made for this purpose; one of the stimulating electrodes was placed on the Achilles tendon, and the other more proximal at 4 mm distance from it. The stimulation with square-wave pulses of 50 c/s frequency and of 1 ms duration lasted for 0.2 s. The value of the stimulus threshold was determined with the aid of the contraction just appearing. The total quantity of the solution perfused during the experiment was measured in each case. The experiments were performed

in a cold room at a temperature of about 0 °C. The K- and Ca-content of the muscles was determined by a Zeiss flame-photometer after drying (at 110 °C) and burning the whole gastrocnemius in a combustion furnace at 450 °C.

Isotonic Ringer's solutions of the following composition were used in our experiments: *a)* normal Ringer's solution: 6.6 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$ sicc., 0.2 g $NaHCO_3$; *b)* K-free Ringer's solution: 6.8 g NaCl, 0.2 g $CaCl_2$ sicc., 0.2 g $NaHCO_3$; *c)* Ca-free Ringer's solution: 6.8 g NaCl, 0.2 g KCl, 0.2 g $NaHCO_3$; *d)* combination of K- and Ca-free Ringer's solution: 7.0 g NaCl, 0.2 g $NaHCO_3$, in 1000 ml of bidistilled water each.

Results

1. As our first experimental task we examined how the excitability of the muscle will change in time when normal Ringer's solution is perfused through the preparation for several days. As a result of 12 experiments we found that the 1 V value of the stimulus threshold measured at the beginning of the experiment changed only very slightly in the first days but, at the end, it rose to 4 V. The K-content of the muscle decreased by about 10 per cent; its Ca-content did not essentially change as compared with the control values, also considering the fluid uptake of the perfused muscle (Table 1).

Table 1

Perfusion of a Läwen–Trendelenburg's frog preparation with normal Ringer's solution at a temperature of about 0 °C for 7 to 10 days

Number	Time, days (d)	Stimulus threshold of perfused muscle measured in V		Stimulus threshold of control muscle measured in V		Gastrocnemius muscle			
						K-content mg		Ca-content mg	
		at the beginning	at the end	at the beginning	at the end	perfused	control	perfused	control
1	7	1	4	1	1	3.02	3.51	0.12	0.11
2	7	1	4	1	1	5.13	6.10	0.15	0.13
3	7	1	5	1	1	5.13	5.89	0.15	0.13
4	8	1	4	1	1	4.21	4.32	0.09	0.07
5	8	1	3	1	1	4.43	4.43	0.10	0.09
6	8	1	4	1	1	3.73	4.21	0.12	0.11
7	9	1	3	1	1	3.78	4.05	0.11	0.11
8	9	1	4	1	2	4.48	4.79	0.11	0.12
9	9	1	7	1	2	3.78	3.94	0.12	0.12
10	10	1	4	1	1	4.70	5.35	0.15	0.08
11	10	1	4	1	1	4.32	4.32	0.14	0.09
12	10	1	5	1	2	4.37	5.02	0.10	0.10
		$\overline{V}_p=1.0$	$\overline{V}_p=4.3$	$\overline{V}_c=1.0$	$\overline{V}_c=1.3$	$\overline{K}_p=4.2$	$\overline{K}_c=4.6$	$\overline{Ca}_p=0.12$	$\overline{Ca}_c=0.11$

2a. Thereafter we examined how the excitability and K-content of the muscle will change when K-free isotonic solution is perfused through the preparation (literature e.g. Ernst, 1963). The experimental conditions are the same as those described above with experiments lasting for 5 to 10 days. We found that the 1 V stimulus-threshold value measured at the beginning changed but very slightly in the first (1 to 5) days; at the same time the K-content of the muscle markedly decreased. With K-free Ringer's solution this loss is about 65 per cent of the total muscle-K; the Ca-content (taking also the fluid uptake into consideration) was perhaps a little increased. About 3500 ml of K-free Ringer's solution was perfused through each frog preparation. (The weight of the perfused muscles in wet condition is 2.17 g on the average; that of the control is 1.28 g.) In the later course of the experiment the value of stimulus-threshold rose to 14 V in contrast with the 2 V value of the control muscle (Table 2a).

Table 2a

Perfusion of a Läwen–Trendelenburg's frog preparation with K-free Ringers' solution at a temperature of about 0 °C for 5 to 10 days

Number	Time, days (d)	Stimulus threshold of perfused muscle measured in V		Stimulus threshold of control muscle measured in V		Gastrocnemius muscle			
						K-content mg		Ca-content mg	
		at the beginning	at the end	at the beginning	at the end	perfused	control	perfused	control
1	5	1	3	1	1	1.32	4.27	0.10	0.06
2	5	1	3	1	1	1.28	4.00	0.12	0.06
3	5	1	4	1	1	1.37	4.10	0.14	0.06
4	7	2	15	1	4	2.49	4.59	0.20	0.14
5	7	2	20	1	5	2.43	4.64	0.26	0.18
6	7	1	20	1	1	2.79	5.67	0.23	0.14
7	7	1	13	1	2	—	—	—	—
8	7	1	15	1	6	—	—	—	—
9	7	1	13	1	1	—	—	—	—
10	8	1	7	1	1	—	—	—	—
11	8	1	7	1	2	—	—	—	—
12	8	1	8	1	1	—	—	—	—
13	9	1	20	1	2	0.50	4.32	0.16	0.12
14	9	1	20	1	2	2.21	4.37	0.16	0.09
15	9	1	20	1	2	1.68	3.78	0.15	0.08
16	10	1	20	1	1	1.29	5.22	0.20	0.09
17	10	1	20	1	2	1.94	5.40	0.20	0.12
18	10	1	20	1	2	1.82	5.40	0.21	0.12
19	10	1	15	1	1	1.79	5.08	0.26	0.10
20	10	1	15	1	3	1.32	4.91	0.21	0.11
21	10	1	15	1	5	2.03	4.91	0.23	0.12
		$\overline{V}_p = 1.1$	$\overline{V}_p = 14.0$	$\overline{V}_c = 1.0$	$\overline{V}_c = 2.2$	$\overline{K}_p = 1.75$	$\overline{K}_c = 4.78$	$\overline{Ca}_p = 0.19$	$\overline{Ca}_k = 0.11$

2b. As another part of this series of experiments we examined what will happen when we perfuse normal Ringer's solution through the preparations-after they had been perfused with K-free Ringer's solution. After perfusing about 500 ml of normal Ringer's solution through each frog preparation the stimulus-threshold value decreased, the K-content again increased considerably with no change in the Ca-content (Table 2b).

Table 2b

Continuation of the experiments of Table 2a. Perfusion with normal Ringer's solution; the initial stimulus threshold is the value obtained in experiments 7 to 12 of Table 2a

Number	Time,	Stimulus threshold of perfused muscle measured in V at the end	Stimulus threshold of control muscle measured in V at the end	Gastrocnemius muscle			
				K-content mg		Ca-content mg mg	
				perfused	control	perfused	control
7	5 hours	13	2	—	—	—	—
8	5 hours	13	6	—	—	—	—
12	1 day	8	4	2.91	3.78	0.17	0.10
9	2 days	2	1	4.77	5.13	0.21	0 11
10	4 days	5	1	4.92	5.02	0.21	0.12
11	4 days	5	2	3.60	4.43	0.15	0.11
				$\overline{K_p}=4.02$	$\overline{K_c}=4.59$	$\overline{Ca_p}=0.19$	$\overline{Ca_k}=0.11$

Table 3a

Perfusion of a Läwen–Trendelenburg's frog preparation with Ca-free Ringer's solution at a temperature of about 0 °C for 2 to 3 days

Number	Time, days (d)	Stimulus-threshold of perfused muscle measured in V		Stimulus-threshold of control muscle measured in V	
		at the beginning	at the end	at the beginning	at the end
1	2	1	15	1	1
2	2	1	20	1	2
3	2	1	15	1	1
4	2	1	16	1	1
5	3	1	40	1	2
6	3	1	32	1	2
7	3	1	35	1	1
8	3	1	50	1	1
9	3	1	30	1	2
10	3	1	43	1	1
11	3	1	35	1	1
12	3	1	45	1	1
		$\overline{V_p}=1.0$	$\overline{V_p}=31.3$	$\overline{V_c}=1.0$	$\overline{V_c}=1.3$

Table 3b

Continuation of the last 4 experiments of Table 3a. Perfusion with normal Ringer's solution; the initial stimulus threshold is the value obtained in the last 4 experiments of Table 3a

Number	Time, days (d)	Stimulus threshold of perfused muscle measured in V at the end	Stimulus threshold of control muscle measured in V at the end
9	2	2	2
10	3	2	1
11	4	2	1
12	4	3	2

Table 4a

Perfusion of a Läwen–Trendelenburg's frog preparation with K- and Ca-free Ringer's solution at a temperature of about 0 °C for 2 to 3 days

Number	Time, days (d)	Stimulus threshold of perfused muscle measured in V		Stimulus threshold of control muscle measured in V		Gastrocnemius muscle			
						K-content mg		Ca-content mg	
		at the beginning	at the end	at the beginning	at the end	perfused	control	perfused	control
1	2	1	20	1	1	—	—	—	—
2	2	1	15	1	1	—	—	—	—
3	2	1	20	1	1	—	—	—	—
4	2	1	20	1	1	—	—	—	—
5	2	1	15	1	1	—	—	—	—
6	2	1	17	1	1	—	—	—	—
7	2	1	20	1	1	—	—	—	—
8	2	1	22	1	2	—	—	—	—
9	2	1	27	1	2	—	—	—	—
10	2	1	20	1	2	—	—	—	—
11	2	1	20	1	2	—	—	—	—
12	2	1	25	1	1	—	—	—	—
13	2	1	15	1	1	—	—	—	—
14	2	1	25	1	1	—	—	—	—
15	2	1	9	1	1	—	—	—	—
16	3	1	50	1	2	—	—	—	—
17	3	1	45	1	2	—	—	—	—
18	3	1	40	1	1	—	—	—	—
19	3	1	40	1	2	2.39	5.29	0.11	0.10
20	3	1	40	1	2	2.84	5.18	0.12	0.11
21	3	1	35	1	1	3.01	4.54	0.10	0.09
		$\overline{V}_p = 1.0$	$\overline{V}_p = 25.7$	$\overline{V}_c = 1.0$	$\overline{V}_c = 1.4$	$\overline{K}_p = 2.75$	$\overline{K}_c = 5.00$	$\overline{Ca}_p = 0.11$	$\overline{Ca}_c = 0.10$

3a. In the next series of experiments Ca-free Ringer's solution was perfused through the frog preparation. The initial 1 V stimulus threshold value of the perfused muscle rose to 31 V during the first 3 days of perfusion in contrast with the 1 V stimulus threshold value of the non-perfused control muscle. The quantity of Ca-free Ringer's solution perfused through each frog preparation was 2000 ml (Table 3a).

3b. Then we perfused normal Ringer's solution through a muscle which had been previously perfused with Ca-free Ringer's solution. After the perfusion of 850 ml of normal Ringer's solution the former high stimulus threshold decreased to a value close to that of the control muscle (Table 3b).

4a. In a further series of experiments K- and Ca-free isotonic Ringer's solution was perfused through the right limb of the frog. The experiments were done in a way similar to that already described; the initial 1 V stimulus threshold value of the perfused muscle rose to 26 V within 2 to 3 days. 50 per cent of the muscle-K was washed out, while the Ca-content did not change (Table 4a).

4b. When a muscle which had already been perfused with K- and Ca-free Ringer's solution was perfused again with Ringer's solution containing K but free of Ca, the already increased stimulus-threshold value rose further on (Table 4b).

Table 4b

Continuation of the experiments in Table 4a. Perfusion with Ca-free Ringer's solution containing K. The initial stimulus threshold is the value obtained in experiments number 1 to 6 of Table 4a

Number	Time, days (d)	Stimulus threshold of perfused muscle measured in V at the end	Stimulus threshold of control muscle measured in V at the end
1	2	55	10
2	2	60	2
3	2	60	3
4	3	70	1
5	3	50	1
6	3	65	1

4c. But when a solution which contained Ca but no K was perfused, the stimulus-threshold value decreased to 2 V agreeing with the control value of 2 V (Table 4c).

Table 4c

Continuation of the experiments of Table 4a. Perfusion with K-free Ringer's solution. The initial stimulus threshold is the value obtained in experiments number 7 to 9 of Table 4a

Number	Time, days (d)	Stimulus threshold of perfused muscle measured in V at the end	Stimulus threshold of control muscle measured in V at the end
7	2	2	1
8	2	2	2
9	2	2	3

4d. Similar result was obtained when the muscle was perfused with normal Ringer's solution after having been perfused with K- and Ca-free solution. The stimulus threshold decreased to a value roughly corresponding to that of the control; the already decreased K-content increased again, and the quantity of Ca was somewhat over that expectable on the basis of fluid absorption (Table 4d).

Table 4d

Continuation of the experiments in Table 4a. Perfusion with normal Ringer's solution. The initial stimulus threshold is the value obtained in experiments number 13 to 18 of Table 4a

Number	Time, days (d)	Stimulus threshold of perfused muscle measured in V at the end	Stimulus threshold of control muscle measured in V at the end	Gastrocnemius muscle			
				K-content, mg		Ca-content, mg	
				perfused	control	perfused	control
13	3	2	3	—	—	—	—
14	3	2	2	—	—	—	—
15	3	2	2	—	—	—	—
16	3	5	3	4.16	6.21	0.23	0.13
17	2	3	2	4.91	5.29	0.17	0.10
18	3	3	2	4.37	5.56	0.18	0.10
		$\overline{V_p}=2.8$	$\overline{V_c}=2.3$	$\overline{K_p}=4.48$	$\overline{K_c}=5.68$	$\overline{Ca_p}=0.19$	$\overline{Ca_c}=0.11$

Discussion

In the course of our experiments we wanted to obtain data concerning the role of K and Ca in connection with the excitability of the frog gastrocnemius muscle. According to the experimental data obtained the marked decrease of K, occurring in a large quantity in the muscle, does not play such a great part in the stimulation process of the gastrocnemius muscle as it does in the *automacy of* the frog *heart*.

In the case of a perfusion with K-free Ringer's solution for several days the excitability of the gastrocnemius muscle decreases to a certain degree as compared with the excitability of the control muscle, and the K-content decreases to a remarkable degree. But frog hearts treated with K-free Ringer's solution stopped beating after a certain period of time; they restarted under the effect of Ringer's solution containing K, or under that of mechanic or electric stimuli (Ernst, 1965; Belágyi, Fór, 1966). A similar restarting effect was found with radioactive radiation (Niedetzky, 1966; Hajnal-Papp, Niedetzky, 1967) or with light irradiation of eosine-treated hearts (Lakatos, Kollár-Mórocz, 1966).

When the muscle was perfused with Ca-free Ringer's solution a very great increase of the stimulus-threshold was found already within a rather short time. When the frog preparation was perfused with normal Ringer's solution after it had been perfused by a Ca-free one, the stimulus-threshold value soon decreased again, and the irritability increased almost to its normal degree.

The frog hearts treated with Ca-free Ringer's solution cannot be stimulated electrically (Belágyi, Fór, 1966). Further on, contraction does not appear either with sensibilization with Na-eosine in the case of irradiation by visible light. But some of the frog-hearts restart under the influence of gamma irradiation of ^{60}Co. (Niedetzky, 1967). These experimental results show that there exists an essential difference between the contractility of muscle and the automacy of the heart.

These results call attention to the role of K and Ca in the excitation process of the muscle; according to data obtained up to now *the effect of these two atoms should be examined in their interrelationship and not separately.* This to emphasize again is the chief aim of the article, and that is why mathematical evaluation of the results is left unregarded.

Furthermore, it would be interesting to see how the increased stimulus-threshold would change, if the K- and Ca-free Ringer's solution initially passed through the muscle were afterwards exchanged with Ringer's solution containing radioactive K- or Ca-isotopes.

We thank Professor Ernst for raising the question.

References

Auber, X. (1963) Handb. Exp. Pharm. Ergänzungswerk XVII. 1. Springer. S. 337
Belágyi, J., Fór. E. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 85
Ernst, E. (1963) Biophysics of the Striated Muscle. Publ. House of Hung. Acad. Sci. Budapest
Ernst, E. (1965) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 61
Ernst, E., Straub, F. B. (1968) Symposium on Muscle. Publ. House of Hung. Acad. Sci. Budapest
Hajnal-Papp, M., Niedetzky, A. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 199
Lakatos, T., Kollár-Mórocz, A. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 413
Niedetzky, A. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 299
Niedetzky, A. (1967) Acta Biochim. Biophys. Acad Sci. Hung. *2* 205

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (3), pp. 327–331 (1969)

A Probability Theory Model of Stimulus-Irritation Processes

G. SZÉPHALMI

Institute of Research in Automatization, Budapest

(Received November 1, 1968)

The model discussed does not approach by mathematical formulae results of measurements gained in an experimental way, but it builds up a way of treating of special aspect on the basis of theoretical reflections on the circumscribed sphere of stimulus-irritation processes.

We raise the question of the model as follows: what is the state (that can be represented by a new stimulus threshold value) an excitation-capable biological system (the state of which is characterized by the probability density function of the distribution of stimuli according to their strength and by the actual value of the stimulus threshold) gets into if excited by a stimulus of definite strength?

The model is built up in the first part of this paper; its application — which is the concretization of the general wordings of the model for the given case — is shown in the second part.

1. The Description of the Model

The model of probability theory presented in this communication only comprises those parameters of the stimulus-irritation processes of living systems which are mentioned in the following verbal circumscription; furthermore, this model strives to follow only the changes in the quantitative value of the parameters but, due to the characteristics of the method, it does not include the physico-chemical processes causing these changes.

We can characterize the state of an excitation-capable biological system — which state belongs to a $t = t_1$ moment — unambiguously, without limiting the generality, by the probability density function of the strength of the stimulus — which function is statically characteristic of the system and is designated with $p(i)$ — and by the momentary value of the stimulus threshold which is designated with i_{k1}.[1] We indicate the stimulus affecting the system in the moment $t = t_1$ by the strength of the stimulus designated with i. The state of the system changes to a certain degree, depending on the parameters up to this point, under the effect of the newly arrived stimulus. Its new state is represented again by a stimulus threshold value, designated with i_{k2} in this $t = t_2$ ($t_2 > t_1$) moment. The central problem of the model is the determination of i_{k2}.

[1] The application of the i_{k1} designation instead of the more correct i_{k_1} designation is justified by simplicity. The same is true for the case of i_{k2}.

According to this wording $i_{k2} < i_{k1}$ means excitation, $i_{k2} > i_{k1}$ means inhibition; as special limitation case $i_{k2} = 0$ represents full excitation (i.e. appearance of stimulus) and $i_{k2} = \infty$ represents full inhibition.[2]

The length of the time intervals (t_1, t_2) (in which the physico-chemical processes elicited by the stimulus take place) is uninteresting from the point of view of the model treated here. The further change $(t > t_2)$ of the stimulus threshold value, namely its access (that can be considered exponential) from the i_{k1} value towards the resting stimulus threshold value (Török, Herpai, 1968), is beyond the scope of the present examination.

Starting from the parameters thus for obtained which are considered adequate characteristics of the complex question to be examined, we apply mathematical concepts of general validity for the interpretation of the excitation-capable biological system and for that of the stimulus reaching it. Accepting the "all or none" principle as an approximation, we distinguish two possible outcomes of the phenomenon under examination, according to which the system either gets into a state of excitation by a $t = t_2$ moment, or does not. Before the stimulus exerts its effect, i.e. at the moments $t = t_2$, this question should be considered as an accidental phenomenon and the answer to it can be but one of a possibility character. According to the usual interpretation of the density function of possibility (e. g. Rényi, 1962) the formula designated with P_1[3]

$$P_1 = \int_{i_{k1}}^{\infty} p(i)di \tag{1}$$

arises as a possibility of the occurrence of irritation. As our conditions distinguish only two possible outcomes, the probability of an arriving stimulus not to be followed by excitation is $1-P_1$. Neglecting the problems connected with the transient phenomena of the post-stimulus period we can assume the new stimulus possibly following the stimulus just examined, i.e. that arriving at the moment $t = t_2$, to have a probability of P_2 in a way analogous to (1). P_2 differs from P_1 in the fact that the lower limit of integration is i_{k2}. In the present treatment P_2 is the characteristic of the excitation-capable biological system in the case of the stimulus examined, having already exerted its influence, i.e. at the moment $t = t_2$.

We remark that

$$p(i) = 0 \text{ if } i < 0 \tag{2}$$

should exist as a result of the nature of the problem, and so P_2 adopts values that can be well interpreted even in the above mentioned limitation cases (e. g. $i_{k2} = 0$ would lead to $P_2 = 1$, i.e. the excitation arises independently from the "following" stimulus).

If the possible outcomes of the process and the possibilities of their realization are known one can characterize the state of the system also with an entropy

[2] The use of infinitely great values is an approximation which makes the treating of the subject and the calculations more simple and which does not bring about significant in accuracies.

[3] The sign P_1 is the abbreviation of the $P(i_{k1})$ designation which contains a relation of dependence too.

of probability calculation. Designating the pre-stimulus entropy of probability calculation of the system with H_1 we obtain, according to the usual interpretation

$$H_1 = -P_1 \operatorname{ld} P_1 - (1 - P_1) \operatorname{ld} (1 - P_1) \tag{3}$$

where (1) interprets P_1 and ld designates $\log_2$.[4] H_2, the characteristic parameter of the post-stimulus state of the system can be given similarly by using P_2.

As our aim is not only the characterization of the states of the system, but also characterization of the stimuli, it is suitable to consider (3), at least formally, to be a special application of the entropy of probability calculation, i.e. to be an entropy of information theory instead of being an entropy of probability calculation. So the expression

$$I = H_1 - H_2 \tag{4}$$

as a change of the entropy of information theory under the effect of the stimulus, can be considered as a characteristic parameter of the stimulus. The I, interpreted by (4) is the amount of information given to (or taken from) the system by the stimulus.

We still have to define the quantitative connection between the i strength of the stimulus and the I quantity of information. To do this we must consider that a stimulus of i strength can stimulate a system of $i_{k1} \leqq i$ actual state, and so we can easily accept the expression below as the information-content of a stimulus of i strength, since, in the case of stimulation, $H_2 = 0$;

$$I(i) = H(i_{k1} = i). \tag{5}$$

After this preparation we can word the model as follows: if a system with an i_{k1} actual stimulus threshold is excited by a stimulus of i strength, the system gets into a state which can be characterized by an i_{k2} stimulus threshold value, to which state

$$H(i_{k2}) = H(i_{k1}) - I(i) \tag{6}$$

is valid, where (1), (3) and (5) interpret the H and I functions. If the initial conditions and the strength of the stimulus are given, the new stimulus threshold value — that can be expected according to the model — can be determined from relation (6).

2. A Possible Application of the Model: Summation of Subthreshold Stimuli

The application of the model to a concrete stimulus-excitation process means that the parameters interpreted in the first part are examined within the intervals characteristic of the analyzed phenomenon. The intervals, corresponding to the

[4] The designation H_1, and $H(i_{k1})$ used further on are the simplified versions of the $H[P(i_{k1})]$ designation. The same is true for the case of H_2 and $H(i_{k2})$, resp.

summation of the subthreshold stimuli as a concrete stimulus-excitation process, are:

$$\begin{aligned} &a)\ 0 < i_{k1} < \infty, \\ &b)\ 0 < i \ < i_{k1}, \\ &c)\ 0 < i_{k2} < i_{k1}. \end{aligned}$$

Condition *a)* expresses that the initial state can be arbitrary, except the $i_{k1} = 0$ and $i_{k1} = \infty$ limitation cases (already interpreted before). *b)* is a double criterium again. On the one hand there should be a stimulus ($i \neq 0$), on the other hand it should be a subthreshold one ($i < i_{k1}$). The *c)* inequality is already the result of the first two both in the reality and the model: the stimulus threshold diminishes ($i_{k2} < i_{k1}$), but no stimulus appears ($i_{k2} \neq 0$).

On the basis of the connections of the model

$$\begin{aligned} &a')\ 0 < H_1 \ \leqq 1, \\ &b')\ 0 < I \ < H_1, \\ &c')\ 0 < H_2 \ < H_1 \end{aligned}$$

is equivalent to the definition by *a)*, *b)* and *c)* of the complex of circumstances.

We must concretize the probability density function of the distribution of stimuli according to their strength, as the second level of specialization. It seems to be justified by the thesis of the central limitation value of the probability calculation (e. g. Gnyegyenko, Kolmogorov, 1949) to choose the logarithmic normal distribution. According to this

$$p(i) = \frac{1}{\sqrt{2\pi}\,\sigma i} \exp\left[-\frac{(\ln i - m)^2}{2\sigma^2}\right], \quad i \geqq 0, \tag{7}$$

where m and σ are the expectable value, and the standard deviation, resp., of the normal distribution belonging to the logarithmic normal distribution mentioned here. We do not go into the details of the calculation; they can be done on the basis of relations (1), (3), (5) and (7). Following the examined topic it is suitable to use the expectable value and standard deviation of the logarithmic normal distribution (i.e. the expectable value and standard deviation of the strength of the stimulus) instead of m and σ. Designating these parameters with $\bar{i}$, and s resp., the following relation arises

$$H(i_k) = -\left[\Phi(x)\,\mathrm{ld}\,\Phi(x) + \Phi(-x)\,\mathrm{ld}\,\Phi(-x)\right] \tag{8}$$

where $\Phi(x)$ is the standard normal distribution function and

$$x = \frac{\ln\left(\frac{i_k}{\bar{i}}\right) \cdot \sqrt{\left(\frac{s}{\bar{i}}\right)^2 + 1}}{\sqrt{\ln\left[\left(\frac{s}{\bar{i}}\right)^2 + 1\right]}} \tag{8'}$$

We remark here that statistical methods are known (Aichison, Brown, 1958) to calculate the $\bar{i}$ and s parameters in (8) if we know the actual sequence of stimuli.

Inasmuch the parameters in (8') are known quantitatively too, — this is the third level of specialization — the new stimulus threshold values appearing under the effect of stimuli of different strength can be given numerically by making use of (6) and (8). Fig. 1 shows the dependence of stimulus threshold diminution on the strength of the subthreshold stimulus, in the case of four — arbitrarily chosen — initial states.

We remark only for the sake of perfection, that the fourth level of specialization, through which we reach to a concrete and elementary stimulation event, consists of indicating a point on the set of curves of Fig. 1.

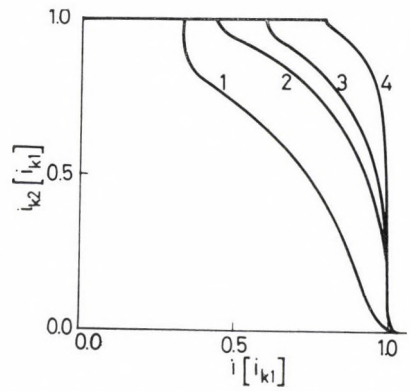


Fig. 1. Dependence of a new stimulus threshold value — appearing under the influence of a subthreshold stimulus — on the strength of the subthreshold stimulus in the case of different initial states. (The values are calculated on the basis of a probability calculation model.) The parameters belonging to individual curves are:

1 $s = 1.0$	$i_{k1} = 0.9\ \bar{i}$;
2 $s = 1.0$	$i_{k1} = 0.7\ \bar{i}$;
3 $s = 0.5$	$i_{k1} = 1.0\ \bar{i}$;
4 $s = 0.5$	$i_{k1} = 0.8\ \bar{i}$.

Finally, we call attention to a phenomenon which comes about from the presented model without a supplementary assumption and which can be seen in the set of curves of Fig. 1: subthreshold stimuli weaker than certain values determined by the initial state of the system are ineffective in the strict sense of the word, i.e. it is not only that they do not cause excitation, but they do not even bring about a diminution of stimulus threshold. To put it in another way: it means that such stimuli, the strength of which is smaller than the value which can be called an absolute stimulus threshold, do not show the phenomenon of summation.

References

Aichison, J., Brown, J. A. C. (1957) The lognormal distribution. Cambridge University Press

Гнеденко, Б. В., Колмогоров, А. Н. (1949) Предельные распределения для сумм независимых случайных величин. Госмехиздат.

Rényi, A. (1962) Wahrscheinlichkeitsrechnung. Deutscher Verlag der Wissenschaften

Török, A., Herpai, S. (1968) Acta Biochim. Biophys. Hung. *3* 452

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (3), pp. 333—335 (1969)

The Action of a Radioprotector (AET) on the Distribution of H-3-Thymidine in Bone Marrow Chromosomes of the Rat

AET-action on the Uptake of Labelled Thymidine by Chromosomes

(Preliminary Report)

J. BRASCH

"Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest

(Received January 11, 1969)

The uptake of H-3-thymidine by chromosomes has been studied in bone marrow cells of S-2-aminoethylisothiourea dihydrobromide (AET)-treated rats. This radioprotector decreased H-3-T uptake to the same extent as did gamma rays; however, without affecting the heterochromatic regions of the chromosomes. A further decrease of DNA synthesis occurred in the AET-treated + irradiated animals. The causes of these differences are being examined.

The uptake of labelled thymidine by animals treated with various sulphydryl containing radioprotective compounds has been studied by many researchers. These earlier studies also revealed the details of the mechanism of action of S,2-amino-ethylisothiourea dihydrobromide (AET). It has been demonstrated that, upon administration of protective agents, the incorporation of labelled thymidine into DNA decreases; at the same time, also the grain count above the nuclei and the mitotic activity decrease (Hell et al. 1960; Bacq, 1965; Sinclair, 1967).

However, no one has so far studied in which way does AET-treatment affect the morphological and functional structure, resp., of the single chromosomes. This still unrevealed point has prompted us to study the distribution of H-3-thymidine in the single chromosomal segments of chromosomes from AET-treated rats.

Ten rats weighing 150 to 200 g were used in the experiments. The animals were divided into 4 groups. One of the rats was given H-3-thymidine alone, three rats only received irradiation, three further rats only AET and the last three animals both AET and irradiation.

AET was administered 15 minutes prior to irradiation in a dose of 200 mg/kg of body weight by the intraperitoneal route.

1 μCi/g of body weight of H-3-thymidine (Amersham, spec. act.: 500 mCi/mM was given to all animals intravenously 10 minutes prior to irradiation. A dose of 100 R Co-60 rays (dose rate 265 R/min) was delivered.

Mitoses were arrested by 100 μg/animal of Colcemid, given two hours after irradiation. The animals were killed two hours later. Accordingly, the cells were exposed to labelled thymidine for a total of 4 hours and 10 minutes. Bone marrow

from both femora was aspired. Following chromosome preparation according to Hungerford and Nowell (1963), the slides were covered by liquid emulsion (Ilford G-5). After exposure for 6 weeks, 150 metaphases were analyzed from each experimental group.

When analyzing the karyograms according to the classification of Hungerford and Nowell (1963) we found no differences between the labelling rates of the individual groups in the case of big size chromosomes; as to the small size chromosomes, the disproportion between the respective sizes of the silver grain and of the chromosome itself renders investigation rather unreliable. Differences were

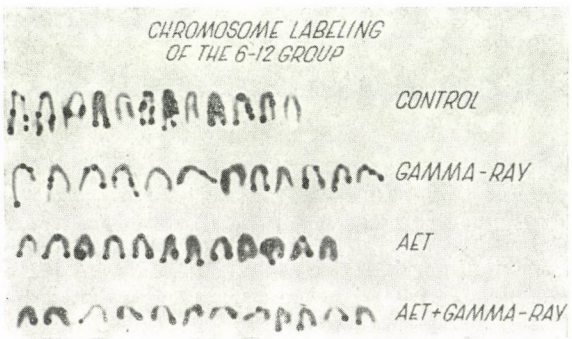


Fig. 1. The distribution of grains in the various experimental groups. (Note that in the AET-treated groups the grains are always located above the ends or centromers)

found in the labelling of the telocentric chromosomes in group 6 to 12. Only this group will be discussed in the following.

No morphological difference was found between the control groups and those only treated with AET. On the other hand, chromatid fragments were as a rule found in the only irradiated and in the irradiated + AET-treated groups. Their number and site displayed a random distribution but they could be demonstrated in all the mitoses (Dubinin, 1965).

In the control group an average of 7 ± 1.4 grains were found per chromosome. On the other hand, two unlabelled chromosomes were found in each case among the chromosomes in group 6 to 12. The 7 grains were distributed at random. However, one was always situated above the centromer and two others above both ends (Fig. 1), resp. In the only irradiated or only AET-treated animals the grain count decreased to 3 ± 0.8 on the average. In the only irradiated group the distribution of these 3 grains was irregular. On the other hand these 3 ± 0.6 grains in the only AET-treated animals were always located above the centromer and above both ends, indicating the sites of DNA synthesis. In the AET-treated + irradiated group the average grain count diminished to 1, and labelling was always found above the intact end or above the centromer. These segments contain the late synthesizing heterochromatic regions. Our observations suggest that AET reduces the metabolic activity of the cells. Thereby it inhibits the cells to enter the S-phase, i.e. that phase of the cycle where DNA synthesis and thus thymidine

incorporation are accomplished, or it delays the accomplishment of DNA synthesis of the cells already in the S phase. Owing to the prolonged cell cycle, 4 hours after the administration of the labelled precursor only those cells were found in labelled mitosis which had been staying at the end of the S phase at the time of H-3-thymidine administration. It is not yet known how the heterochromatin reacts with the radioprotectors. We are carrying out experiments to find out if there is any change in the DNA replication of the heterochromatin.

References

Racq, Z. M. (1965) Chemical protection against ionizing radiation. Springfield, Charles C. Thomas.
Dubinin, N. P. (1965) Doklad. Akad. Nauk. S. S. S. R. *164* 1405
Hell, E., Berry, R. J., Lajtha, L. G. (1960) Nature *185* 47
Hungerford, D. A., Nowell, P. C. (1963) J. Morphol. *113* 275
Sinclair, W. K. (1967) Proc. of Natl. Acad. Sci. *58* 115

Book Reviews

Fortschritte der experimentellen und theoretischen Biophysik. Heft 8. *Biophysikalische Prinzipien der Populationsdynamik in der Mikrobiologie.* Edited by W. Beier. VEB Georg Thieme, Leipzig, 1968, 100 pages. Price: 24.60 MDN

The publication appearing under the editorship of Prof. Dr. W. Beier breaks with its former traditions and aims at making us acquainted with some selected chapters of biophysics in the form of lectures instead of short original communications under a new title ("Fortschritte der experimentellen und theoretischen Biophysik" instead of the old "Physikalische Grundlagen der Medizin — Abhandlungen aus der Biophysik"), taking the rapid development of biological sciences and the new needs brought about by this development into consideration. A guarantee of the realization of the aim at an appropriate level is — beside W. Beier — the staff of the cooperating famous theoretical and experimental biophysicians (L. Bertalanffy, H. Drischel, E. Ernst, H. Pauly, B. Rajewsky, N. Rashevsky).

We are very glad to be able to welcome this intention because it offers a very great help to all those who want to become acquainted with the branches of biophysics rapidly developing at present. This need of cognition is much more inevitable for those who want not only to gather informations and to educate in this field but to contribute to the cultivation of this new area creatively.

In the first lecture of the series D. Noack sets forth the biophysical theories of population dynamics in his theoretical work of 100 pages with 23 figures. The work is divided into nine chapters including the introduction. In the introduction the author circumscribes the question to be examined; the treatment of population dynamics is limited to the field of microbiology. The second chapter briefly surveys the interactions figuring in microorganism populations. A more detailed evolving of the interactions listed here forms a separate chapter of the booklet each.

The third chapter makes known the chemostat, an apparatus suitable for cultivating bacterial colonies under appropriate experimental circumstances; further on, this is the chapter where the basic connections to be used of in the subsequent chapters are introduced.

The fourth chapter deals with certain cases of interaction of indirect character occurring in microbiology; the fifth chapter introduces us to the simplest direct interaction with which the individuals of the population are mutually influenced by each other through the exciting and inhibiting effects induced by metabolic products. It also makes us acquainted with the statistical method with the aid of which the interaction can be analyzed mathematically; the sixth chapter deals with a kind of mixed population model containing two sorts of entities with which the multiplication of one entity is subjected to the metabolic product of the other one; the seventh chapter makes known the so-called robber-prey interaction; the dynamics of the parasite-interaction is examined theoretically in the eighth chapter; and finally, in the ninth chapter the author discusses the influence of the factors of external environment on the behaviour of microorganism populations.

The style of the lecture is clear and simple. It is a special merit of the author that beside the elegant treatment of problems the work can be easily understood from the beginning to the end. The list of references affixed contains about 100 citations which fact helps the work of the reader interested in the details.

J. Belágyi

Symposium on Modern Methods in the Investigation of Protein Structure. Edited by F. B. STRAUB and P. FRIEDRICH. Publ. House of Hung. Acad. Sci. Budapest, 1967. pp. 93

The book covers papers read at the Socialist Countries' Symposium held in Budapest from 6—8th October, 1965. It offers a comprehensive review of topical problems, worded in a rather concise form, and bearing on protein structure research. In spite of the speedy development in the field of protein structure research, the publication has maintained its modern character due to the thorough compilation and the suitable selection of articles with an up-to-date view of the problems dealt with. It contains one article in German and five articles in English. However, handling of this book would be rendered more easy if it contained an Index.

The individual chapters have been written by outstanding experts of the methods dealt with. In general the articles adhered to the common principle of compilation, i.e. the authors present their topic on the basis of their own experimental findings and results, after giving a short review of the pertaining literature.

H. Hanson and S. Fittkau discuss traditional methods of structure investigation by means of exopeptidases. Among these they deal with leucynaminopeptidase which has been isolated by them from bovine lens. W. Mejbaum-Katzenellenbogen offers an insight into a rather privileged and peculiar field of protein chemistry. Starting from the tannin micromethod which has been developed by the author for the determination of protein, he offers information on a series of practical utilizations of reactions involving tannin as well as on the probable mechanism of this reaction. The report of K. Vainshtein closely follows the far-reaching interest of the author. He demonstrates on the basis of his own X-ray diffraction experiments the states of conformation of the monotonous peptid-chain; he reports the results obtained up to 1967 on the field of X-ray diffraction analysis of the atomic structure of globular proteins and some methods of large scale structure investigation of macro-molecules (small angle scattering of X-rays, electronmicroscopy).

In the short chapter dealing with the practical application of optical rotatory dispersion P. Elődi asks what kind of conclusions may be drawn from optical rotatory dispersion studies of peptides and proteins with respect to absolute conformation of peptide chains. In accordance with a number of literary data the author also proves that the method in its present form cannot be applied automatically for the determination of protein helix-contents. In an excellent compilation of the research in the field of amino acid side chain modification of enzymes in the Institute of Biochemistry of the Hungarian Academy of Sciences, G. Szabolcsi presents a thorough discussion of this question (namely, effect of these modifications on the conformation of enzymes).

A. E. Braunstein deals with the most thrilling question of enzyme structure research, namely the structure of the active center as well as the inter-relation of structure and catalytic function. This is the point where enzymology and protein chemistry are most closely related; the various approaches, including methods discussed in the foregoing chapters, are so to say inexhaustible. On the basis of existing data and examples Braunstein analyses the problem with utmost accuracy.

L. GRÁF

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója Műszaki szerkesztő: Farkas Sándor

A kézirat nyomdába érkezett: 1969. VI. 18. — Terjedelem: 10,50 (A/5) ív, 57 ábra

69.67839 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

Reviews of the Hungarian Academy of Sciences are obtainable at the following addresses:

ALBANIA

Ndermarja Shtetnore e Botimeve
Tirana

AUSTRALIA

A. Keesing
Box 4886, GPO
Sydney

AUSTRIA

Globus Buchvertrieb
Salzgries 16
Wien I

BELGIUM

Office International de Librairie
30, Avenue Marnix
Bruxelles 5
Du Monde Entier
5, Place St. Jean
Bruxelles

BULGARIA

Raznoiznos
1, Tzar Assen
Sofia

CANADA

Pannonia Books
2, Spadina Road
Toronto 4, Ont.

CHINA

Waiwen Shudian
Peking
P. O. B. 88

CZECHOSLOVAKIA

Artia
Ve Směčkách 30
Praha 2
Poštovná Novinová Služba
Dovoz tisku
Vinohradská 46
Praha 2
Mad'arská Kultura
Václavské nám. 2
Praha I
Poštová Novinová Služba
Dovoz tlače
Leningradská 14
Bratislava

DENMARK

Ejnar Munksgaard
Nörregade 6
Copenhagen

FINLAND

Akateeminen Kirjakauppa
Keskuskatu 2
Helsinki

FRANCE

Office International de Documentation et Librairie
48, rue Gay Lussac
Paris 5

GERMAN DEMOCRATIC REPUBLIC

Deutscher Buch-Export und Import
Leninstraße 16
Leipzig 701

Zeitungsvertriebsamt
Fruchtstrasse 3—4
1004 Berlin

GERMAN FEDERAL REPUBLIC

Kunst und Wissen
Erich Bieber
Postfach 46
7 Stuttgart S.

GREAT BRITAIN

Collet s Holdings Ltd.
Dennington Estate
London Rd.
Wellingborough. Northants.
Robert Maxwell and Co. Ltd.
Waynflete Bldg. The Plain
Oxford

HOLLAND

Swetz and Zeitlinger
Keizersgracht 471—487
Amsterdam C.
Martinus Nijhof
Lange Voorhout 9
The Hague

INDIA

Current Technical Literature
Co. Private Ltd.
India House OPP
GPO Post Box 1374
Bombay I

ITALY

Santo Vanasia
Via M. Macchi 71
Milano
Libreria Commissionaria Sansoni
Via La Marmora 45
Firenze

JAPAN

Nauka Ltd
92, Ikebukuro O-Higashi 1-chome
Toshima-ku
Tokyo
Maruzen and Co. Ltd
P. O. Box 605
Tokyo Central
Far Eastern Booksellers
Kanda P. O. Box 72
Tokyo

KOREA

Chulpanmul
Phenjan

NORWAY

Johan Grundt Tanum
Karl Johansgatan 43
Oslo

POLAND

Ruch
ul. Wronia 23
Warszawa

ROUMANIA

Cartimex
Str. Aristide Briand 14—18
Bucure,ti

SOVIET UNION

Mezhdunarodnaya Kniga
Moscow G—200

SWEDEN

Almquist and Wiksell
Gamla Brogatan 26
Stockholm

USA

Stechert Hafner Inc.
31, East 10th Street
New York, N. Y. 10003
Walter J. Johnson
111, Fifth Avenue
New York, N. Y. 10003

VIETNAM

Xunhasaba
19, Tran Quoc Toan
Hanoi

YUGOSLAVIA

Forum
Vojvode Mišića broj 1
Novi Sad
Jugoslovenska Knjiga
Terazije 27
Beograd

45,— Ft

Contents

6. XI. 1969 Index: 26.030

VOLUME 4
NUMBER 4
1969

Acta biochimica et biophysica

ACADEMIAE SCIENTIARUM HUNGARICAE

AKADÉMIAI KIADÓ, BUDAPEST

Acta Biochimica et Biophysica

Academiae Scientiarum Hungaricae

Szerkeszti:

STRAUB F. BRUNÓ és ERNST JENŐ

Technikai szerkesztők:

SAJGÓ MIHÁLY és NIEDETZKY ANTAL

Szerkesztőség címe: Budapest XI., Karolina út 29. (biokémia);
Pécs, Rákóczi út 80. (biofizika)

Az *Acta Biochimica et Biophysica*, a Magyar Tudományos Akadémia idegen nyelvű folyóirata, amely angol nyelven (esetleg német, francia vagy orosz nyelven is) eredeti tanulmányokat közöl a biokémia és a biofizika — fehérjék (struktúra és szintézis), enzimek, nukleinsavak, szabályozó és transzport-folyamatok, bioenergetika, izom-összehúzódás, radiobiológia, biokibernetika, funkcionális és ultrastruktúra stb. — tárgyköréből.

A folyóirat negyedévenként jelenik meg, a négy füzet évente egy kb. 400 oldalas kötetet alkot. Kiadja az Akadémiai Kiadó.

Előfizetési díja belföldre 120,— Ft, külföldre 165,— Ft kötetenként. Megrendelhető az Akadémiai Kiadónál (Bp. V., Alkotmány u. 21), a külföld részére pedig a Kultúra Könyv és Hírlap Külkereskedelmi Vállalatnál (Budapest I., Fő u. 32).

The *Acta Biochimica et Biophysica*, a periodical of the Hungarian Academy of Sciences, publishes original papers, in English, on biochemistry and biophysics. Its main topics are: proteins (structure and synthesis), enzymes, nucleic acids, regulatory and transport processes, bioenergetics, excitation, muscular contraction, radiobiology, biocybernetics, functional structure and ultrastructure.

The Acta Biochimica et Biophysica is a quarterly, the four issues make up a volume of some 400 pp, per annum. Manuscripts and correspondence with the editors and publishers should be sent to

Akadémiai Kiadó, Budapest 24, P.O.B. 502.

The subscription rate is 165,— Ft per volume. Orders may be placed with *Kultúra* Trading Co. for Books and Newspapers (Budapest 62, P.O.B. 149) or with its representatives abroad, listed on p. 3 of the cover.

Acta Biochimica et Biophysica — журнал Академии Наук Венгрии, публикующий на английском языке (возможно и на немецком, французском и русском языках) оригинальные статьи по проблемам биохимии и биофизики — белков (структура и синтез), энзимов, нуклеиновых кислот, процессов регуляции и транспорта, биоэнергетики, мышечного сокращения, радиобиологии, биокибернетики, функциональной структуры и ультраструктуры и т. д.

Журнал выходит ежеквартально, выпуски каждого года составляют том объемом около 400 страниц. Журнал выпускает Издательство Академии Наук Венгрии.

Рукописи и корреспонденцию просим направлять по следующему адресу:

Akadémiai Kiadó, Budapest 24, P.O.B. 502.

Подписная цена — 165 Фт за том. Заказы принимает:

Предприятие по внешней торговле книгами и газетами «Kultúra» (Budapest 62, P.O.B. 149) или его заграничные агентства.

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (4), pp. 339—348 (1969)

Regulation of the Transfer of Amino Acids to Ribosomes by Testosterone in the Seminal Vesicle of the Rat

M. TÓTH, R. MACHOVICH

Institute of Medical Chemistry, University Medical School, Budapest

(Received May 30, 1969)

The high speed supernate of the homogenized seminal vesicle of the rat stimulates the transfer of ^{14}C-labelled amino acids from aminoacyl transfer RNA into protein in a system containing rat liver polysomes. This activity of the vesicular supernate is decreased after castration. After replacement of testosterone a lag period of about 12 hrs can be observed which is followed by a progressive increase in the amino acid transferase activity of the vesicular supernate.

Among the several steps involved in the incorporation of an amino acid into the polypeptide chain two consecutive processes can clearly be differentiated: First, the amino acid is coupled with the specific transfer RNA molecule and this is followed by the transfer of the aminoacyl moiety from aminoacyl-tRNA to peptidyl-tRNA. The first process takes place in the cell sap while the site of the second is on the ribosome-messenger RNA complex. Experiments with high speed supernatant fractions of rat liver homogenates have revealed the presence of enzymatic factors for both reaction series (Hoagland et al., 1958; Grossi, Moldave, 1959; Nathans, Lipmann, 1960; Takanami, 1961; Fessenden, Moldave, 1962, 1963).

We have reported that acceleration of protein synthesis induced by testosterone in the seminal vesicle of castrated rats is accompanied by an increase in the levels of various amino acid activating enzymes (Tóth, 1968; Tóth, Mányai, 1968). Recent data, especially in relation to some differentiation processes and to the mechanism of action of certain hormones (Tata, 1966; Gross, 1968) have suggested that the translation process may also be involved in the regulation of the rate of protein synthesis in the vesicles. It was of interest therefore to investigate the activity of the transfer enzymes in the course of the testosterone induced growth and differentiation of this male accessory sexual gland.

In the experiments presented in this paper the high speed supernates of the homogenized seminal vesicles of normal and castrated rats and of testosterone

Abbreviations used:

ATP — Adenosine 5' triphosphate; GTP — Guanosine 5' triphosphate; RNA — Ribonucleic acid; DNA — Deoxyribonucleic acid; tRNA — transfer ribonucleic acid; TCA — Trichloro-acetic acid; EDTA — Ethylene-diamine-tetraacetate

treated castrates were assayed in a rat liver polysome system for their stimulatory activity in the aminoacyl transfer reaction.

A preliminary report on some of these experiments has been published (Tóth, Machovich, 1967).

Materials and Methods

Adult male rats of 200—300 g body weight obtained from the National Fodor Sanatorium (Budapest) were used. The animals were fed on a normal laboratory diet and were supplied with water ad libitum. Castration was performed *via* the scrotal route, under ether anaesthesia. Testosterone phenylpropionate (Retandrol, Richter, Budapest) was administered in the form of intraperitoneal injections, usually in a single dose of 5 mg.

Animals were killed by a blow on the head, the seminal vesicles were removed and collected in an ice cold isotonic NaCl solution. The pooled seminal vesicles were freed from the coagulating gland and adhering secretory fluid. The glands were then blotted, weighed, chopped with scissors and about 1 gram of the wet vesicular tissue was homogenized in a Potter-Elvehjem all glass homogenizer with 10—12 ml of an ice cold medium containing 2 mM mercaptoethanol, 1 mM EDTA and 20 mM Tris-HCl, pH 7.6. Finally the homogenate was spun down at 150 000 *g* for 60 min in the No. 50 rotor of the Spinco Model L-2 ultracentrifuge. The supernatant was used for transfer determinations. In each experiment freshly prepared supernates were assayed.

The assay of aminoacyl transfer into material resistant to hot 10 per cent TCA was performed using a rat liver polysome system. In order to prepare polysomes about 80 g of liver tissue was homogenized in the Waring blender with 160 ml ice cold 0.33 M sucrose containing 0.1 M KCl. After centrifugation at 10 000 *g* for 15 min the supernatant fluid was adjusted to 0.002 M with respect to Mg acetate and ten minutes later one tenth volume of 5 per cent deoxycholate was added. The deoxycholate-treated supernate was centrifuged at 150 000 *g* for 60 min and the sedimented ribosomes were suspended in a 0.33 M sucrose, 0.002 M Mg-acetate solution. The washing with 0.5 per cent deoxycholate was repeated and the ribosomes were suspended again in sucrose-Mg acetate medium. To this mixture 4 M NH_4Cl was added to attain a final concentration of 0.5 M and the ribosomes were pelletted by centrifuging at 150 000 *g* for 90 min. Finally the ribosome pellet was suspended in 0.33 M sucrose containing 0.002 M Mg-acetate.

An aliquot of the ribosome suspension was layered on top of a 25 ml linear gradient (10 to 40 per cent w/w sucrose in 0.025 M KCl, 0.005 M Mg-acetate, 0.02 M Tris-HCl, pH 7.6 solution). The tubes were centrifuged in a Spinco No. SW 25 rotor at 22 500 r.p.m. for 2 hrs. The distribution of ribosomes in the sucrose gradient was determined by using an ISCO model D density gradient fractionator. Collection was made from the top of the tubes at a syringe speed of 2 ml/min and with continuous recording at a wavelength of 254 mμ.

For full activity in the aminoacyl transfer system the ribosomes needed GTP, mercaptoethanol, inorganic ions and transfer enzymes of mammalian origin. If kept frozen the ribosomes retained their activity for about one month.

Transfer RNA was isolated from Escherichia coli B cells by the method of v. Ehrenstein and Lipmann (1961) or from yeast as described by Zubay (1966). E. coli transfer RNA's were charged with the amino acids of a ^{14}C-labelled algal hydrolysate, yeast transfer RNA was charged with ^{14}C-valine and other 19 unlabelled amino acids, as described by Zabos et al. (1967). The preparations were lyophilized unless used immediately, and stored in sealed vials at —20°C.

The polysome system for transfer assay contained in 0.5 ml final volume: 0.3—0.5 mg ribosomal protein, 0.4—0.6 mg ^{14}C-aminoacylated tRNA (30 000—70 000 c.p.m.), 0.5 μmole ATP, 0.1 μmole GTP, 6 μmoles Mg acetate, 5 μmoles mercaptoethanol, 25 μmoles KCl, 25 μmoles NH_4Cl, 25 μmoles Tris-HCl, pH 7.8 and vesicular supernatant protein as indicated. Compared to the RNA content of the vesicular supernate tested for aminoacyl transferase activity, the amount of the exogenous tRNA was at least in 50-fold excess in the assay mixtures.

Controls without added vesicular supernate were also included in each experiment. The reaction was started by the addition of the charged tRNA after a 2 min preincubation. Incubations were carried out at 37°C for 15 min unless otherwise stated. The reaction was stopped by precipitating proteins and nucleic acids with 2 ml of 10 per cent TCA. RNA was extracted from the precipitated material by 10 per cent TCA at 90°C for 15 min. Following successive washings with cold TCA and 96 per cent ethanol, the protein precipitate was suspended in distilled water, layered on aluminium planchets and dried under an infrared lamp. The amount of plated protein was weighed directly. The radioactivity of the samples was measured in a Friesecke—Hoepfner methane gas flow counter.

Specific radioactivities were calculated, unless otherwise stated, as counts/min/mg ribosomal protein.

Protein contents were determined with the Lowry method (Lowry et al., 1951), horse serum albumin served as a standard.

DNA was separated from RNA by the procedure of Schmidt and Thannhäuser (cf. Leslie, 1955) and the DNA content was estimated according to Dische (1955).

Acetyl phosphate was prepared according to the method of Stadtman (1957).

Mercaptoethanol was purchased from Fluka A.G.

^{14}C-labelled algal hydrolysate (214 mCi/g), (U-^{14}C)-L-valine (90 mCi/mmole), and (U-^{14}C)-L-lysine (91 mCi/mmole) were the products of the Institute for Research, Production and Utilization of Radioisotopes, Prague. Crystalline ATP and GTP as Na salts and other chemicals were obtained from Reanal (Budapest).

Results

Characterization of the transfer system

Rat liver ribosomes prepared as described in Materials and Methods were analyzed for polysome distribution with sucrose density gradient centrifugation. Fig. 1 shows a typical sedimentation profile of the preparations: high percentage of the ribosomes exists in the polymeric form.

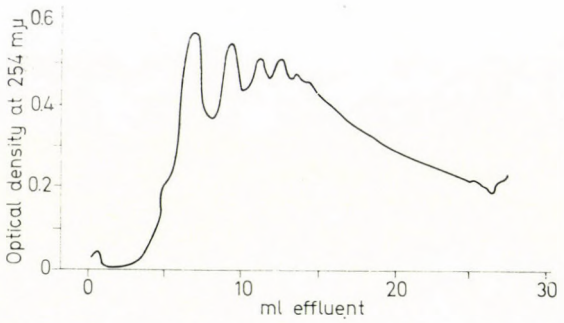


Fig. 1. Sedimentation profile of rat liver ribosomes used for transfer assay. Analysis was performed as described in Methods. Monitoring of absorbancy started at the top of the tube

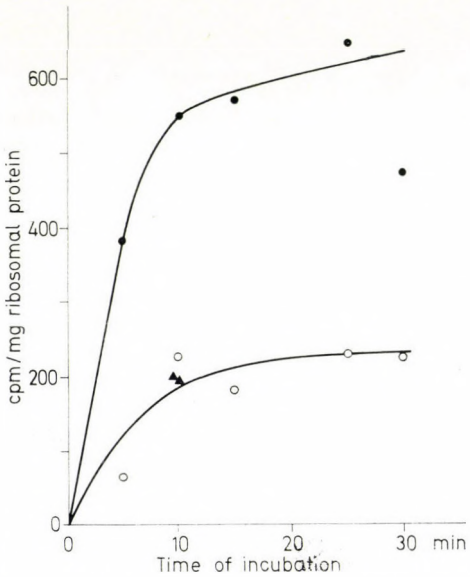


Fig. 2. Catalysis of transfer of amino acids to ribosomes by the vesicular supernate. Reaction mixtures contained in 3.0 ml final volume: 1.8 mg ribosomal protein; 3.0 mg yeast transfer RNA charged with ^{14}C-valine (37 500 c.p.m.) and additional unlabelled amino acids; 3.0 μmoles ATP; 0.6 μmole GTP; 30 μmoles mercaptoethanol; 150 μmoles KCl; 150 μmoles NH_4Cl; 36 μmoles Mg acetate; 200 μmoles Tris-HCl (pH 7.6) and 720 μg vesicular supernatant protein from 5 normal rats. At the points indicated 0.5 ml of the incubation mixture was pipetted into ice cold 10 per cent TCA. Determination of radioactivity resistant to hot acid was carried out as described in Methods. Filled circles: incubation with vesicular supernate. Open circles: incubation without supernate. Triangles: incubation with a supernate (180 μg protein in 0.5 ml reaction mixture) stored at 2° C for a week prior to the experiment

Freshly prepared, unfractionated high speed supernate of the homogenized seminal vesicles was used as a transfer enzyme preparation. This simple extraction procedure was preferred to minimize a differential loss of enzyme activity in various preparations. Storage of the supernate for a week at 2°C completely de-

stroyed its activity in the aminoacyl transfer system (Fig. 2). In addition to the ribosomes the assay mixtures contained optimal amounts of Mg ion and other inorganic ions and they were repleted with charged transfer RNA, ATP and GTP.

Some residual aminoacyl transferase activity of the polysome preparations showed that the removal of endogenous transfer factors was incomplete. However, the addition even of minor amounts of high speed supernate stimulated the rate of the transfer of amino acids to ribosomes. Conditions were established where this stimulatory activity was proportional to the amount of the supernatant protein added.

Partial characterization of the system (Table 1) revealed that the rate of the aminoacyl transfer was increased by the vesicular supernate even if GTP, ATP or the inorganic ions and mercaptoethanol were omitted from the system. The increase in these cases was about half as much as in the presence of the above compounds. This fact suggested that these components were partially replaced by similar factors present in the supernate. Residual activity of the polysome preparation was significant in this case, nevertheless, the amount of radioactivity transferred in the presence of high levels of supernatant protein showed that aminoacyl transfer could be increased nearly sixfold over the "background incorporation".

Table 1

Partial characterization of the aminoacyl transfer system

The vesicles and the livers of six normal rats were used. The complete system contained 315 μg ribosomal protein, 400 μg of E. coli tRNA charged with a mixture of ^{14}C-labelled amino acids (68 000 c p.m.) and the other components as described in Methods. Incubations were carried out at 37°C for 15 min

	counts/min/mg ribosomal protein
complete system (without supernate)	1060
+ supernate from liver (2 mg protein)	5940
+ vesicular supernate (370 μg protein)	3000
+ vesicular supernate, -ATP, GTP	2130
+ vesicular supernate, -Mg acetate, KCl, NH_4Cl, mercaptoethanol	2125

It was shown that the mixed vesicular supernates of normal and castrated rats as well as the mixed supernates of castrated and testosterone treated animals had an additive stimulatory effect on the rate of the transfer reaction. When the vesicular supernate was kept, however, for 5 min in a 90°C water bath, this stimulatory effect was completely abolished.

The time course of the aminoacyl transfer was studied (Fig. 2). The rate of transfer was found to fall rapidly during incubation at 37°C. The early decline in the rate of transfer is due largely to loss of amino acids from tRNA. When continuous generation of aminoacyl tRNA's was promoted during the incubation, a linear kinetics of amino acid incorporation into protein was obtained, at least up to a 30 min incubation period (Fig. 3).

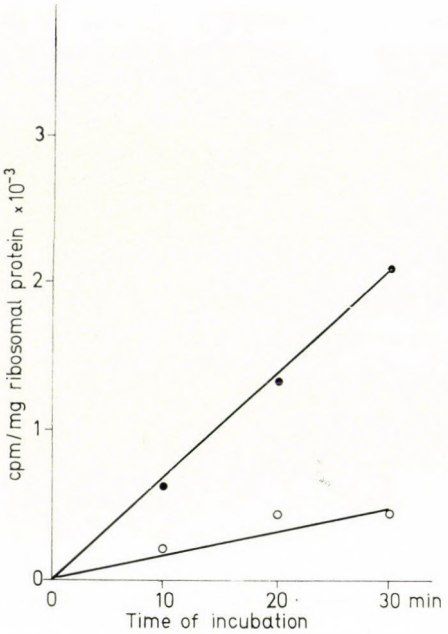


Fig. 3. Stimulation of amino acid incorporation into protein by the vesicular supernate. Aliquots of vesicular high speed supernate from six normal rats (180 μg protein), E. coli 105 000 *g* supernate (200 μg protein), 800 μg acetyl phosphate, 150 μg E. coli carrier RNA, 1—1 μCi ^{14}C-valine and ^{14}C-lysine, additional unlabelled amino acids and the other components as described in Methods were incubated at 37°C with rat liver ribosomes (275 μg protein). Filled circles: vesicular supernate added. Open circles: without addition of supernate

Effects of castration and testosterone treatment

In order to demonstrate the alterations caused by castration and subsequent testosterone administration in the ability of the vesicular supernate to catalyze the transfer of amino acids to ribosomes, the supernates prepared from the seminal vesicles of normal and castrated animals and from testosterone treated castrates were tested for transferase activity (Fig. 4). Although the rate of transfer did not show a linear relationship with time of incubation, conditions could be established where the amount of radioactivity transferred within a 15 min incubation was fairly linear when plotted against the amounts of supernatant protein added to the assay mixtures.

These data indicated that transferase activity relative to the protein content of the supernate was decreased following castration, while testosterone injection to castrates induced a marked increase within 48 hrs.

In the next series of experiments devoted to the study of early effect of testosterone on the vesicular transfer enzymes, the results were related to 1 mg DNA of the original homogenate (Fig. 5). It had been assumed that DNA content is roughly in direct proportion to the number of cells in the tissue. In this way, therefore, a relative change of transfer enzyme activity in the vesicular cells could be estimated. It has been found that after testosterone administration to castrated

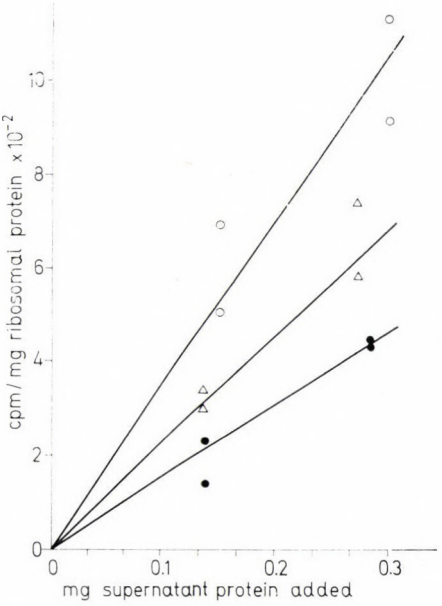


Fig. 4. Dependence of the rate of aminoacyl transfer on the amount of supernatant protein Twelve rats castrated 7 days earlier and 5 normal litter mates were used. Five castrated animals were intraperitoneally injected with 5 mg testosterone phenylpropionate two days prior to killing. Incubation mixtures contained 350 μg ribosomal protein, 600 μg E. coli transfer RNA charged with a mixture of ^{14}C-labelled amino acids (30 000 c.p.m.), and the other components as described in Methods, in a volume of 0.5 ml. Incubations were carried out at 37°C for 15 min. Assays without supernate: 193 and 207 counts/min/mg ribosomal protein (mean value: 200 was subtracted). Open circles: supernate from normal rats. Filled circles: supernate from castrates. Triangles: supernate from castrates injected with testosterone

rats no change in the total amount of DNA per seminal vesicle can be detected until 48 hours, when a small increase is evident (Wicks, Villee, 1964; Tóth, 1968a).

A typical experiment is shown by Fig. 5. Adult rats were castrated a fortnight prior to the experiment. Three groups of 4—7 castrated rats each received testosterone injections at different time intervals before killing. High speed supernates were prepared from the pooled seminal vesicles of each group of similarly treated animals as well as from the vesicles of the castrates. Intact litter mates of the castrated rats were the source of a control enzyme preparation. Supernates were assayed simultaneously and with each preparation 3 parallel incubations were made. Results calculated as counts/min/mg ribosomal protein were corrected for background incorporation and related to 1 mg DNA of the homogenate serving as the source of the supernate.

Subsequent to testosterone injection to castrated rats a lag period of about 12 hours could be observed followed by a progressive rise in the amount of radioactivity transferred to the ribosomes. As a result of a two days action of testosterone *in vivo*, the transferase activity of the vesicular supernate was approaching the normal value.

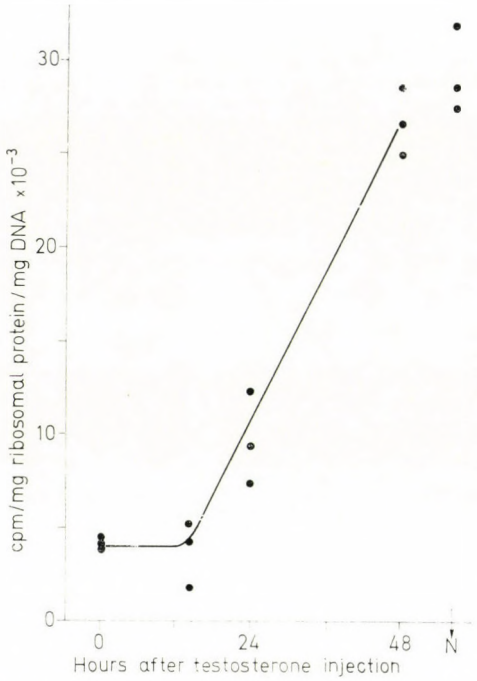


Fig. 5. Decrease in aminoacyl transferase activity of the vesicular high speed supernate following castration and the increase of the same activity after replacement of testosterone. Castration was performed two weeks before the experiment. Supernates were prepared from 4—6 rats per group. Animals (three groups) were given 5 mg testosterone phenylpropionate intraperitoneally at various intervals prior to killing. The assay system contained 380 μg ribosomal protein, E. coli soluble RNA charged with the amino acids of a ^{14}C-labelled algal hydrolysate (30 000 c.p.m.), vesicular supernatant protein (175 to 370 μg) and the other components as described in Methods, in a volume of 0.5 ml. The tubes were incubated at 37°C for 15 min. Background incorporation (210 c.p.m. per mg ribosomal protein) had been subtracted and figures were related to 1 mg DNA of the original homogenate. Each point corresponds to a separate determination. Values obtained with supernates from normal rats are marked by N on the abscissa

Discussion

According to the experiments presented in this paper the high speed supernate of the vesicular homogenate has a catalytic effect on the incorporation of labelled amino acids from aminoacyl-tRNA's into protein in a system containing rat liver polysomes. In the experiments the easily available E. coli and yeast tRNA's were used. There is ample evidence at present that aminoacyl transferring enzymes do not show species specificity toward the aminoacyl-tRNA's but considerable specificity of that kind exists with respect to the ribosome. In line with this, aminoacylated carrier RNA's from E. coli and yeast were readily utilized for protein synthesis by polysome systems of mammalian origin (Nathans, Lipmann, 1960, 1961; v. Ehrenstein, Lipmann, 1961; Lamfrom, Squires, 1962; Rendi, Ochoa, 1962; Okamoto, 1964).

Denaturation of proteins in the vesicular supernate by heating or storage of the supernate at 2°C for a week completely abolish its catalytic activity. The presence of GTP, ATP, mercaptoethanol and certain inorganic ions promote the transfer reaction, which is consistent with the requirements described recently for the action of purified mammalian transfer enzymes in aminoacyl transfer systems (Sutter, Moldave, 1966; Ibuki, Moldave, 1968). All these findings support the assumption that the vesicular transfer enzymes are responsible for the catalytic activity observed with the vesicular supernate in our system.

Experiments reported in the past few years pointed to the translation of messenger RNA as a possible level for action of certain hormones (Rampersad, Wool, 1965; Garren et al., 1965; Gorski, Padnos, 1965; Korner, Gumbley, 1966; Garren et al., 1967). The activity of transfer enzymes appears to be involved in the alterations induced by hydrocortisone (Breuer, Davies, 1963), ACTH (Farese, 1965; Scriba, Reddy, 1965) and thyroxine (Unsworth, Cohen, 1968) in the respective target organs. It is now widely held that the action of sexual steroid hormones on the rate of protein synthesis in the male accessory sexual glands and in the uterus is mediated by their primary action on RNA synthesis (Williams-Ashman, 1965; Liao et al., 1966; Ching-Sung-Teng, Hamilton, 1967). The regulation of the aminoacyl transferase activity in these organs has not yet been studied, however.

According to the experiments described in this paper the decrease of the transfer enzyme activity in the vesicular supernate is greater following castration than that of the soluble protein content of the vesicles. On the other hand, after testosterone injection to castrated rats the specific transferase activity (i.e. enzyme activity per mg protein) increases in the vesicular supernate.

The time course of the early change of vesicular transferase activity in castrates subsequent to the injection of testosterone is analogous to the response of the amino acid activating enzymes in this tissue to androgens (Tóth, Mányai, 1968). These activities, as related to the number of cells (per mg DNA) begin to increase progressively after a lag period of about 12 hours. In addition, the start in the increase of the transferase activity takes place together with an abrupt rise in the rate of protein synthesis (Tóth, Mányai, 1968). Compared to this rise the increase in the activity of the transfer enzymes is relatively slow which suggests that the levels of these enzymes are being altered in the vesicular cells and activation of these enzymes, presumably, does not play a significant role in their regulation. The observation concerning an additive transferase activity of mixed vesicular supernates is in good agreement with this suggestion.

With respect to the primary action of testosterone in the vesicular cells the increase in the activity of the soluble transfer enzymes appears to be a secondary phenomenon that is due to the accelerated protein synthesis. The possibility is not excluded, however, that the increase in the activities of the transfer enzymes as well as the amino acid activating enzymes may have a bearing on the regulation of function of the protein synthesizing machinery in this gland.

The authors wish to thank Prof. F. B. Straub for his interest and valuable suggestions.

References

Breuer, C. B., Davies, F. F. (1963) Biochim. Biophys. Acta *76* 650
Ching-Sung-Teng, Hamilton, T. H. (1967) Biochem. J. *105* 1101
Dische, Z. (1955) in The Nucleic Acids (Eds: Chargaff, E. Davidson, J. N.), Acad. Press, New York, Vol. 1, p. 285
v. Ehrenstein, G., Lipmann, F. (1961) Proc. Nat. Acad. Sci. U.S. *47* 941
Farese, R. V. (1965) Endocrinology *76* 795
Fessenden, J. M., Moldave, K. (1962) Biochemistry *1* 485
Fessenden, J. M., Moldave, K. (1963) J. Biol. Chem. *238* 1479
Garren, L. D., Ney, R. L., Davies, W. W. (1965) Proc. Natl. Acad. Sci. U.S. *53* 1443
Garren, L. D., Richardson, A. P., Grocco, R. M. (1967) J. Biol. Chem. *242* 650
Gorski, J., Padnos, D. (1965) Fed. Proc. *24* 600
Grossi, L. G., Moldave, K. (1959) Biochim. Biophys. Acta *35* 275
Gross, P. R. (1968) in Annual Review of Biochemistry, Vol. 37, p. 631
Hoagland, M. B., Stephenson, M. L., Scott, J. F., Hecht, L. I., Zamencik, P. C. (1958) J. Biol. Chem. *231* 241
Ibuki, F., Moldave, K. (1968) J. Biol. Chem. *243* 791
Korner, A., Gumbley, J. M. (1966) Nature *209* 505
Lamfrom, H., Squires, R. F. (1962) Biochim. Biophys. Acta *61* 421
Leslie, I. (1955) in The Nucleic Acids (Eds: Chargaff, E., Davidson, J. N.) Acad. Press, New York, Vol. 2, p. 1
Liao, S., Barton, R. W., Lin, A. H. (1966) Proc. Nat. Acad. Sci. U.S. *55* 1593
Lowry, O. H., Rosebrough, N. J., Farr. A. L., Randall, R. J. (1951) J. Biol. Chem. *193* 265
Nathans, D., Lipmann, F. (1960) Biochim. Biophys. Acta *43* 126
Okamoto, T. (1964) J. Biochem. *55* 19
Rampersad, O. R., Wool, I. G. (1965) Science *149* 1102
Rendi, R., Ochoa, S. (1962) J. Biol. Chem. *237* 3707
Scriba, P. C., Reddy, W. J. (1965) Endocrinology *76* 745
Stadtman, E. R. (1957) in Methods in Enzymology (Eds: Colowick, S. P., Kaplan, N. O.) Acad. Press, New York, Vol. 3, p. 228
Sutter, R. P., Moldave, K. (1966) J. Biol. Chem. *241* 1698
Takanami, M. (1961) Biochim. Biophys. Acta *51* 85
Tata, J. R. (1966) in Progress in Nucleic Acid Research and Molecular Biology (Eds: Davidson, J. N., Cohn, W. E.), Acad. Press, New York—London, Vol. 5, p. 191
Tóth, M., Machovich, R. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. Suppl. *2* 49
Tóth, M. (1968) Abstracts of the 5th Meeting of FEBS, Praha, p. 86
Tóth, M. (1968a) Acta Biol. Acad. Sci. Hung. Appendix, *19* 519
Tóth, M., Mányai, S. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 337
Unsworth, B. R., Cohen, P. P. (1968) Biochemistry *7* 2581
Wicks, W. D., Villee, C. A. (1964) Arch. Biochem. Biophys. *106* 353
Williams-Ashman, H. G. (1965) Cancer Res. *25* 1096
Zabos, P., Hatfaludi, F., Machovich, R. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 263
Zubay, G. (1966) in Procedures in Nucleic Acid Research (Eds: Cantoni, G. L., Davies, D. R.) Harper and Row Publ., New York—London, p. 375

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (4), pp. 349–356 (1969)

Effect of Monovalent Cations on Myosin ATPase

Gabriella Sz. KELEMEN, A. MÜHLRAD

Biochemistry Group, L. Eötvös University, Budapest

(Received June 2, 1969)

The effect of monovalent cations (NH_4^+, Li^+, Na^+, K^+) on the ATPase activity of native and covalently modified myosins was studied in the presence of different effectors (Ca^{2+}, Mg^{2+}, EDTA).

The inhibitory effect of monovalent cations decreases in the presence of Ca^{2+} in the following order: $Li^+ > NH_4^+ > Na^+ > K^+$; in the presence of Mg^{2+}: $Li^+ \cong NH_4^+ \cong Na^+ > K^+$. The differences between the activities measured in the presence of different monovalent cations are relatively small.

The order of inhibition in the presence of EDTA is $Na^+ > Li^+ > K^+ > NH_4^+$. The differences between the activities measured in the presence of different monovalent cations are enormously large (e.g. the ratio of Na^+ to NH_4^+ is 1 : 3550). The order of relative affinities of these cations to a protein center influencing myosin ATPase in the presence of EDTA is $NH_4^+ > Na^+ > K^+$.

Covalent modification (blocking of SH and NH_2 groups influencing enzymic activity) does not change the order of inhibition but reduces the sensitivity of myosin to the monovalent cation present.

The importance of cations in connection with the EDTA activation of myosin ATPase has been shown by several authors. EDTA activation of myosin ATPase was first observed in solutions with relatively high (0.5 M) concentration of KCl (Friess, 1954; Bowen, Kerwin, 1954) and shown to be due to the removal of Mg^{2+} from the solution (Mühlrad et al., 1964; Offer, 1964; Kiely, Martonosi, 1968). The observed strong dependence of the EDTA-activated myosin ATPase on the species of the monovalent cation present in the solution (Bowen, Kerwin, 1954; Kielley et al., 1956) shows the inhibitory effect to decrease in the order $Na^+ > Li^+ > Rb^+ > K^+ > NH_4^+$.

The data on the EDTA activated myosin ATPase seem to be inconsistent with the results of Warren et al. (1966). These authors observed the inhibition of the activity of very different enzymes, including Ca^{2+} activated myosin ATPase, at high (0.3—3.0 M) concentrations of monovalent cations and found the inhibitory effect of the monovalent cations to be always of the same order for any of the enzymic activities studied, namely, $Li^+ > Rb^+ > Na^+ > Cs^+ > (CH_3)_4N^+$ in the case of the cations included in their investigations.

It seemed of interest therefore to study the behaviour of monovalent cations in the presence of different effectors of myosin ATPase. The experiments were

* Abbreviations: TBS = 2,4,6-trinitrobenzene sulfonate; TNP = trinitrophenyl; HEDD = β-hydroxyethyl 2,4-dinitrophenyl disulfide.

performed with the cations NH_4^+, Li^+, Na^+, K^+ using Ca^{2+}, Mg^{2+} or EDTA as effectors by measuring the ATPase activity of native and covalently modified myosins.

TBS and HEDD were chosen as modificators since either of them reacts selectively with only one type of side chain groups (HEDD with SH-groups: Kakol et al., 1964; TBS with NH_2-groups: Kitagawa et al., 1961; Fábián, Mühlrad, 1968) and the modification of relatively few amino acid residues produces major changes in the ATPase activity.

Materials and Methods

Myosin was prepared essentially as described by Portzehl et al. (1950). After the last precipitation it was dissolved in 0.5 M KCl + 0.02 M borate buffer (pH 7.4) and centrifuged for 1 hr at 105 000 *g* in the Spinco preparative ultracentrifuge.

Reagent grade chemicals were used throughout. TBS was a product of Sigma Chemical Co. HEDD prepared by the method of Böhme and Stachel (1957) was put at our disposal by the courtesy of Dr Kakol (Nencki Institute, Warsaw).

Myosin was trinitrophenylated at 20°C for 10 min. The test solution contained 10 mg of protein per ml, 20 mM Tris-HCl buffer (pH 7.4), 0.5 M KCl to which TBS was added in 5×10^{-4} M concentration. The pH was kept at a constant value by performing the reaction in a Radiometer pH stat (TTT1/SBR2) and using 1.0 M KOH for neutralization.

Myosin was precipitated at the end of the reaction by dilution with 12 vol. of ice cold distilled water and the pH was immediately adjusted to 6.2—6.4 in order to stop further trinitrophenylation. The suspension was centrifuged at 3°C at 10 000 *g* for 30 min. The precipitated myosin was dissolved in 0.5 M KCl + + 0.02 M borate buffer (pH 7.4) and left to stand overnight, then it was centrifuged again to obtain the clear supernatant used in the experiments. The number of TNP-lysine groups formed was evaluated by the method of Okuyama and Satake (1960) from the absorbance change at 346 mμ ($\Delta\varepsilon = 1.45 \times 10^4$). In each case the number of TNP-lysine groups formed during the treatment was 5.4—5.6 mole per 5×10^5 g myosin.

Myosin was treated with HEDD at 20°C for 70 min. The test solution contained 10 mg of protein per ml, 0.05 M Tris-acetate buffer (pH 6.8), 0.5 M KCl and 0.08 mg HEDD per 10 mg of protein. On termination of the incubation myosin was precipitated and dissolved in the same way as in the case of trinitrophenylation.

The number of blocked SH groups was calculated from the quantity of 2,4-dinitrophenol liberated during the treatment, as determined by the absorbance change at 408 mμ ($\Delta\varepsilon = 1.306 \times 10^4$). In each case the number of blocked SH groups was 11.0—12.0 mole per 5×10^5 g myosin.

The ATPase activity of myosin was measured in a solution containing 1 mg of myosin per ml, 4 mM ATP, 20 mM Tris-HCl buffer (pH 8), and alternatively $CaCl_2$, $MgCl_2$ or EDTA in different concentrations specified in the figures. KCl, NaCl, NH_4Cl or LiCl were present alternatively in the test solution at a concentration of either 0.1 or 0.6 M. The measurements were carried out at 20°C on 2 ml

samples. Incubation was terminated by addition of 2 ml of 10 per cent TCA. P_i was measured by the method of Fiske and SubbaRow (1925). ATPase activity was evaluated as μmole P_i per mg myosin per min. The time of incubation was chosen so as to obtain a decomposition of the terminal phosphate of ATP to less than 25 per cent.

Protein content was measured by the biuret method of Gornall et al. (1949).

Fresh myosin (not older than 4 days) was used throughout the experiments.

Results

ATPase activity of native, trinitrophenylated and HEDD-treated myosins, respectively, as specified by the symbol of the monovalent cation present, is shown for Ca^{2+} activator in Figs 1—3, for Mg^{2+} in Figs 4—6 and for EDTA in Table 1.

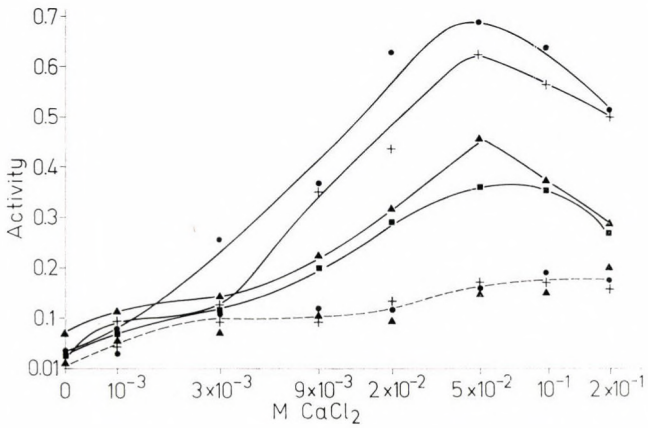


Fig. 1. The effect of monovalent cations at 0.1 M concentration on the ATPase activity of native and trinitrophenylated myosins in the presence of Ca^{2+}. Experimental conditions see in Methods. ■ LiCl + NaCl; ▲ NH_4Cl; ● KCl; full line, native myosin; dotted line, modified myosin

In the case of Ca^{2+} activator for non-treated myosin and at low concentration of monovalent cations the order of inhibition is $Li^+ > NH_4^+ > Na^+ > K^+$ at any of the Ca^{2+} concentrations used (Fig. 1). At high concentration (0.6 M) of these ions this order changes to $Li^+ > Na^+ > NH_4^+ > K^+$ if the Ca^{2+} concentration is lower than 5×10^{-3} M (Fig. 2).

Trinitrophenylation and HEDD-treatment do not change the order of inhibition but seem to reduce the sensitivity of myosin to the species of the cation present, especially at a 0.1 M concentration of the cations (Fig. 1).

It is of interest to note that the blocking of SH groups enhances the Ca^{2+} activation of myosin ATPase at any of the Ca^{2+} concentrations used (Fig. 3), while the blocking of NH_2 groups enhances the activation only at low ($< 5 \times 10^{-3}$ M) concentrations of Ca^{2+} and reduces the extent of Ca^{2+} activation at higher

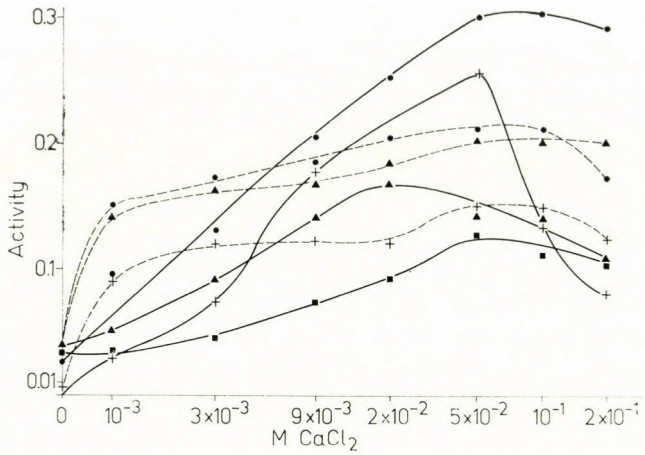


Fig. 2. The effect of monovalent cations at 0.6 M concentration on the ATPase activity of native and trinitrophenylated myosins in the presence of Ca^{2+}. Experimental conditions see Methods. Symbols as in Fig. 1

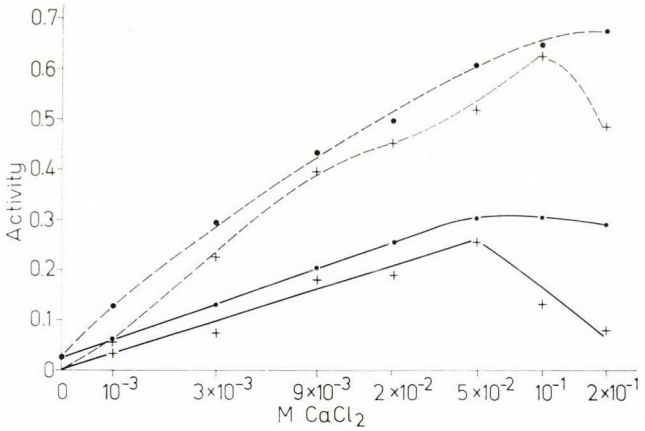


Fig. 3. The effect of monovalent cations at 0.6 M concentration on the ATPase activity of native and HEDD treated myosins in the presence of Ca^{2+}. Experimental conditions see Methods. Symbols as in Fig. 1

concentrations of Ca^{2+}. This effect is especially apparent at high ionic strength (0.6 M) as shown in Figs 2 and 3.

For Mg^{2+} an inhibitor of myosin, the order of inhibition for non-treated myosin ATPase is $Na^+ > Li^+ > K^+ > NH_4^+$ at low Mg^{2+} concentration and changes to $Li^+ \cong Na^+ \cong NH_4^+ > K^+$ at high Mg^{2+} concentration at both concentrations of the monovalent cation (Figs 4 and 5). For the modified myosins the order of inhibition is $Na^+ > NH_4^+ > K^+$.

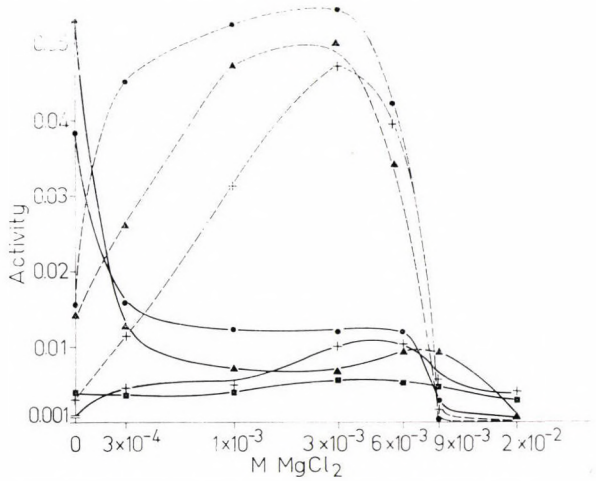


Fig. 4. The effect of monovalent cations at 0.1 M concentration on the ATPase activity of native and trinitrophenylated myosins in the presence of Mg^{2+}. Experimental conditions see Methods. Symbols as in Fig. 1

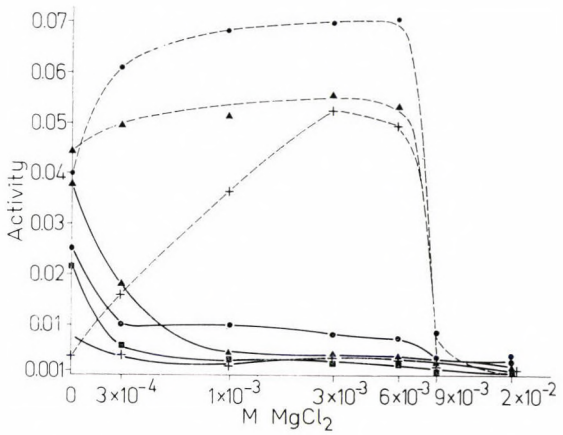


Fig. 5. The effect of monovalent cations at 0.6 M concentration on the ATPase activity of native and trinitrophenylated myosins in the presence of Mg^{2+}. Experimental conditions see in Methods. Symbols as in Fig. 1

As expected from earlier results (Kitagawa et al., 1961; Fábián, Mühlrad, 1968), the trinitrophenylation of NH_2 groups causes a remarkable increase in myosin ATPase activity in the presence of Mg^{2+} up to 6×10^{-3} M concentrations of the latter. Above this value a sharp decrease is observed in the ATPase activity of myosin. This phenomenon is independent of the species and concentration of the monovalent cation present. The Mg^{2+} activation of myosin ATPase is less enhanced by the blocking of SH groups and the sharp decrease in activity observed at high Mg^{2+} concentrations in the case of trinitrophenylation is not apparent on HEDD-treatment (Fig. 6).

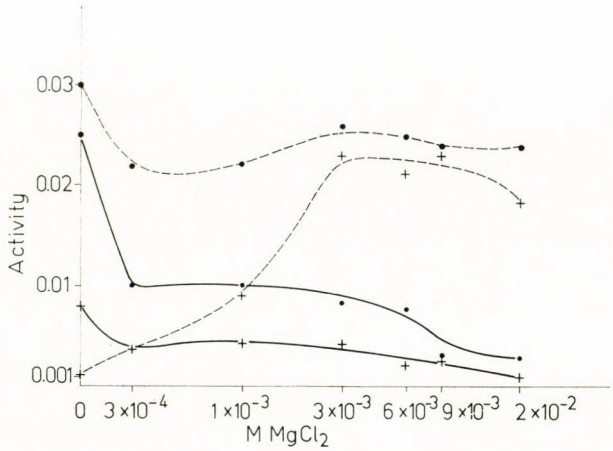


Fig. 6. The effect of monovalent cations at 0.6 M concentration on the ATPase activity of native and HEDD treated myosins in the presence of Mg^{2+} Experimental conditions see Methods. Symbols as in Fig. 1.

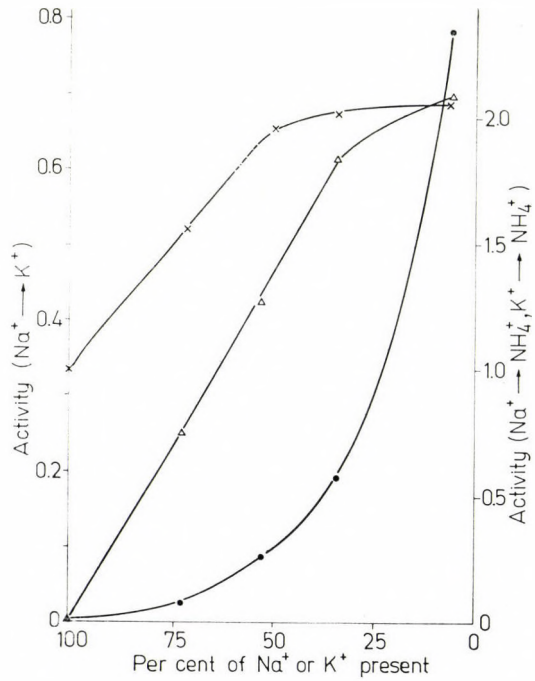


Fig. 7. The dependence of myosin ATPase on the relative concentration of monovalent cations. Experimental conditions: EDTA 5 mM; total concentration of monovalent cations 0.6 M (chloride salts). ● = $Na^+ \rightarrow K^+$ competition; △ = $Na^+ \rightarrow NH_4^+$ competition; × = $K^+ \rightarrow$ $\rightarrow NH_4^+$ competition. Abscissa: per cent of Na^+ present in $Na^+ \rightarrow K^+$ and $Na^+ \rightarrow NH_4^+$ competitions; per cent of K^+ present in $K^+ \rightarrow NH_4^+$ competition

In the case of EDTA-activated myosin ATPase the measured values show the effect of the monovalent cations to be markedly different from that observed for Ca^{2+} or Mg^{2+} activation. The differences between the specific values of activity are very great (Table 1). The highest value obtained for NH_4^+ is reduced by 50 per cent for the next highest K^+, very low for Li^+ and almost non-measurable (0.0006 μMP_i per minute per mg myosin) for Na^+ at 0.6 M concentration of the cations. The relative difference between the values for NH_4^+ and K^+ is even higher at 0.1 M concentration.

Table 1

The effect of monovalent cations on myosin ATPase in the presence of EDTA

(Experimental conditions see in Methods)

Myosin	Monovalent cation	ATPase activity in the presence of EDTA at molar concentrations of					
		0	10^{-3}	3×10^{-3}	9×10^{-3}	2×10^{-2}	5×10^{-2}
Native	0.1 M NH_4Cl	0.082	0.627	0.71	0.517	0.451	0.704
	0.1 M KCl	0.0175	0.0204	0.0243	0.0202	0 0187	0.016
	0.1 M LiCl	0.0074	0.0054	0.0047	0.0031	0.0028	0.0045
	0.1 M NaCl	0.00102	0.00136	0.00128	0.00124	0 0008	0.0006
	0.6 M NH_4Cl	0.0378	0.955	1.32	2 09	2.09	2.13
	0.6 M KCl	0.0252	0.617	1.06	1.01	0.76	0.789
	0.6 M LiCl	0.0212	0.0296	0.0403	0 0328	0.0313	0.0366
	0.6 M NaCl	0.0038	0.00048	0.00062	0.00051	0.0003	0.0006
TBS treated	0.1 M NH_4Cl	0.013	0.019	0.0237	0.0179	0.0125	0.019
	0.1 M KCl	0.0	0.0	0.0	0.0	0 0	0.0
	0.1 M NaCl	0.0	0.0	0.0	0.0	0.0	0.0
	0.6 M NH_4Cl	0.042	0.062	0.107	0.128	0.139	0.142
	0.6 M KCl	0.0419	0.0465	0.0605	0.063	0 042	0 07
	0.6 M NaCl	0.0	0.0	0.0	0.0	0.0	0.0
HEDD treated	0.6 M KCl	0.0303	0.065	0.075	0.06	0.046	0.072
	0.6 M NaCl	0.0	0.0	0.0	0.0	0.0	0.0

On both trinitrophenylation and HEDD-treatment there is a marked decrease in EDTA activated myosin ATPase as compared to the values measured with non-treated myosin for any of the monovalent cations studied.

The great differences between the specific inhibitory effects of the monovalent cations in the case of EDTA activation suggest some highly specific interaction of these ions with a given protein center in (or near to) the active site. In order to evaluate the relative affinities of the cations studied to this assumed center, competition experiments were performed. In these runs the total concentration of monovalent salts was kept at a constant value of 0.6 M to avoid unspecific ionic strength effects, while varying the relative concentration of the two cations. The results are shown in Fig. 7. It is seen that 50 per cent activation is obtained for the competitions $Na^+ \rightarrow K^+$, $Na^+ \rightarrow NH_4^+$ and $K^+ \rightarrow NH_4^+$ at the ratios 1 : 3, 1 : 0.64 and 1 : 0.33, respectively. These results show that the order of relative affinities is $NH_4^+ > Na^+ > K^+$.

Discussion

The specific values of myosin ATPase activity measured for the monovalent cations in the presence of the divalent cations Ca^{2+} and Mg^{2+} show essentially the same order of inhibition as that observed by Warren et al. (1966). The inhibitory effect of NH_4^+, which was not studied by these authors, lies between that of Li^+ and Na^+. The differences between the specific values are not remarkably great and the data are consistent with the criteria by which the above authors attribute the inhibition of the activity of a given enzyme to the disruption of the structure of the macromolecule by the monovalent cation. The modification of myosin in spite of the great activity changes does not alter essentially the above effect of cations on myosin ATPase, but reduces the sensitivity of myosin to the cation present.

On the other hand, the extremely great differences in the specific values (e.g. the ratio of Na^+ to NH_4^+ is 1 : 3550) measured in the case of EDTA activation cannot be explained by an indirect effect of the monovalent cations *via* structural disruption. The very recent results of Seidel (1969) also strongly support our view that the monovalent cations have a direct effect on the enzymic center of myosin in the presence of EDTA. Because of the great differences in activities observed in the absence of divalent cations, it seems highly probable that there exists a specific cation binding site which directly influences the rate of ATP splitting. If NH_4^+ or K^+ is bound to this site the activity is high, while in the case of Na^+ there is hardly any activity at all. The relative affinities of the monovalent cations to this site (cf. Fig. 7) were found to be $NH_4^+ > Na^+ > K^+$. The hypersensitivity to the species of the monovalent cations ceases on the addition of Ca^{2+} or Mg^{2+}. This can be explained by the higher affinity of the divalent cations to this binding site as compared with that of the monovalent cations.

The authors are indebted to Dr N. A. Biró for his kind interest and helpful suggestions during the preparation of the paper and to Mrs M. Magyar for her skilled technical assistance. We are also grateful to Dr I. Kakol who put the HEDD preparation at our disposal.

References

Bowen, W. J., Kerwin, T. D. (1954) J. Biol. Chem. *211* 237
Böhme, H., Stachel, M. D. (1957) Z. Anal. Chem. *154* 27
Fábián, F., Mühlrad, A. (1968) Biochim. Biophys. Acta *162* 596
Fiske, C. H., SubbaRow, J. (1925) J. Biol. Chem. *66* 375
Friess, E. T. (1954) Arch. Biochem. Biophys. *51* 17
Gornall, A., Bardawill, C. J., David, N. M. (1949) J. Biol. Chem. *177* 751
Kakol, I., Gruda, J., Bitny-Szlachto, S. (1964) Acta Biochim. Polon. *11* 411
Kielley, W. W., Kalckar, H. M., Bradley, L. B. (1956) J. Biol. Chem. *218* 653
Kiely, B., Martonosi, A. (1968) J. Biol. Chem. *243* 2273
Kitagawa, S., Yoshimura, J., Tonomura, Y. (1961) J. Biol. Chem. *236* 902
Mühlrad, A., Fábián, F., Biró, N. A. (1964) Biochim. Biophys. Acta *89* 186
Offer, G. W. (1964) Biochim. Biophys. Acta *89* 566
Okuyama, T., Satake, K. (1960) J. Biol. Chem. *47* 454
Portzehl, H., Schramm, G., Weber, H. H. (1950) Z. Naturforsch. *5b* 61
Seidel, J. C. (1969) J. Biol. Chem. *244* 1142
Warren, J. C., Stowring, L. Morales, M. F. (1966) J. Biol. Chem. *241* 309

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (4), pp. 357—363 (1969)

The Effect of pH on the ATPase Activity of the Unicellular Alga Scenedesmus obtusiusculus CHOD

Gabriella MESZES, L. ERDEI

Department of Plant Physiology, L. Eötvös University, Budapest

(Received June 26, 1969)

The properties of the ($Mg^{2+}-K^{+}-Na^{+}$)-activated and ouabain-sensitive ATPase of the unicellular alga Scenedesmus obtusiusculus were compared in intact cells and in isolated chloroplasts.

In intact cells the enzyme activities observed at different pH-values indicate the presence of several enzyme species or of different, pH-dependent conformational states of one enzyme.

The assumption that the same enzyme operates in both intact cells and chloroplasts can be excluded on the basis of enzyme activity measurements at pH 7.2, since ouabain only inhibited the ($Mg^{2+}-K^{+}-Na^{+}$)-activated ATPase of the intact cells, but failed to influence that of the chloroplasts.

In our earlier investigations we have studied the properties of the ATPase activity found in the unicellular green alga Scenedesmus obtusiusculus. We have shown the presence of Mg^{2+}-activated ATPase and the further activating effect of the combined application of the monovalent cations K^{+} and Na^{+} at pH 7.2. The specific inhibitor of "transport ATPase", ouabain, inhibited about 1/4 to 1/3 of the (Mg^{2+}—K^{+}—Na^{+})-ATPase activity of the intact alga cell. The functioning of (Mg^{2+}—K^{+}—Na^{+})-activated ATPase was found to be dependent on light. Illumination was shown to increase the activity. The increase in enzymic activity, when plotted against illumination, exhibited a saturation-type curve (Meszes, Erdei, 1969).

The above results prompted us to study the intracellular distribution of this ATPase activity. Thus it seemed desirable to clarify how much of the total ATPase activity of the intact cell is comprised in the chloroplast fraction and to characterize the different ATPase enzymes. In the present paper we describe the pH-dependence of ATPases found in the intact cells and in the isolated chloroplasts.

Materials and Methods

The experiments were carried out with the unicellular alga Scenedesmus obtusiusculus CHOD, obtained from the alga collection of the Biological Research Institute of the Hungarian Academy of Sciences, Tihany. The algae were grown under conditions described in our previous paper (Meszes, Erdei, 1969).

For ATPase assay cells were collected by centrifugation, washed with distilled water and after centrifugation disrupted by ethylacetate. The ATPase activ-

ity of the suspension was measured in the presence and absence of Mg^{2+}, K^+ and Na^+ as described earlier (Meszes, Erdei, 1969), but in various pH-ranges. As inhibitor 0.1 mM ouabain was applied.

The disruption of cells was carried out by grinding in a ball and tube mill with constant cooling. (Enzymic breakdown, sonication, or the application of blade homogenizers proved to be inefficient.) The algae collected from the culture medium by centrifugation were washed in tris-HCl buffer, pH 7.2, containing 0.3 M mannitol. After washing three times the cells were suspended in the same solution, put into a mill and shaken for 3.5 to 4 hours in a shaker (New Brunswick Sci. Co. Inc.) at 5°C.

Intact cells and cell debris were separated from the chloroplasts by sucrose gradient centrifugation. Sucrose solution of 1.35 density proved to be satisfactory. After centrifugation at 6000 *g* for 45 min with cooling, the intact cells and cell debris were found in the supernatant fluid, whereas the chloroplasts sedimented to the bottom of the tubes. This sediment was used for the enzyme assay. The chloroplast is in fact a spherical or oval fragment of about uniform size of the chromatophore.

For the enzyme assay the chloroplasts were prepared by washing three times with buffer of the appropriate pH, while care was taken to keep the temperature low (5° C). After washing the precipitate was suspended in the appropriate buffer and a portion of this suspension was used for the enzyme assay. The following mixtures were tested at the different pH-values:

1. ATP
2. Chloroplasts
3. ATP + chloroplasts
4. ATP + chloroplasts + 10 mM Mg^{2+} + 20 mM Na^+ + 10 mM K^+
5. The same as 4. + 10^{-4} M ouabain.

A pH range from 4.8 to 9.2 was studied using 0.4 pH unit intervals. In the lower pH region tris-maleic acid NaOH buffer, whereas in the higher pH region tris-HCl buffer was used.

The enzyme activity of intact cells was examined parallel to that of chloroplasts with cells obtained from the same culture. The enzyme assay was carried out as already described (Meszes, Erdei, 1969). The amount of inorganic phosphate split off by ATPase was determined spectrophotometrically at 700 mμ by the method of Fiske—SubbaRow (1925). The absorbancy values were converted into μg P per 100 μg chlorophyll units by the aid of a standard curve.

Chlorophyll was extracted with hot methanol. After extraction the light absorption of the chlorophyll solution was read at 663 and 644 mμ. The concentration of chlorophyll was calculated on the basis of the following molar extinction coefficients:

chlorophyll a	ε_{663}	= 81 000
	ε_{664}	= 10 600
chlorophyll b	ε_{663}	= 425
	ε_{644}	= 52 600.

Results

In the present experiments we compared the (Mg^{2+}—K^+—Na^+)-activated and ouabain sensitive ATPase activities of Scenedesmus obtusiusculus in intact alga cells and in the chloroplasts. At the optimal concentrations of the cations Mg^{2+}, K^+ and Na^+, given in our previous paper (Meszes, Erdei, 1969), the enzyme activity was measured in the pH range of 4.8 to 9.2. For such a wide pH-range two buffers were used because of the limitations of buffer capacity. At the higher pH-values, between pH 7.2 and 9.2, tris-HCl, whereas in the acidic range, between pH 4.8 and 8.6, tris-maleic acid-NaOH buffer were employed. To eliminate possible interfering effects between the buffer and the material examined and to be

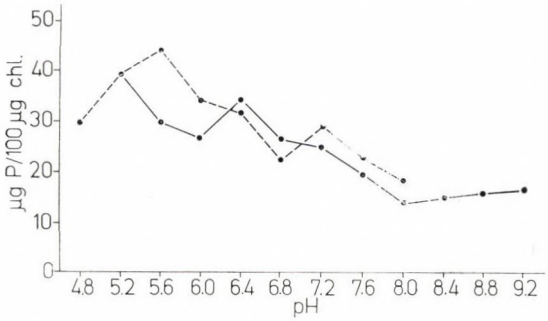


Fig. 1. Effect of pH on the ATPase activity of intact Scenedesmus obtusiusculus cells. In tris-HCl buffer: ● ——— ● and in tris-maleic acid-NaOH buffer: — — — — —

able to compare the results obtained in the two buffer systems, even at low pH-values (pH 5.2, 5.6 and 6.0) tris-HCl buffer was applied where this buffer had already no buffer capacity. Fig. 1 shows the comparison of results obtained by the use of the two media.

As seen in Fig. 1, there is no significant difference between the two curves between pH 6.4 and 8.0. In the acidic region data obtained in tris-maleic acid--NaOH medium should be taken into account, since tris-HCl has no buffer capacity in this region. As shown in the figure, activity maxima were observed at three pH values in tris-maleic acid-NaOH: pH 5.6, 6.4 and 7.2. These findings suggest the presence of different enzymes or different conformational forms, depending on the pH, of one enzyme.

The ATPase activity of intact cells was then measured in the two buffer systems with and without the cations Mg^{2+}, K^+ and Na^+. Figs 2 and 3 show the results obtained in tris-HCl buffer and tris-maleic acid-NaOH buffer, respectively. The activating effect of ions is conspicuous in both pH-profiles. The increase in activity in tris-HCl buffer is about 12 to 25 per cent in the acidic region, and somewhat higher in the alkaline region. The greatest activating effect, 42 per cent, occurred at pH 7.2. The same pattern was also observed in tris-maleic acid-NaOH buffer, where the activating effect of ions was in general 10 to 12 per cent. The maximal effect, 26 per cent enhancement, was detected again at pH 7.2.

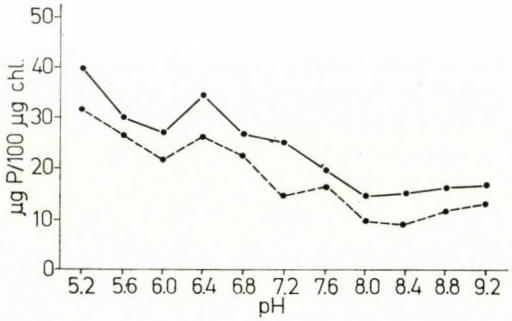


Fig. 2. Effect of Mg^{2+}, K^+, and Na^+ on the ATPase activity of intact Scenedesmus obtusiusculus cells in tris-HCl buffer. Ions added: ————; control (no ions added): — — —

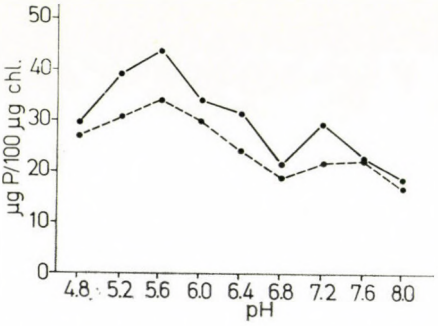


Fig. 3. Effect of Mg^{2+}, K^+ and Na^+ on the ATPase activity of intact Scenedesmus obtusiusculus cells in tris-maleic acid buffer. Ions added: ————; control (no ions added): — — —

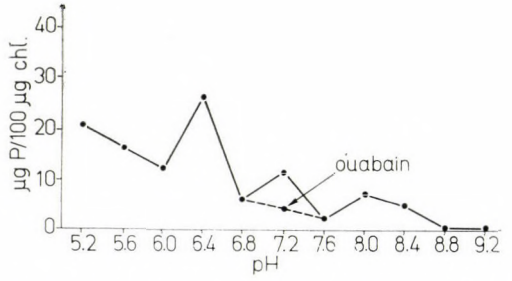


Fig. 4. Effect of ouabain on the (Mg^{2+}, K^+, Na^+)-activated ATPase activity of intact Scenedesmus obtusiusculus cells in tris-HCl buffer

To decide whether the (Mg^{2+}—K^+—Na^+)-activated and ouabain sensitive ATPase activity of Scenedesmus obtusiusculus belongs to the same enzyme functioning at pH 7.2, we examined the inhibitory effect of ouabain at all pH-values in both buffers. As seen in Figs 4 and 5, inhibition occurred only at pH 7.2, where it amounted to 30 to 40 per cent in the intact cells. Thus it appears to be proved that the ATPase present in the intact cells of Scenedesmus obtusiusculus is sensitive to ouabain and maximal inhibition reduces enzyme activity by 1/3 to 1/4.

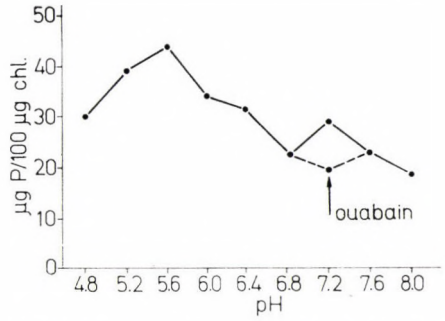


Fig. 5. Effect of ouabain on the (Mg^{2+}, K^+, Na^+)-activated ATPase activity of intact Scenedesmus obtusiusculus cells in tris-maleic acid, NaOH buffer

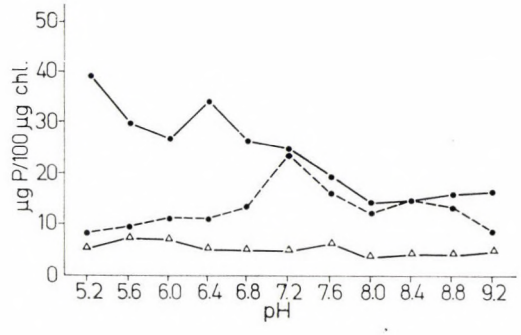


Fig. 6. Effect of pH on the ATPase activity of the chloroplast fraction of Scenedesmus obtusiusculus. (Mg^{2+}, K^+, Na^+)-activated ATPase activity of intact cells: ————; enzyme activity of chloroplast fraction without ions: ——△——△——; and with ions: — — — — —

In the next series of experiments we examined the pH-dependence of ATPase activity of the chloroplasts isolated from Scenedesmus obtusiusculus cells. To obtain comparable results, the method described for intact cells was applied in the enzyme assay. A more detailed study of the ATPase localized in the chloroplast will be undertaken in a further stage along with the electron microscopic analysis of the chromatophore of Scenedesmus obtusiusculus. Therefore in the present paper we only deal with experiments carried out under conditions identical with those for intact cells.

The ATPase activity, measured in the presence of Mg^{2+}, K^+ and Na^+, of intact cells and of the chloroplast fraction were compared in tris-HCl medium. As can be seen in Fig. 6, the pH-dependence of curve of the chloroplast enzyme differs from that of intact cells. The chloroplast enzyme is active only at pH 7.2, and very slightly around pH 8.4. At pH values where the intact cell preparation exhibited marked activity (pH 6.4, 5.2 and 5.6), the chloroplast fraction did not split ATP. Thus, it can be assumed that the enzymes of pH optima 6.4 and 5.6 are localized in other cell components.

As shown in Fig. 6, the ATPase activities of the intact cells and chloroplasts are quantitatively almost identical. This coincidence may suggest that the enzyme activity of the intact cells at this pH-value originates from the enzyme located in the chloroplast fraction. Since at this pH the (Mg^{2+}—K^+—Na^+)-activated ATPase of the intact cells was inhibited by ouabain, ouabain would inhibit also the chloroplast enzyme at pH 7.2 if the enzyme was involved in both cases. Therefore we examined the effect of ouabain on the ATPase activity of the chloroplasts. However, since ouabain had no effect on the ATPase activity of the chloroplast fraction at various pH values there are presumably two enzymes both functioning optimally at pH 7.2: an ouabain-sensitive enzyme outside the chloroplasts and an ouabain-insensitive one located in the chloroplasts. The verification of this assumption requires further experiments.

Fig. 6 shows also the enzyme activity of chloroplasts without added ions. There is practically no enzyme activity at any pH value. This can be due to the fact that the ionic concentration required for the activity of ATPase has been lost in the course of washing during the isolation of chloroplasts.

The same result was obtained when tris-maleic acid-NaOH buffer was used. On the addition of ions ATPase activity could be detected only at pH 7.2. Ouabain had no inhibitory effect.

Discussion

According to literary data ATPase enzymes in plants have widely different pH optima. Thus e.g. Dodds and Ellis (1966) have examined the cation-stimulated ATPase activity in the cell-wall fraction of carrot and pea roots in the presence of $MgCl_2$, KCl, and ATP in imidazole-acetate buffer. The optimum was observed at pH 4.5 (Dodds, Ellis, 1966). A Mg^{2+}-dependent enzyme, activated by Na^+ and inhibited by ouabain, i.e. similar to the transport ATPase of erythrocytes, has been found in the root tips of pine-tree. However, the pH optimum of this enzyme was different from that of animal ATPases: the optimum was at pH 5.5 instead of 7.2 (McClurkin, McClurkin, 1967). The pH optimum of the Mg^{2+}-dependent ATPase of the chloroplast of Euglena gracilis, one of the active ATPase from this source, was foundto be 5.5 (Carell, Kahn, 1967). On the alkaline side the pH optima of the ATPases of chloroplasts fall between pH 7.4 and 8.4 (Bennun, Avron, 1964; Hoch, Martin, 1963; McCarthy, Racker, 1968). The Mg^{2+}-activated ATPase of Escherichia coli had its pH optimum at 8.7 in the absence of KCl. In the presence of KCl the specific activity increased and the optimum shifted to pH 9.1 (Hafkenscheid, Bonting, 1969). Günther and Dorn (1966) studied the pH-dependence of ATPase activity in intact E. coli cells and observed the optimum at pH 8.5.

In our present experiments we compared the (Mg^{2+}—K^+—Na^+)-activated ATPase activities of Scenedesmus obtusiusculus in intact alga cells and in the chloroplasts at various pH values. Our results have shown that the ATPase activity of intact cells was strongly dependent on the pH. The highest activities were measured at pH 5.6, 6.4 and 7.2. Ouabain exhibited an inhibitory effect only at pH 7.2. Consequently it seems reasonable to assume that there are more than one ATPase

enzymes in the cell, which can be separated on the basis of Mg^{2+}-requirement, pH optimum, and ouabain sensitivity.

The ATPase activity of the chloroplast fraction when plotted as a function of pH, showed only one maximum at pH 7.2. This activity, however, was resistant to ouabain, therefore it could not be identical with the enzyme activity of the intact cell observed at the same pH. The combined addition of Mg^{2+}, K^+ and Na^+ brought about a marked increase in enzyme activity in both the intact cells and the chloroplasts.

On the basis of our results it seems to be important to study the properties of the various ATPases, their role and intracellular localization. To this end further experiments have to be conducted on the effect of light, various ions and inhibitors on the enzyme activity in chloroplasts and other cell constituents. Such experiments may reveal the interrelated, mutually dependent processes of ion uptake, the mechanism of ATPase action, and photosynthesis.

References

Bennun, A., Avron, M. (1964) Biochim. Biophys. Acta *79* 646
Carell, E. F., Kahn, J. S. (1967) Biochim. Biophys. Acta *131* 571
Dodds, J. J. A., Ellis, R. J. (1966) Biochem. J. *101* 31 P
Fiske, C. H., SubbaRow, Y. (1925) J. Biol. Chem. *66* 375
Günther, T., Dorn, F. (1966) Z. Naturforsch. *21b* 1076
Hafkenscheid, J. C. M., Bonting, S. L. (1969) Biochim. Biophys. Acta *178* 128
Hoch, G., Martin, I. (1963) Biochem. Biophys. Res. Comm. *12* 223
McCarthy, R. E., Racker, E. (1968) J. Biol. Chem. *243* 129
McClurkin, I. T., McClurkin, D. C. (1967) Plant Physiol. *42* 1103
Meszes, G., Erdei, L. (1969) Acta Biochim. Biophys. Acad. Sci. Hung. *4* 131

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (4), pp. 365—378 (1969)

Effect of Diethylpyrocarbonate on the Conformation and Enzymic Activity of D-Glyceraldehyde-3-phosphate Dehydrogenase

Judit OVÁDI, T. KELETI

Institute of Biochemistry, Hungarian Academy of Sciences, Budapest

(Received July 8, 1969)

In denatured D-glyceraldehyde-3-phosphate dehydrogenase all (11 per subunit) histidyl residues react with diethylpyrocarbonate. Six residues react if 100 mole equivalents and four if 25 mole equivalents per subunit of this reagent are used.

The apparently first order rate constant of decrease in enzymic activity is practically identical with the rate constant of the formation of carbethoxy-histidyl groups in the presence of 25 moles diethylpyrocarbonate per subunit.

D-glyceraldehyde-3-phosphate dehydrogenase, which is completely inactivated by the formation of 4 carbethoxy-histidyl bonds per subunit in the absence of substrates, may be partially protected from inactivation if treatment with diethylpyrocarbonate is carried out in the presence of NAD and/or phosphate. Moreover, incubation with substrates and mercaptoethanol partially reactivates the fully inactive carbethoxy-enzyme. However, in each case the same number of carbethoxy-histidyl groups are formed.

The peptide containing two out of four carbethoxylated histidyl residues was isolated and analyzed.

The dissociation of two out of the four firmly bound NAD molecules increases after carbethoxylation of the enzyme. The fluorescence of the carbethoxylated enzyme increases indicating the loosening of the steric structure of D-glyceraldehyde-3-phosphate dehydrogenase.

These results suggest that carbethoxylation of four histidyl residues per subunit alters the conformation of the enzyme. Some of the modified residues may be in the neighbourhood of the active centre, however, probably are not directly involved in the catalytic activity.

It has been shown by Ovádi et al. (1967) that DEP* reacts specifically with the histidyl groups of proteins at pH 6.

In the present investigation we have examined the effect of DEP on the structure and enzymic activity of GAPD. Our results suggest that carbethoxylation of some histidyl residues alters the conformation of GAPD. The modified residues may not be directly involved in the catalytic activity of the enzyme, however, some of them may be in the neighbourhood of the active centre.

* Abbreviations used: DEP = diethylpyrocarbonate; GAPD = D-glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase, phosphorylating, EC. 1.2.1.12); GAP = D-glyceraldehyde-3-phosphate; GA = D-glyceraldehyde; p-NPA = p-nitrophenylacetate; p-MB = p-(hydroxy)-mercuribenzoate; ME = β-mercaptoethanol.

Materials and Methods

Four times recrystallized swine muscle GAPD isolated according to Elődi and Szörényi (1956) was used. The enzyme was dissolved in 0.1 M phosphate or 0.05 M tris-HCl buffer, pH 6.0.

The molecular weight was taken as 140 000 (Elődi, 1958) for 4 subunits (Watson, Banaszak, 1964; Harris, Perham, 1965; Harrington, Karr, 1965).

NAD, firmly bound to swine muscle GAPD, was removed by charcoal treatment and the quantitative removal was controlled by measuring the E_{280}/E_{260} ratio of the protein (= 1.85 to 1.98).

The protein content of the solutions was determined spectrophotometrically at 280 mμ [$E^{1\ mg/ml}_{280\ m\mu}$ = 1.0 for GAPD(NAD)$_4$ and = 1.1 for NAD-free GAPD].

The solutions of DEP were prepared daily by diluting "Baycovin" (Bayer Co., Leverkusen, BRD) with 95 per cent ethanol. Therefore ethanol was added in the same (two per cent) final concentration to all assay media of the controls.

Carbethoxylation was carried out at 0°C. When 1×10^{-5} to 2.5×10^{-5} moles GAPD were treated with 100-fold molar excess of DEP, carbethoxylation was completed after 30—40 minutes of incubation.

The number of histidyl groups which reacted in the protein was determined using the molar extinction coefficient of the absorption difference at 240 mμ of carbethoxy-histidyl complex and of histidine: $\Delta\varepsilon = 3.2 \times 10^3$ (Ovádi et al., 1967).

Enzymic activity of GAPD was measured by Warburg's optical test with 2×10^{-3} M GAP or 3×10^{-2} M GA substrate in the presence of 2×10^{-3} M NAD and 1×10^{-2} M inorganic phosphate or with 5.5×10^{-4} M p-NPA, using in the latter case charcoal treated enzyme.

The number of SH-groups was determined according to Boyer (1954).

In order to isolate the peptides containing carbethoxy-histidine, 100—150 mg protein at a concentration of 3.5 mg/ml was treated with DEP. The reaction mixture was gel-filtered on Sephadex G-50 column (2.5 × 60 cm). Elution was performed with distilled water. After gel-filtration the modified protein was heat-denatured and digested with papain (1/50 w/w) at pH 6.5—7.0 in the presence of 0.01 M mercaptoethanol and 0.001 M EDTA for 2 hours at 37°C. After digestion the amount of carbethoxy-histidine was checked spectrophotometrically at 240 mμ.

The peptides containing the carbethoxylated histidine were separated by the diagonal method of Sajgó (1969) in the following way: The digest was freeze-dried, dissolved in distilled water and subjected to paper electrophoresis at pH 5.0, in pyridine—acetic acid—water 10 : 10 : 980 (by volume), at 35 V/cm for 2 hours in a flat-plate, water-cooled apparatus. After electrophoresis a guide strip was cut off and exposed to the vapour of 6 N ammonia, for 16—20 hours. This ammonia-treated strip was stitched to another sheet of Whatman 3MM paper and a second electrophoresis was carried out at pH 5.0, perpendicular to the strip. Since ammonia treatment split off the carbethoxy-group, the imidazole side chain of the histidyl residue regained its positive charge and this resulted in the moving of this histidine peptide off the diagonal position.

The diagonal fingerprint was developed with ninhydrin and Pauli-reagent. On the basis of this diagonal fingerprint the carbethoxylated histidyl peptides were

located in the neutral band. This band was cut out from the preparative electrophoretogram, treated with ammonia and rerun at pH 5. The Pauli-positive peptides were further purified at pH 1.9, by using formic acid–acetic acid–water 50 : 150 : : 800 at 80 V/cm in a Michl-apparatus for 1 hour, followed by chromatography in butanol–pyridine–acetic acid–water 150 : 100 : 30 : 120 or pyridine–isoamyl-alcohol–water 35 : 35 : 30 systems. The peptides were eluted with 0.05 M ammonia, the N-terminal residue was determined with the "dansyl"-method of Gray and Hartley (1963). For amino acid analysis the homogeneous peptide was hydrolyzed in 20 per cent hydrochloric acid at 105°C, for 16 hours. Amino acid analysis was performed in a Beckman Unichrom analyser using the single-column method of Dévényi (1968).

GAP was prepared from fructose-1,6-diphosphate (Reanal) according to Szewczuk et al. (1961). NAD and NADH were Reanal preparations of 90 and 70 per cent purity, respectively. All other chemicals were commercial preparations of reagent grade.

Opton PMQ II spectrophotometer and spectrofluorimeter were used.

Results

1. The reactivity of histidyl residues of GAPD

GAPD contains 11 histidyl residues per subunit (Harris, Perham, 1968). All of the histidyl residues react with DEP in 8 M urea only (Ovádi et al., 1967). Six histidyl groups per subunit react with DEP in the native protein if 100 moles of DEP are applied per mole of subunit (Fig. 1).

When GAPD is treated with 25 moles of DEP per mole of subunit, only 4 histidyl groups per subunit are blocked in the presence or absence of substrates (NAD and/or phosphate). The formation of 4-carbethoxy-histidyl groups per subunit in the absence of substrates results in the complete inactivation of the enzyme. Therefore in the following experiments we used enzyme which contained 4 carbethoxy-histidyl groups per subunit and this was called GAPD-CT_4*.

If 4 histidyl groups per subunit react with DEP — even in the presence or in the absence of substrates — the kinetics of the overall process are apparently first order (Fig. 2A).

Apparently first order kinetics with a similar rate constant are obtained for the inactivation of the enzyme during DEP treatment in the absence of substrates (Fig. 2B).

These data would suggest that loss of enzymic activity is a consequence of the formation of carbethoxy-histidyl groups.

* We do not know whether GAPD-CT_4 is a well defined compound or not, i.e. in all cases (in tris or in phosphate buffer, in the presence or in the absence of substrates) the same four histidyl residues are blocked in the subunits or not. GAPD-CT_4 means only the same number of carbethoxy-histidyl groups formed in the presence of 25 moles of DEP per subunit.

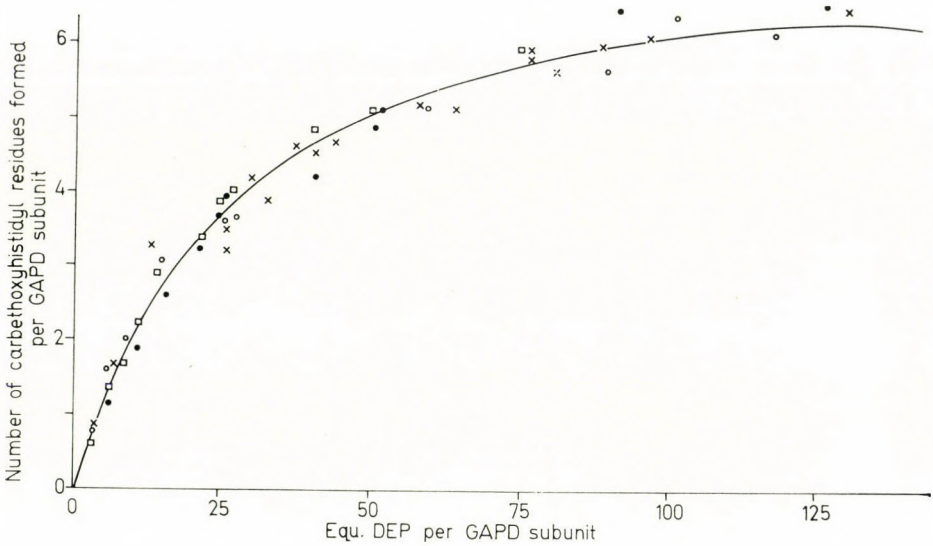


Fig. 1. Titration of histidyl residues in GAPD with DEP. 1×10^{-5} M GAPD was treated with DEP for 30 minutes at 0°C in 0.05 M tris-HCl buffer, pH 6.0 in the presence (×) or absence (●) of 4 mole equivalents of NAD or in 0.1 M phosphate buffer, pH 6.0, in the presence (○) or absence (□) of 4 mole equivalents of NAD

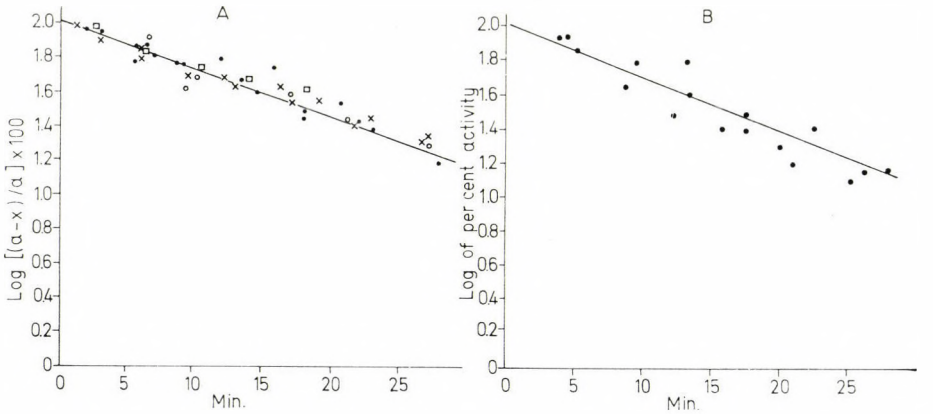


Fig. 2. Changes in histidine content and in enzymic activity during the carbethoxylation of GAPD. *A)* Loss of histidyl residues: 1×10^{-5} M native GAPD, containing about 4 moles of firmly bound NAD per mole of protein, was incubated at 0°C with 1×10^{-3} M DEP (25 equivalents per subunit) for 30 minutes in 0.05 M tris-HCl buffer, pH 6.0 (×) or in 0.1 M phosphate buffer, pH 6.0 (○), as well as charcoal treated enzyme was used in tris buffer (●) or in phosphate buffer (□). *B)* Decrease in enzymic activity: 1×10^{-5} M charcoal treated GAPD was incubated with 1×10^{-3} M DEP in 0.05 M tris-HCl buffer for 30 minutes, pH 6.0, at 0°C. The enzymic activity was measured in 0.1 M glycine buffer at pH 8.5 with GAP substrate, using about 10^{-8} M protein concentration, as described in Methods

2. Isolation of the peptide containing carbethoxylated histidines

Two out of the four histidines which react with DEP may be isolated from the papainic digestion mixture of GAPD-CT_4 in a unique peptide, using the diagonal method of Sajgó (1969), as described in Methods. The amino acid analysis of this peptide is shown in Table 1, compared with the composition of histidyl peptides of swine GAPD.

The data in Table 1 show that the amino acid composition of the peptide containing the two carbethoxy-histidyl residues of GAPD-CT_4 corresponds to that peptide which contains the histidyl residues number 50 and 54 in linear sequence.

Up to now we have been unable to isolate the peptide(s) containing the other two histidyl residues which are able to react with DEP applied in the concentration of 25 moles per mole GAPD subunit.

Table 1

Amino acid composition of peptides in GAPD, containing two histidyl residues

Amino acid	Peptide isolated from carbethoxylated enzyme (average of 5 experiments*)	Peptide in the linear sequence of GAPD analyzed by Harris and Perham (1968)			
		from No. 48 to 62	from No. 37 to 51	from No.157 to 171	from No.163 to 177
Lys	1	2	0	1	0
His	2	2	2	2	2
Asp	2	2	1	1	1
Met	0	0	2	0	1
Thr	2	2	1	0	2
Ser	2	1	1	0	0
Glu	1	1	1	1	1
Gly	2	2	1	2	2
Ala	1	1	0	1	1
Val	1	1	1	2	2
Ile	0	0	0	2	1
Leu	0	0	1	2	1
Tyr	0	0	3	0	0
Phe	1	1	1	1	1

* Two experiments were performed with native GAPD in phosphate buffer, one with charcoal treated GAPD in tris buffer and two with GAPD previously treated with 4 equivalents of p-MB. The pentadecapeptides isolated contain two histidyl residues.

3. Protection with substrates against inactivation

The number of histidyl groups which react in the presence of 25 mole DEP per mole GAPD subunit is identical whether or not NAD and/or phosphate are present during carbethoxylation. However, the decrease in enzymic activity is different (Table 2).

Table 2

Carbethoxylation and decrease in enzymic activity of GAPD

1×10^{-5} M GAPD was incubated with 25 mole equivalents of DEP per subunit at 0°C, during 30 minutes. The enzymic activity was measured after dilution in 0.1 M glycine buffer, pH 8.5. The coenzymes — if added — were present during the DEP treatment in the amount of 4 mole equivalents. No change in the number of SH-groups was found upon either of these treatments

Added materials during DEP treatment	Average number of carbethoxylated histidyl residues per subunit*	Per cent of original activity		
		GAP oxidation	GA oxidation	p-NPA hydrolysis
none**	3.75	0—5	25—30	70
0.1 M phosphate**	3.92	20—30	—	—
NAD**	3.90	20—30	60—70	—
NADH**	3.0	0—5	—	—
NAD + 0.1 M phosphate**	3.80	45—55	—	—
none***	3.98	20—30	60—70	90
NADH***	3.1	35—45	—	—
NAD***	4.13	40—50	60—70	—
NAD*** the samples were gel-filtered after DEP treatment in tris buffer on Sephadex G-50 column	3.97	20—30	—	—

* The average number of carbethoxylated histidyl residues was determined from 7—12 experiments.

** In 0.05 M tris-HCl buffer, pH 6.0

*** In 0.1 M phosphate buffer, pH 6.0

The GAPD-CT_4 formed in the absence of substrates is completely inactive. The data in Table 2 reveal that GAPD is partially protected by substrates against inactivation by DEP. The protective effect is similar with both NAD and phosphate, but that of the two substrates added simultaneously is greater.

From these data it may be assumed that almost two out of four carbethoxylated histidyl residues may be in the neighbourhood of the coenzyme and phosphate ion binding groups.

We measured the change in enzymic activity and the number of reacted histidyl groups with the following methods:

a) 4 to 25 moles of DEP per mole of subunit were added to GAPD and the enzymic activity and the number of reacted histidyl residues were measured after the completion of the reaction;

b) the change in enzymic activity and in the number of reacted histidyl residues of GAPD treated with 25 moles of DEP per mole of subunit was measured as a function of time.

The results of both types of experiments are summarized in Fig. 3.

The results show a linear relation between the reaction of histidines with DEP and the loss of enzymic activity in the absence of substrates, as well as the

protective effect of substrates against inactivation. This protection is marked during the reaction of the first two histidines. The loss of enzymic activity as a function of time during the reaction of the second two histidines with DEP is nearly parallel in each case.

These results suggest the possibility that the reactive histidyl residues may be in the neighbourhood of the active centre, the conformation of this being influenced by the binding of substrate. This change in the steric structure may cause the difference in the enzymic activity of GAPD-CT_4 formed in the absence and in the presence of substrates.

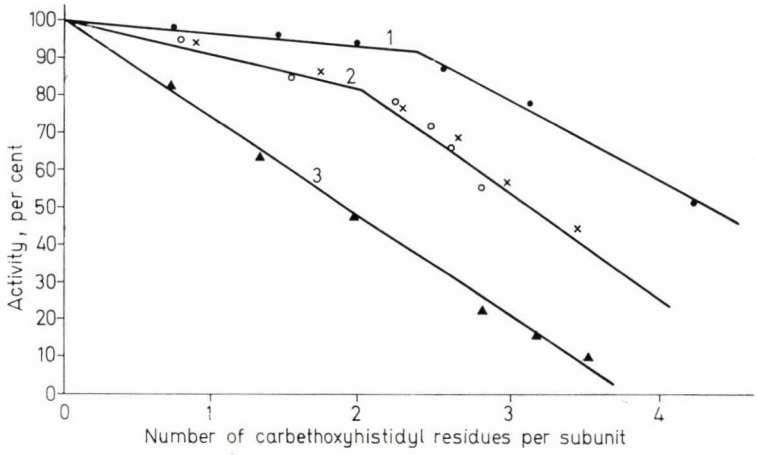


Fig. 3. Changes in the enzymic activity of carbethoxylated GAPD as a function of blocked histidyl groups. 1×10^{-5} M native or charcoal treated enzyme was dissolved in 0.1 M phosphate or 0.05 M tris-HCl buffer at pH 6.0, as indicated. Other conditions as in the legend to Fig. 2. *1.* Native GAPD carbethoxylated in phosphate buffer; *2.* Native GAPD carbethoxylated in tris-HCl buffer (×); charcoal treated GAPD carbethoxylated in phosphate buffer (○); *3.* Charcoal treated GAPD carbethoxylated in tris-HCl buffer

4. Reactivating effect of substrates

The substrates are able not only to protect the enzyme from the inactivating effect of DEP but they can also partially reactivate the fully inactive carbethoxylated enzyme.

When the inactive GAPD-CT_4 was incubated with substrates and mercaptoethanol during 16—20 hours, the enzyme was partially reactivated (Table 3).

The data in Table 3 reveal that neither phosphate, nor mercaptoethanol alone can reactivate the inactive enzyme and the effect of mercaptoethanol + + NAD + phosphate cannot be increased with Zn ion.

It is known that after blocking the reactive SH-groups with specific reagents (e.g. p-MB) the conformation of the enzyme is altered (Szabolcsi, 1958). We blocked the 4 reactive SH-groups per mole of GAPD with p-MB and after this the protein was treated with 25 mole equivalents of DEP per subunit, in the ab-

sence of substrates. Following the removal of p-MB by the addition of mercaptoethanol the number of carbethoxylated histidyl groups was found to be 4 per subunit (GAPD-CT_4^7), however, about 60 per cent of the original activity was regained. Upon further incubation of GAPD-CT_4^7 for 20 hours in the presence of mercaptoethanol and substrates (NAD and phosphate) the enzyme regained practically 100 per cent of the original activity (Table 3).

Table 3

Reactivating effect of substrates

Charcoal treated GAPD was carbethoxylated with 25 mole equivalents of DEP per subunit in 0.05 M tris-HCl buffer, pH 6.0. The fully inactivated GAPD was gel-filtered on a Sephadex G-50 column equilibrated with 0.1 M phosphate buffer, pH 6.0. The gel-filtered samples (protein concentration about 10^{-5} M) were incubated with substrates and ME during 20 hours at 0°C. After incubation the number of blocked histidyl residues was checked (3.7—4.1 per subunit in each case). The enzyme activity was measured, after dilution in 0.1 M glycine buffer, pH 8.5, with GAP substrate

Added materials per mole of GAPD		Per cent of original activity
before treatment with DEP	after treatment with DEP	
—	—	1—4
—	4 equ. NAD	15—17
—	4 equ. NAD + 20 equ. ME	23—27
—	4 equ. ME	4—5
—	4 equ. NAD + 20 equ. ME + + 4 equ. Zn^{++}	24—27
4 equ. p-MB	20 equ. ME*	50—60
4 equ. p-MB	20 equ. ME* 4 equ. NAD + 20 equ. ME	70—80**
4 equ. p-MB	20 equ. ME* 100 equ. NAD + 100 equ. ME	90—100
4 equ. Ag^+	20 equ. ME*	50—60

* The samples — incubated with p-MB or Ag^+ — were reactivated with ME before gel-filtration.

** In these cases the concentration of incubated carbethoxylated samples was about 10^{-6} M.

It has been shown that native GAPD forms a ternary Ag-GAPD-NAD complex with Ag ion in which the reactive thiols are blocked by Ag ion (Boross, 1965). After the DEP treatment of GAPD in the presence of Ag ion, a similar effect was observed as with GAPD blocked with p-MB (Table 3).

It is known that GAPD partially blocked with p-MB or with Ag ion shows the phenomenon of disproportionation (Szabolcsi et al., 1960; Boross, Keleti, 1965). With protein treated with DEP no such process could be demonstrated.

5. Dissociation of NAD from, and changes in conformation of, carbethoxylated GAPD

GAPD-CT_4 was partially freed from NAD by gel-filtration on a Sephadex G-50 column. By carbethoxylation and gel-filtration the E_{280}/E_{260} ratio of the protein is increased from 1.1 to 1.5 (Fig. 4, Fig. 5B).

The elution profile in Fig. 4 shows that about half of the bound NAD dissociates completely from the enzyme as determined after gel-filtration (Fig. 5) and about half remains firmly bound to the enzyme.

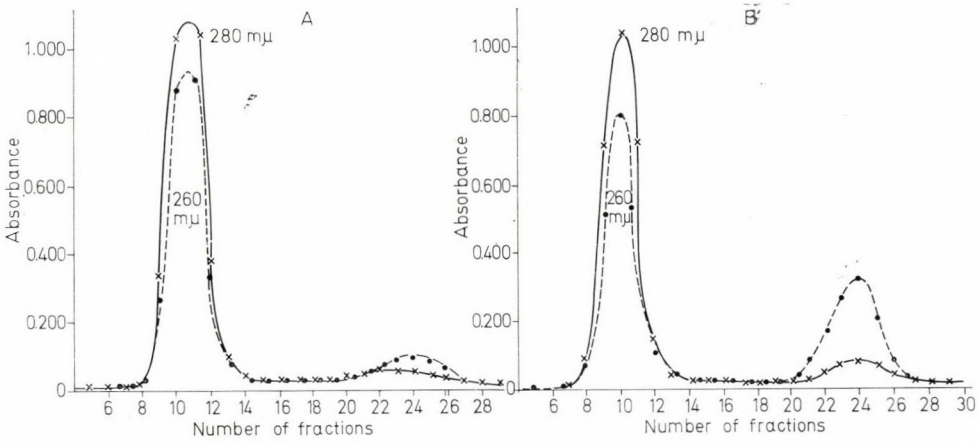


Fig. 4. Gel-filtration profile of GAPD and GAPD-CT_4. *A:* GAPD; *B:* GAPD-CT_4. 4.5 ml of 2×10^{-5} M GAPD was incubated with 2×10^{-3} M DEP in 0.1 ml 95 per cent ethanol and with 0.1 ml 95 per cent ethanol, respectively, in 0.05 M tris-HCl buffer, pH 6.0 at 0°C for 30 minutes. The samples were gel-filtered on a 1.5×20 cm Sephadex G-50 column with 0.05 M tris-HCl buffer, pH 6.0, as eluent. Fractions of 1.5 ml were collected and their optical density was measured in silica cuvettes of 0.5 cm light path at 280 mμ (solid line) and 260 mμ (dotted line). Taking the molar extinction coefficient of NAD as $\varepsilon_{260m\mu} = 1.8\times10^4$, the amount of dissociated NAD was calculated to be 0.7 equivalents in GAPD and 2.4 equivalents in GAPD-CT_4, respectively. In the calculation of the amount of dissociating NAD the molar extinction coefficient at 260 mμ was used and correction was made for the carbethoxylated enzyme, the maximum absorbancy of carbethoxy-histidine being at 240 mμ

Irrespective of the concentration of GAPD — in the concentration range from 3.68×10^{-7} to 2.5×10^{-5} M — only two out of the four bound NAD per mole enzyme are reducible as determined in the presence of excess GAP and arsenate, in tris-HCl buffer, pH 6.0. Two reducible NAD may be determined by dilution of GAPD-CT_4 with glycine buffer, pH 8.5, in the concentration range from 2.5×10^{-5} to 3.2×10^{-6} M after gel-filtration. In this case the enzyme only contains about two equivalents of bound NAD. However, by diluting GAPD-CT_4 in tris-HCl buffer, pH 6.0, up to the same concentration as above after gel-filtration, no reducible bound NAD is detectable (Fig. 5).

Changes in the intensity of protein fluorescence at 340 mμ are usually ascribed to alterations in steric structure assuming that buried tyrosines and trypto-

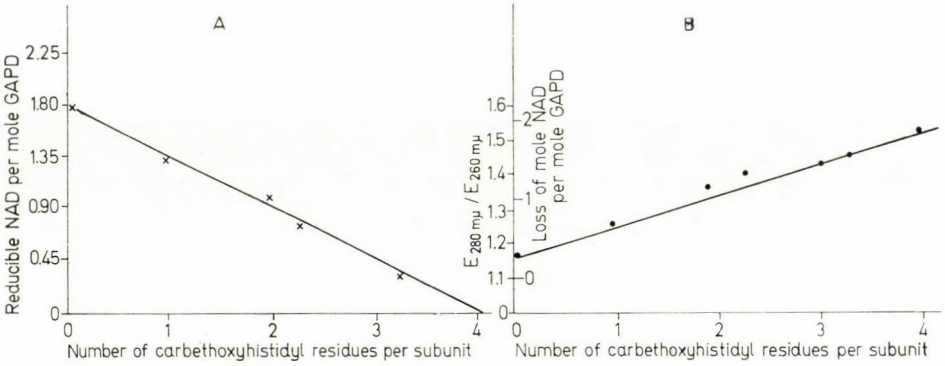


Fig. 5. Relation between the dissociation and reducibility of firmly bound NAD after gel-filtration of GAPD-CT_4. *A:* amount of reducible firmly bound NAD; *B:* loss of firmly bound NAD. Treatment and gel-filtration of the enzyme as in the legend to Fig. 4. The amount of reducible bound NAD was measured in tris-HCl buffer, pH 6.0 in the presence of excess GAP and arsenate (1×10^{-5} M gel-filtered GAPD-CT_4 + 1×10^{-3} M GAP + 1×10^{-3} M arsenate measured at 340 mμ in a cuvette of 4 cm light path) and calculated with the molar extinction coefficient $\varepsilon_{340m\mu} = 6.22 \times 10^3$

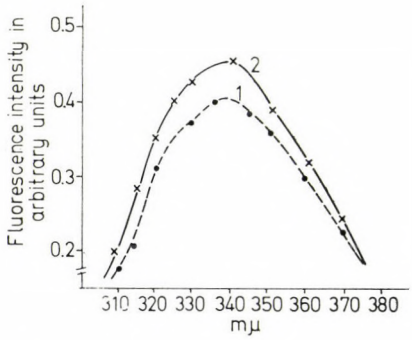


Fig. 6. Fluorescence spectra of GAPD and GAPD-CT_4. *1.* GAPD in tris-HCl buffer, containing 2 per cent ethanol; *2.* GAPD-CT_4 in tris-HCl buffer. 50 μg/ml enzyme concentration was used. Activation at 290 mμ

phans become unmasked and ionized and thus excitable at 280 mμ, or *vice versa* (Joly, 1965). The maximum intensity of fluorescence spectrum at 340 mμ increased upon carbethoxylation (Fig. 6).

Marked changes in fluorescence intensity with changes in pH in the range of pH 5.0 to 8.5 are ascribed to the existence of histidine-tryptophan complexes in the protein (Shinitzky, Goldman, 1967). No such changes in fluorescence intensity were observed either with GAPD or with GAPD-CT_4 in the range of pH 6.0 to 9.0.

It is known that the carbethoxy-histidyl bond can be disrupted at alkaline pH (Mühlrad et al., 1967). GAPD-CT_4 formed in the presence of substrates was

incubated at pH 9.3 in 0.1 M glycine buffer. As measured with the difference spectrum at 240 mμ the carbethoxy groups were broken quantitatively but the enzyme remained inactive. Further incubation of these samples in the presence of substrates and mercaptoethanol does not lead to the reactivation of the enzyme.

Discussion

Severin and Nagradova (1958; Nagradova, 1958) were the first to suggest some role of the histidyl residues in the catalytic activity of GAPD. Later, applying the reagent Roussin-salt, Dobry-Duclaux (1961, 1961a) reached the same conclusion. Friedrich et al. (1964) demonstrated by photo-oxidizing GAPD that the enzyme contains slowly and rapidly photo-oxidizable histidyl residues. The rate constant of the photo-oxidation of the "fast" histidines equals the rate constant of inactivation of the enzyme in the GAP-oxidation reaction. The pH-dependence of Michaelis constants and of maximum velocities suggest the role of ionizable histidyl residues in the catalysis of GAP-oxidation (Keleti, Batke, 1967). Park and co-workers (Olson, Park, 1964; Mathew et al., 1967) postulated that histidine also participates in the esterolytic activity of GAPD.

Our results show that the histidyl residues of GAPD may be classified into two categories:

1. six residues react if 80—120 mole equivalents of DEP per subunit are used;
2. five further residues per subunit only react in the denatured protein.

From these results it may be concluded that five histidines are buried in the enzyme-subunit. Six histidyl groups are on the surface of the protein and four from these react when 25 mole equivalents of DEP per subunit are applied*.

The rate constant of carbethoxy-histidine formation is practically the same as that of the inactivation of the enzyme treated with DEP in the absence of substrates. However, the four histidyl residues in GAPD-CT_4 are not directly involved in the catalytic activity, since:

1. GAPD-CT_4 may be partially protected from inactivation if the treatment with DEP is carried out in the presence of substrate;
2. the partially active GAPD-CT_4 is partially inactivated by dissociation of one of the substrates from the enzyme;
3. completely inactive GAPD-CT_4 may be partially reactivated by incubating the enzyme with substrates and mercaptoethanol;

* These data probably do not indicate a particular structure for GAPD since qualitatively the same results are obtained with α-glycerophosphatedehydrogenase (GDH) crystallized from rabbit muscle (Telegdi, 1964). Assuming two subunits per 76 000 molecular weight (Telegdi, Keleti, 1968), two histidyl residues per subunit react when 50 mole equivalents of DEP per subunit are used, 5 residues if 200 mole equivalents of the reagent are used and 10—11 histidyl residues per subunit when the reaction is carried out in 8 M urea. This is in good agreement with data showing in GDH 9 histidines per subunit of 35 000 molecular weight (van Eys et al., 1964). The enzymic inactivation of GDH is proportional to the number of carbethoxylated histidines.

4. partially inactive GAPD-CT_4^1 which is formed after blocking the reactive SH-groups with p-MB, and treating with mercaptoethanol, may be completely reactivated by incubating the enzyme with mercaptoethanol and substrates.

It is to be noted that the process of reactivation is a very slow one, it takes place in about 20 hours.

The substrates protect GAPD from the inactivation caused by the blocking of two histidines. It may be assumed that these histidyl residues are in the neighbourhood of the active center.

One peptide containing two carbethoxylated histidyl residues was isolated, purified and its amino acid composition determined. The reacting histidyl residues were identified as those of number 50 and 54 in the linear sequence of swine muscle GAPD (Harris, Perham, 1968).

It is possible that two of the four histidyl residues which react under different conditions (in the presence and absence of substrates and/or p-MB) are not the same residues. Moreover, we have no evidence whether only four well defined histidyl residues react in the presence of 25 mole equivalents DEP per subunit or two residues are well defined and two react statistically from the remaining four residues reacting with a similar rate constant in the native protein. These possibilities are under investigation.

Another possibility is that the reaction of DEP with SH-groups is partly responsible for the inactivating effect of carbethoxylation. However, with p-MB titration no loss was measured in the number of SH-groups after DEP treatment. A further possibility would be the competition of p-MB and DEP, assuming that DEP cannot split off the S—Hg bond, but p-MB can split off the S-carbethoxy bond. However, no experimental data about the reaction of DEP at pH 6.0 with cysteinyl residues were obtained either in this work or in the literature (Mühlrad et al., 1967; Ovádi et al., 1967), and our data also show that mercaptoethanol alone is not able to reactivate the carbethoxylated enzyme. Therefore these results suggest that:

1. mercaptoethanol protects the reactive SH-groups from oxidation during the long incubation with substrates;

2. the conformation of the active site of GAPD is partially protected by substrates;

3. substrates may partially reconstitute the conformation of the active site.

However, the reactive histidyl residues are not directly involved in the catalytic activity. These assumptions are confirmed by the results showing the loosening of protein structure after DEP-treatment.

Concerning the assumptions on the role of histidyl residues in the catalytic activity of GAPD we suggest the following alternatives:

1. the histidyl residues are not directly involved in the catalysis, but take part only in the stabilization of the native structure;

2. the histidyl residues which are able to react with 25 mole equivalents of DEP per subunit are not those which are directly involved in the catalytic activity;

3. the role in the catalysis of the histidyl residues which take part in the formation of the active center and are able to react with DEP is not absolute i.e. other groups may substitute them if they are blocked (Keleti, 1969a).

It is known from sequence analysis that six histidyl residues of swine muscle GAPD (Harris, Perham, 1968) are absent in the lobster enzyme (Davidson et al., 1967). However, the enzymic activity of the two GAPD-s is the same. The histidines in native swine muscle GAPD which can react with DEP without irreversible loss of enzymic activity may be those which are absent in lobster muscle GAPD.

The histidine residue No. 50 in the linear sequence is present also in the lobster enzyme, whereas His No. 54 is absent.

It is to be noted that NADH does not protect the enzyme against inactivation by DEP, however, the protecting effect of NADH + phosphate is similar to that of NAD + phosphate. This is in agreement with data showing that the binding of NADH differs from that of NAD (Tucker, Grisolia, 1962; Nagradova, 1965; Keleti, Batke, 1965; Keleti, 1966, 1969), with those demonstrating the stabilizing effect of phosphate (Velick, Hayes, 1953; Előd i, 1958; Dévényi et al., 1960; Telegdi, Keleti, 1961; Keleti, 1965, 1969), as well as with those pointing to an interaction of the different substrate binding sites in the same subunit (Keleti, Batke, 1965; Batke, Keleti, 1968).

Batke (1968) has demonstrated that the reducibility of firmly bound NAD is dependent on GAPD concentration if measured in the presence of arsenate in glycine buffer, pH 8.5. We have found that, irrespective of protein concentration, only two firmly bound NAD molecules are reducible by substrate in the presence of arsenate if native enzyme is tested in tris buffer, pH 6.0. Our experiments with carbethoxylated and gel-filtered GAPD show that there is an increase in the dissociation constant of the firmly bound NAD-s which remain bound to the GAPD--CT_4 and are reducible only in glycine buffer, pH 8.5. These data are in agreement with those of Batke (1968) showing the possibility of unsymmetrical binding of the 4 identical subunits of GAPD.

If histidines are, indeed, involved in the stabilization of the steric structure of GAPD, this cannot be due to the formation of a histidine-tryptophan complex since spectrofluorimetric data indicate the absence of the postulated interaction in GAPD.

The authors wish to thank Dr T. Dévényi and the technical assistant, Mrs J. Báti for the amino acid analysis. The valuable suggestions of Dr M. Sajgó in the course of the isolation of peptides containing the carbethoxy-histidyl residues were most helpful. The technical assistance of Mrs I. Batke and Mrs M. Nuridsány is gratefully acknowledged.

References

Batke, J. (1968) FEBS Letters *2* 81
Batke, J., Keleti, T. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 385
Boross, L. (1965) Biochim. Biophys. Acta *96* 52
Boross, L., Keleti, T. (1965) Acta Physiol. Acad. Sci. Hung. *27* 397
Boyer, P. D. (1954) J. Am. Chem. Soc. *76* 4331
Davidson, B. E., Sajgó, M., Noller, H. F., Harris, J. I. (1967) Nature *216* 1181
Dévényi, T. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 429
Dévényi, T., Előd i, P., Keleti, T., Szabolcsi, G. (1960) Biol. Közl. *8* 3
Dobry-Duclaux, A. (1961) Biochim. Biophys. Acta *54* 76

Dobry-Duclaux, A. (1961a) Biochim. Biophys. Acta *54* 84
Elődi, P. (1958) Acta Physiol. Acad. Sci. Hung. *13* 199
Elődi, P., Szörényi, E. T. (1956) Acta Physiol. Acad. Sci. Hung. *9* 339
Eys, J. van, Judd, J., Ford, J., Womack, W. B. (1964) Biochemistry *3* 1755
Friedrich, P., Polgár, L., Szabolcsi, G. (1964) Acta Physiol. Acad. Sci. Hung. *25* 217
Gray, W. R., Hartley, B. S. (1963) Biochem. J. *89* 379
Harrington, W. F., Karr, C. M. (1965) J. Mol. Biol. *13* 885
Harris, J. I., Perham, R. N. (1965) J. Mol. Biol. *13* 876
Harris, J. I., Perham, R. N. (1968) Nature *219* 1025
Joly, M. (1965) A Physico-chemical Approach to the Denaturation of Proteins. Acad. Press, New York
Keleti, T. (1965) Acta Physiol. Acad. Sci. Hung. *28* 19
Keleti, T. (1966) Acta Physiol. Acad. Sci. Hung. *29* 101
Keleti, T. (1969) Reaktionskinetik, Wirkungsmechanismus und aktives Zentrum [in Dévényi, T., Elődi, P., Keleti, T., Szabolcsi, G.: Strukturelle Grundlagen der biologischen Funktion der Proteine]. Akadémiai Kiadó, Budapest
Keleti, T. (1969a) Symposium on NAD-dependent Dehydrogenases. Konstanz (in press)
Keleti, T., Batke, J. (1965) Acta Physiol. Acad. Sci. Hung. *28* 195
Keleti, T., Batke, J. (1967) Enzymologia *33* 65
Mathew, B., Meriwether, B. P., Park, J. H. (1967) J. Biol. Chem. *242* 5024
Mühlrad, A., Hegyi, G., Tóth, G. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 19
Nagradova, N. K. (1958) Biokhimiya *23* 511
Nagradova, N. K. (1965) Biokhimiya *30* 50
Olson, E. J., Park, J. H. (1964) J. Biol. Chem. *239* 2316
Ovádi, J., Libor, S., Elődi, P. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 455
Sajgó, M. (1969) in preparation
Severin, S. E., Nagradova, N. K. (1958) Dokl. Akad. Nauk SSSR *121* 519
Shinitzky, M., Goldman, R. (1967) European J. Biochem. *3* 139
Szabolcsi, G. (1958) Acta Physiol. Acad. Sci. Hung. *13* 213
Szabolcsi, G., Biszku, E., Sajgó, M. (1960) Acta Physiol. Acad. Sci. Hung. *17* 183
Szewczuk, A., Wolny, E., Wolny, M., Baranowski, T. (1961) Acta Biochim. Polon. *8* 201
Telegdi, M. (1964) Acta Physiol. Acad. Sci. Hung. *25* 177
Telegdi, M., Keleti, T. (1961) Acta Physiol. Acad. Sci. Hung. *18* Suppl. p. 49
Telegdi, M., Keleti, T. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 131
Tucker, D., Grisolia, S. (1962) J. Biol. Chem. *237* 1068
Velick, S. F., Hayes, J. E., Jr. (1953) J. Biol. Chem. *203* 545
Watson, H. C., Banaszak, L. J. (1964) Nature *204* 918

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (4), pp. 379–383 (1969)

Fractionation of Crystallized Ovalbumin on DEAE-Cellulose

Á. FURKA, F. SEBESTYÉN

Department of Organic Chemistry, L. Eötvös University, Budapest

(Received July 9, 1969)

A method for preparative scale separation of the components of crystallized ovalbumin was developed. No difference was found either in the amino acid composition or in the carbohydrate content of the components A_1 and A_2.

Introduction

Ovalbumin, comprising about three-fifths of the total protein content of egg white, was first crystallized by Hofmeister (1889). A standard method of crystallization was developed (Sørensen, Høyrup, 1917) and widely used since that time for the preparation of the crystalline protein. Although in 1932 ovalbumin was regarded as one of the two proteins which could be considered as pure substances (Schmidt, 1932), later it was shown to be electrophoretically inhomogeneous (Longsworth et al., 1940). Its three components, termed A_1, A_2 and A_3, were found to be present in different amounts. 81 to 84 per cent A_1, 14 to 16 per cent A_2 and 2 to 4 per cent A_3 have been reported (Perlman, 1950). As a possible explanation for the electrophoretic inhomogeneity, it has been suggested (Linderstrøm-Lang, Ottesen, 1949) and substantiated by dephosphorylation studies (Perlman, 1952) that A_1, A_2 and A_3 differ in their phosphorus content. A_1 has two phosphorus atoms per molecule, A_2 has one, while A_3 has none.

Both A_1 and A_2 were isolated in pure form by stepwise elution from CM-cellulose (Rhodes et al., 1958) and shown to contain 1.99 and 0.93 atoms of phosphorus per molecule, respectively. DEAE-cellulose was used for the separation of proteins of egg white and eleven peaks were found, two of which are identified as A_1 and A_2 (Mandeles, 1960).

This paper reports a procedure for preparative scale separation of the components of ovalbumin by ion exchange chromatography on DEAE-cellulose.

Experimental

Crystalline ovalbumin was prepared from fresh hen eggs and six times crystallized according to a standard procedure (Sørensen, Høyrup, 1917).

Chromatographic procedure. 1 g of ovalbumin in 14 ml of starting buffer was applied to a column (4 × 60 cm) of DEAE-cellulose (Whatman DE 11, 100—325 mesh) previously equilibrated with 0.017 molar acetate buffer, pH 4.4 (adjust-

ed with sodium hydroxide). Elution was done with a linear gradient of 2 l each of 0.017 molar acetate and 0.075 molar acetate at a pH of 4.4. The flow rate was 120 ml per hour, 15 ml fractions being collected. The optical density of the fractions was measured at 280 mμ. The pooled fractions were dialyzed against five changes of distilled water, then freeze dried. All operations were performed at 4°C.

Amino acid analysis. The samples were hydrolyzed with 6 N hydrochloric acid at 105°C for 20 hrs, the amino acids were determined by means of EEL automatic amino acid analyzer.

Reduction and aminoethylation were performed according to the method of Raftery and Cole (1966).

Performic acid oxidation was also used for the determination of the total half cystine + cysteine content (Moore, 1963).

Results and Discussion

To find a procedure suitable for preparative scale separation of the components of ovalbumin, a wide variety of experimental conditions were tested. In general, chromatographic runs on DEAE-cellulose gave much better results than

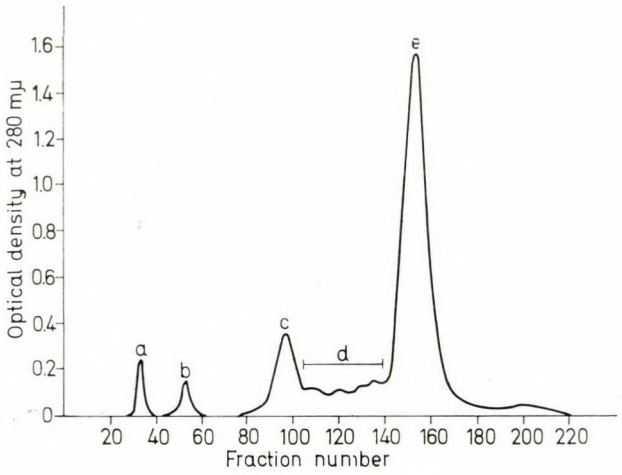


Fig. 1a

those on CM-cellulose column. The best separation of the three components was obtained at pH 4.4, using a linear gradient of acetate buffer from 0.017 M to 0.075 M. The chromatogram is presented in Fig. 1a. Based on optical density units, 91 per cent of the material applied to the column was recovered. Peak *a* was an impurity. Its quantity varied from one run to the other with different preparations of crystallized ovalbumin. Peaks *b*, *c*, and *e* were identified as A_3, A_2 and A_1, respectively. Under somewhat different experimental conditions these three components were eluted far behind peak *a*. The quantities of the fractions, calculated on the basis of optical density units, were 64.6 per cent A_1, 11.3 per cent A_2, 2.2 per cent A_3, 2.6 per cent impurity under *a* and 12.4 per cent unidentified

material under *d*. Partial denaturation of the native proteins was also observed at each run. The chromatographic separations were found to be reproducible, but very sensitive to pH changes.

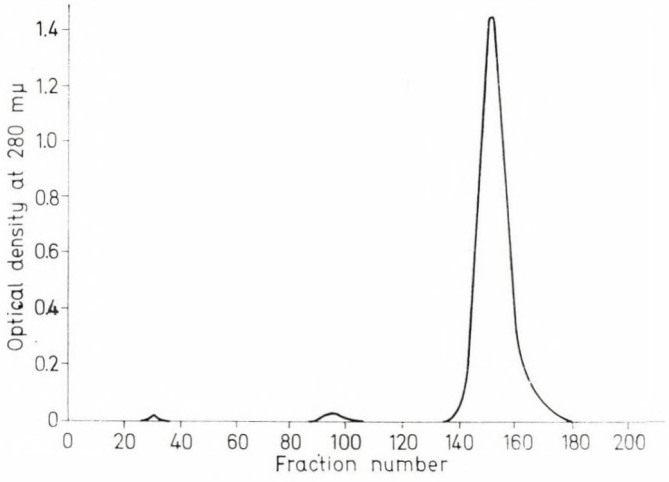


Fig. 1b

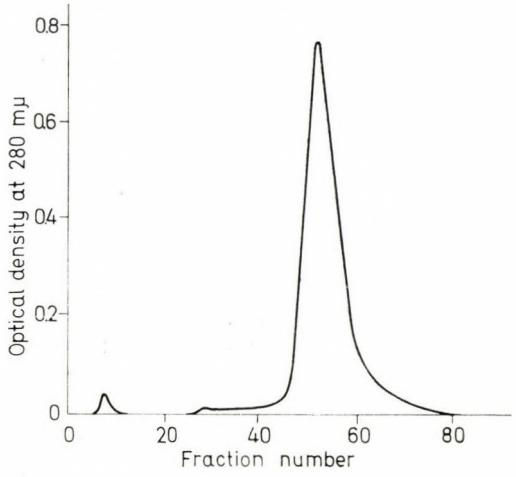


Fig. 1c

Fig. 1. Chromatography of crystalline ovalbumin on DEAE-cellulose. *a)* Fractionation of crystallized ovalbumin on DEAE-cellulose column. For details see experimental part. *b)* Rechromatography of fraction "e" (A_1). 760 mg of A_1 was eluted from a 4×60 cm column. Gradient: 0.018 molar acetate buffer (3 l, pH 4.4), to 0.065 molar acetate buffer (3 l, pH 4.4). Flow rate, 120 ml/hour; fraction size, 15 ml. *c)* Rechromatography of fraction "c" (A_2). 87 mg of A_2 was eluted from a 1.7×25 cm column. Gradient: 0.01 M to 0.1 M acetate buffer at pH 4.4, 700 ml each. 5 ml fractions per 5 minutes were collected

A_1 and A_2 were rechromatographed (Figs 1b and 1c). The results showed that no transformation of one component to the other took place, at least under the experimental conditions used. It can also be seen that the first run gave practically pure fractions.

Table 1

Amino acid composition

Residues/mole

	Crystallized ovalbumin	A_1	A_2
Lys	20.2	21.0	21.0
His	7.7	7.0	6.2
Arg	16.5	16.4	17.1
Asp	32.0	32.1	33.7
Ser	34.0	34.7	35.0
Thr	16.1	15.6	15.2
Glu	47.1	48.3	49.0
Pro	14.4	14.6	14.6
Gly	20.2	19.8	20.3
Ala*	35.0	35.0	35.0
Val	30.6	28.1	28.1
Met	15.4	14.3	15.9
Ile	24.2	22.3	21.0
Leu	32.6	31.4	32.4
Tyr	10.1	9.6	9.5
Phe	18.5	19.6	19.7
Cys**		7.1	
Cys***		7.4	

* Ala arbitrarily fixed as 35.
** Total half cystine + cysteine determined as aminoethyl cysteine.
*** Total half cystine + cysteine determined as cysteic acid.
Tryptophan was not determined.

The amino acid composition of the rechromatographed fractions and that of the six times crystallized protein were determined. The data summarized in Table 1 show practically no difference between A_1 and A_2 in amino acid composition, both of them being similar to that of the crystallized ovalbumin.

The glucosamine content of the two components was also found to be similar (2.5 and 3.2 moles of glucosamine per mole of A_1 and A_2, respectively). Assay by the anthron method (Yemm, Willis, 1954) revealed approximately 5 moles of mannose in both A_1 and A_2.

Thus, it can be concluded that the only difference between A_1 and A_2 remains in their different phosphorus content as suggested earlier.

Thanks are due to Prof. V. Bruckner for his interest in this work, to Dr H. Medzihradszky and S. Kutassy for amino acid analysis, and to Mrs I. Császár and Mrs Zs. Gémes for technical assistance.

References

Hofmeister, F. (1889) Z. physiol. Chem. *14* 165
Linderstrøm-Lang, K., Ottesen, M. (1949) Compt. Rend. Trav. Lab. Carlsberg *26* No. 16
Longsworth, L. G., Cannan, R. K., MacInnes, D. A. (1940) J. Am. Chem. Soc. *62* 2580
Mandeles, S. (1960) J. Chromatog. *3* 256
Moore, S. (1963) J. Biol. Chem. *238* 235
Perlman, G. E. (1950) Nature *166* 870
Perlman, G. E. (1952) J. Gen. Physiol. *35* 711
Raftery, M. A., Cole, R. D. (1966) J. Biol. Chem. *241* 3457
Rhodes, M. B., Azari, P. R., Feeney, R. E. (1958) J. Biol. Chem. *230* 399
Schmidt, C. L. A. (1932) Ann. Rev. Biochem. *1* 160
Sørensen, S. P. L., Høyrup, H. (1917) Compt. Rend. Trav. Lab. Carlsberg *12* 164
Yemm, E. W., Willis, A. J. (1954) Biochem. J. *57* 508

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (4), pp. 385–389 (1969)

Distribution of the Sulfhydryl Groups of Rabbit Muscle Aldolase in the Polypeptide Chain

(Preliminary Communication)

M. SAJGÓ

Institute of Biochemistry, Hungarian Academy of Sciences, Budapest
(Received September 19, 1969)

Rabbit muscle aldolase is composed of four subunits (Kawahara, Tanford, 1966) which are either identical or differ by pairs. In the latter case, however, the difference is only slight and is confined to a small section of the polypeptide chain (Lai, 1968).

In our studies on the primary structure of aldolase, first we intended to establish the amino acid sequence around the SH-groups and the distribution of SH-groups along the polypeptide chain.

As a starting material aldolase carboxymethylated with ^{14}C-bromoacetate was used. The molecular weight of the protein subunit was taken to be 40 000.

Aldolase prepared according to Taylor et al. (1949) was dissolved in 0.05 M Tris buffer pH 8.5, containing 8 M urea, to a final concentration of 10 mg per ml (0.25 μmoles per ml). The protein solution was incubated with dithioerithritol (2 moles per mole of protein SH) at room temperature for 1 hour, then ^{14}C-bromoacetate (2.5 moles per mole of total SH) was added and the mixture was incubated for another two hours at room temperature. The excess bromoacetate was removed by incubation for 30 minutes with an equimolar amount of dithioerithritol, then the solution was dialyzed salt-free against 0.001 M hydrochloric acid. To the dialyzed solution chymotrypsin-free trypsin (in a trypsin : protein weight ratio of 1 : 50) was added and the pH adjusted to 8.5 with solid ammonium bicarbonate. Digestion was carried out at 37°C for 3 hours, the hydrolysate was freeze-dried and the peptides were fractionated by three-dimensional electrochromatography (Fig. 1). The radioautograms of the electrophoretogram and chromatogram thus obtained (Fig. 1a and 1b) show that only fraction N 1 contained two radioactive components (N 11 and N 12), the others contained only one radioactive peptide.

Because of the great number of components formed during tryptic digestion only peptides B', N 2 and N 3 could be isolated in a homogeneous form, using the electrochromatographic method only. To purify the other radioactive components, the mixture first had to be fractionated by gel-filtration on a Sephadex G–25 column.

To determine the amino acid composition of the seven radioactive peptides thus obtained each peptide (0.05–0.1 μmole) was hydrolyzed in 6 N hydrochloric acid in vacuo for 16 hours. The amino acid composition was determined

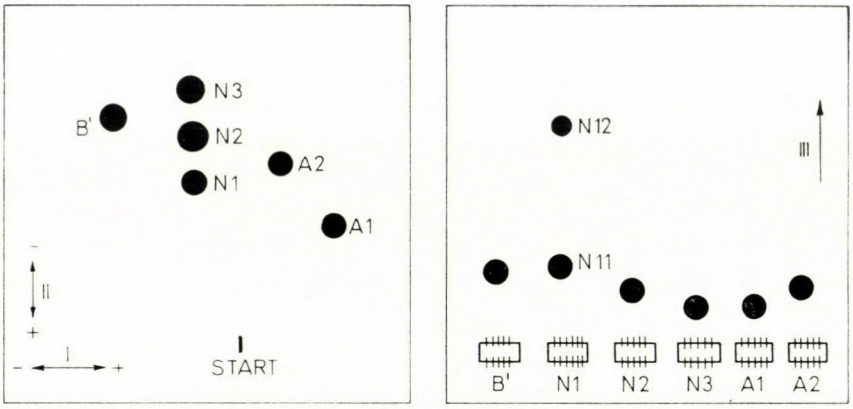


Fig. 1. Three-dimensional pattern of the radioactive peptides of the tryptic hydrolysate of ^{14}C—CM-aldolase On the basis of the radioautogram made after the two dimensional electrophoresis (a) the paper strips containing the radioactive components were cut out, sewn onto a new sheet of paper and subjected to ascending chromatography (b).
I: electrophoresis in pyridine-acetic acid buffer, pH 6.5, 45 V per cm, 90 minutes.
II: electrophoresis in formic acid-acetic acid buffer, pH 1.9, 65 V per cm, 90 minutes.
III: ascending chromatography in isoamylalcohol-pyridine-water (35:35:30) system

in a Beckman-Unichrom amino acid analyzer, by using Dévényi's (1968) single column method. Sequence analyses were performed by the "dansyl"-Edman procedure (Gray, Hartley, 1963), the "dansyl"-amino acids were identified by thin-layer electrophoresis.

The total or partial sequences of the seven peptides are shown in Table 1.

Table 1

CM-cysteinyl peptides isolated from the tryptic hydrolysate of carboxymethylated aldolase

N 11	Ala.Leu.Ala.CMC.Ser.$(Asn_1Gln_1Gly_1Ala_1Leu_1)$Lys
N 12	CMC.Pro.Leu.$(Pro_1Leu_1Trp_1)$Lys
N 2	CMC.Ala.Gln.Tyr.Lys
N 3	CMC.Val.Leu.Lys
A 1	Ala.$(His_1CMC_1Asx_1Ser_1Glx_1Pro_2Gly_2Val_{1-2}Ile_{2-3}Leu_2Tyr_2)$Lys
A 2	CMC.Gln.Tyr.$(Thr_1Glu_1Val_1)$Lys
B'	Met.Val.Thr.Pro.Gly.His.Ala.CMC.Thr.Gln.Lys

Next we intended to determine the approximate distribution of SH-groups along the polypeptide chain. Fully carboxymethylated aldolase was cleaved by cyanogen bromide at the methionine residues: 100 mg of modified aldolase was dissolved in 20 ml of 70 per cent formic acid and incubated with 200 mg of cyanogen bromide at room temperature for 40 hours. The reaction mixture was dried in vacuo. Since aldolase contains three methionines per subunit, cyanogen bromide-treatment results in four fragments (Lai, 1968). The fragments are large and therefore poorly soluble. To increase solubility, the mixture of fragments was acylated with maleic anhydride (Butler et al., 1969) as follows: 100 mg of dry

peptide-mixture was dissolved in 10 ml of 0.1 M pyrophosphate-8 M urea. Two hundred mg of maleic anhydride was added with continuous stirring to the solution in three equal portions. The pH was kept at 9 with 10 per cent sodium hydroxide. After the completion of the reaction the urea and the salts were removed by gel-filtrations on a Molselect G – 25 (coarse) column, and the peptide fraction was freeze-dried.

The maleylated peptide mixture was then gel-filtered on a Sephadex G – 75 (fine) column equilibrated with 0.1 M ammonium bicarbonate. In this way the cyanogen-bromide-treated protein could be resolved into four fractions which corresponded to four fragments of different size. Though the separation of maleylated derivatives was much less sharp than found by Lai (1968) under other conditions, the fractions proved to be homogeneous.

To determine the distribution of the seven tryptic peptides, the four fragments obtained after cyanogen bromide cleavage and maleylation were incubated in 5 per cent acetic acid – 2 per cent pyridine at 60°C for 16 hours. By this treatment maleyl groups were removed and all ε-amino groups were recovered. The deacylated fragments were digested with trypsin in 1 per cent ammonium bicarbonate buffer at 37°C for 3 hours. The tryptic hydrolysate was freeze-dried, then subjected to three-dimensional electrochromatography. The radioautographic pattern of the electro-chromatograms indicated that the various fragments contained the following tryptic CM-cysteinly peptides: fragment 1: N 3 and N 2; fragment 2: N 11 and N 12; fragment 3: A 1 and A 2; fragment 4: B'.

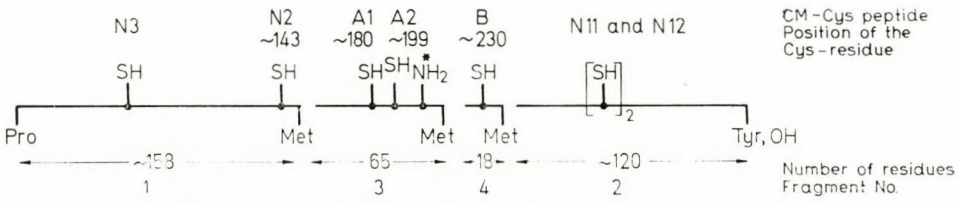
The approximate position of the SH-group of peptide N 2 was deduced from the analysis of the tryptic hydrolysate of maleylated fragment 1. On hydrolysis there also appeared a smaller fragment which contained homoserine as C-terminal and comprised peptide N 2 as well. From the sequence analysis of this peptide we could derive the approximate position of the above cysteinyl residue, taking into account that fragment 1 consisted of about 158 amino acid residues.

Similarly, peptides A 1 and A 2 were located by analyzing the tryptic hydrolysate of maleylated fragment 3. The position of the SH-group in peptide B' came from the residue-number of fragments 1 and 3, and from the amino acid sequence determined by Lai et al. (1965). The SH-group of peptides N 3, N 11, and N 12 could not be located yet.

Consequently, the approximate positions of SH-groups in the polypeptide chain are the following:

The SH-groups of peptide B' and those of peptides N 11 and N 12 (the position of which is still unknown) are of special interest.

Szajáni et al. (1969, 1969a) have shown that three out of the seven SH-groups of aldolase can be selectively carboxymethylated in the native protein.



Kinetic analysis of the alkylation reaction and of the concomitant loss of enzyme activity has shown that carboxymethylation of the SH-group that reacts at the highest rate does not affect enzymic activity. The loss of activity appears to run parallel with the alkylation of a slower-reacting SH-group, whereas carboxymethylation of the third SH-group has no effect on enzyme activity.

The SH-group carboxymethylated at the highest rate is identical with that of peptide B' and is at a distance of 10 amino acids from the lysine residue that forms the Schiff-base with dihydroxyacetone phosphate. The SH-group associated with enzyme activity is found in peptide N 11, which in turn is located in the C-terminal part of the polypeptide chain. The third SH-group that can be carboxymethylated in the native enzyme is also found in the C-terminal half and is comprised in peptide N 12.

References

Butler, P. J. E., Harris, J. I., Hartley, B. S., Leberman, R. (1969) Biochem. J. *112* 679
Dévényi, T. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 429
Gray, W. R., Hartley, B. S. (1963) Biochem. J. *89* 59P
Kawahara, K., Tanford, C. (1966) Biochemistry *5* 1578
Lai, C. Y. (1968) Arch. Biochem. Biophys. *128* 202
Lai, C. Y., Hoffee, P., Horecker, B. L. (1965) Arch. Biochem. Biophys. *112* 567
Szajáni, B., Friedrich, P., Szabolcsi, G. (1969) Acta Biochim. Biophys. Acad Sci. Hung. *4* 265
Szajáni, B., Sajgó, M., Biszku, E., Friedrich, P., Szabolcsi, G. (1969a) in preparation
Taylor, J. F., Green, A. A., Cori, G. T. (1949) J. Biol. Chem. *173* 591

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (4), pp. 389—394 (1969)

Submicroscopic Transversal Structure of the Striated Muscle

K. TROMBITÁS

Central Laboratory, Medical University, Pécs

(Received February 27, 1969)

The submicroscopic transversal structure connecting the longitudinal elements was investigated in a longitudinal section of striated muscle. A periodic transversal structure similar to the transversal lines occasionally seen in stained electron microscopic preparations was demonstrated with the aid of metal vapour treatment.

Introduction

Early electron microscopic investigations performed on fibrils have already found a submicroscopic cross striation of 400 Å periodicity in striated muscle (Hall, Jakus, Schmitt, 1946). Later on, this observation has been corroborated by Draper and Hodge (1949, 1950), Huxley (1951, 1953), Ernst and Benedetzky (1962), Ernst et al. (1969), Pepe, (1967). The unequivocal solution of the problem is rendered difficult by the fact that, in normal electron micrographs, the transversal structure often fails to appear clearly and many times it cannot be observed at all. On the other hand, Robertson (1956), Ernst and Benedetzky (1962), presented photographs where a transversal line system dominated in the longitudinal section, instead of the longitudinal structure.

In the present paper some electron micrographs of stained thin section will be presented which clearly show a periodic transversal structure. In our opinion the examination of this question needs the introduction of other methods.

Materials and Methods

Frog sartorius *(Rana esculenta)* was used in the experiments. The muscle was slightly spanned and placed at once into a 1 per cent OsO_4 fixer buffered to pH 7.4 according to Palade's method (1952). The material was kept in the fixer at 0 °C for one hour. The fixation was followed by washing with buffer and dehydration in an alcohol series of increasing concentration. The dehydrated material was embedded in a 4 : 1 mixture of butyl–methyl methacrylate. Longitudinal sections were made from the embedded material with an LKB microtome; the sections were placed on a grid with a formvar film. With a part of the sections double contrasting with 2.5 per cent uranyl acetate in 50 per cent alcohol was performed for one hour. Therefore, the sections were kept for 30 minutes in lead hydroxide prepared according to the method of Karnovsky (1961).

With the other part of the sections no contrasting by staining was used. Here, the embedding material was dissolved out of the electron microscopic sections placed on the formvar film by an amylacetate treatment for 10 to 60 minutes, and it was steamed in a Zeiss type vacuum steamer with gold or platinum at an angle of 25°. The steaming was done in two directions: a part of the sections was steamed perpendicular and the other part parallel to the fibrils. The direction of steaming was secured as follows: the block was sectioned in such a way that the longitudinal axis of the section cut down in the form of a band was parallel to that of the fibrils. The band of section placed on the grid can be well seen in reflex-light. A part of the grids was placed in the steamer in such a way that the longitudinal axis of fibrils fell together with the direction of steaming, i.e. the steaming was parallel to the longitudinal axis of fibrils. The other part was placed in such a way that the steaming was perpendicular to the longitudinal axis of fibrils. The photographs were made with a Zeiss Elmi D_2 type electron microscope with an accelerator voltage of 50 kV.

Results

1. *Stained preparations.* Fig. 1 shows a photograph of normal contrast and Fig. 2 an overcontrasted one. In the latter photograph a continuous system of transversal lines parallel to Z can be well seen both in the I and the A bands while

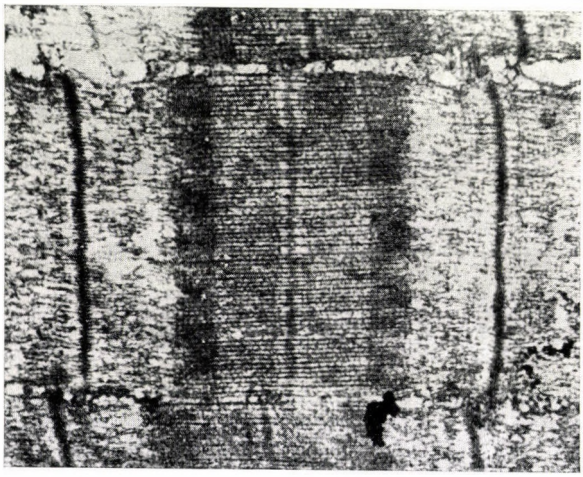


Fig. 1. Longitudinal section of frog sartorius; picture of normal contrast; longitudinal structure is dominating (× 30 000)

no marked system of transversal lines is present in the picture of normal contrast. In Pepe and Huxley's opinion (1963), an antiactin treatment reveals a system of transversal lines with a periodicity of 400 Å on the thin filaments in the I band. Pepe (1968) demonstrated this periodicity in the A band too under the effect of an antiactin treatment. Pepe (1967) demonstrated in chicken breast muscle that an antimyosine treatment gives rise to a system of transversal lines of the same periodicity and of the same number on both sides of the A band. Fig. 3 shows both *phenomena to occur together also in a muscle not treated with antibody.* The picture

Fig. 2. Photograph of an overcontrasted section with a transversal structure clearly seen both in the I and in the A band (× 30 000)

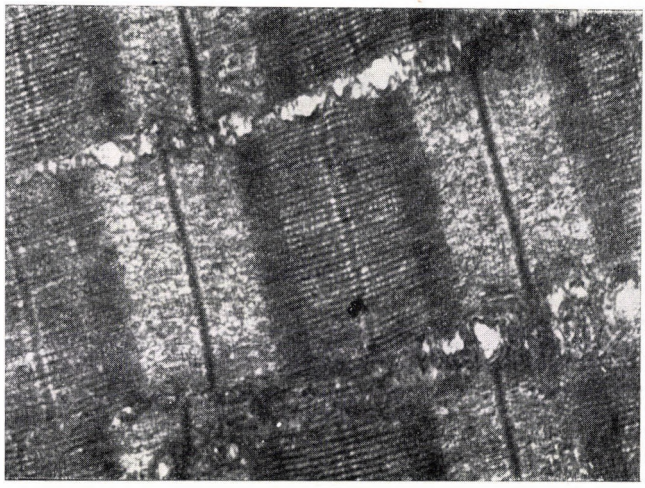


Fig. 3. Transversal lines of the same number of lines and periodicity on both sides of the A band. There is a system of transversal lines of the same periodicity also in the I band (× 30 000)

indicates that there are periodic structures of transversal lines with the same periods and the same number of lines on both sides of the A band; and these systems are parallel to the I—A border. At the same time there exists a system of transversal lines of the same periodicity parallel to the Z line in the I band. Fig. 4 shows a continuous structure of transversal lines parallel to the Z line. The number of lines in the system of transversal lines is 58; it is permanent within a sarcomere, its periodicity is the same both in the I and the A band. Fig. 4 shows that the "pseudo H" zone is bordered with a transversal line on both sides; this area is halved by the M band. Both halves of the "pseudo H" zone are halved by a transversal line.

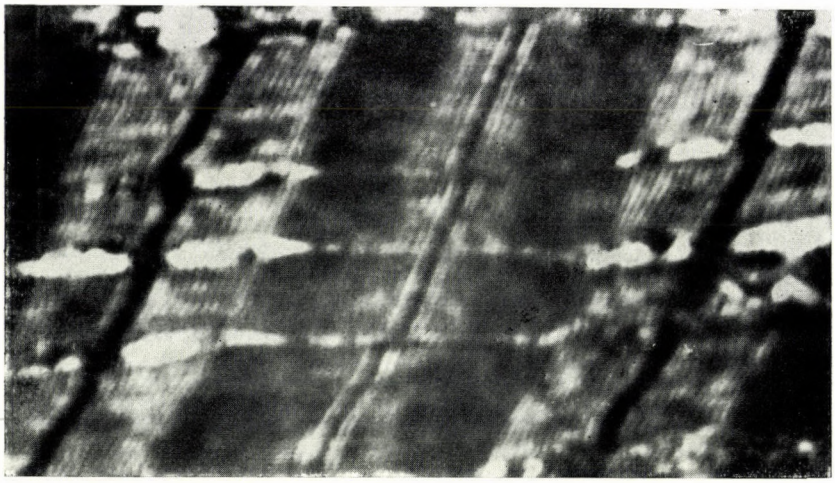


Fig. 4. Periodic system of transversal lines in the whole sarcomere. The "pseudo H" zone is halved by a transversal line (×36 000)

Fig. 5a. Unstained section; the embedding material was dissolved with amylacetate and then he section perpendicular to the fibrils was steamed with gold. Only the longitudinal structure can oe seen (×30 000)

2. *Preparations treated with metal vapour.* The longitudinal sections placed on formvar film were shadowed in vacuum steamer. We expected the direction of steaming to accentuate the longitudinal structure in the one case (when the direction of steaming is perpendicular to the longitudinal axis), and the transversal structure in the other (when the direction of steaming is parallel to the longitudinal axis). The soundness of our idea is proved by Figs 5a, b. Fig. 5a indicates a longitudinal structure clearly visible both in the I and the A bands and, in Fig. 5b, a transversal structure can be well seen.

We succeeded in identifying the system of transversal lines found in photographs made with the aid of staining and in those made with the aid of steaming.

Fig. 5b. The contrasting of the section is the same as that in Fig. 5a, but the direction of steaming is parallel to the direction of the fibrils. The longitudinal structure can hardly be recognized, but there appears a well recognizable transversal structure (× 30 000)

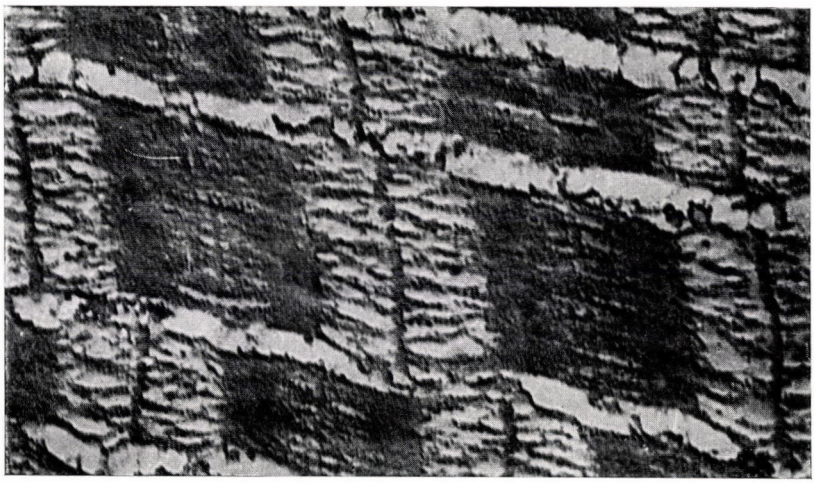


Fig. 6. The preparation of the section is the same as that of Fig. 5b. Shadowing accentuates a continuous system of transversal lines in the A band. The remainder of a continuous system of transversal lines can be seen in the I band (× 30 000)

In the material shown by Fig. 6 (steamed parallel to the longitudinal axis *after incomplete solution*) shadowing accentuated a continuous system of transversal lines in the A band. The remainder of this system of transversal lines can be found in the I band too; though the continuity of transversal lines ceases here, the transversal lines of the I and the A bands are of the same period. It can be stated that the *periodicity of the systems of transversal lines obtained by either staining or steaming is the same.*

Discussion

We have observed in previous experiments that, when the solvent of the dye used for contrasting the electron microscopic sections dissolved the embedding material, we obtained a very intense staining within a very short period of time (e.g. phosphotungstic acid in absolute alcohol dissolved the sections made of badly polymerized araldyte in one or two minutes; these sections already showed full contrast after staining for one second instead of the usual two minutes). We wanted to make use of this phenomenon when staining the sections of material embedded in acrylate with uranyl acetate dissolved in 50 per cent alcohol for an hour and then staining them by the method of Karnovsky (1961) for 30 minutes. The alcohol treatment namely swelled the acrylate, and so we obtained a very intense staining. In our opinion, the system of transversal lines appears clearly only in the case when the sections are overcontrasted, because the stainability of transversal lines is much lower than that of the longitudinal elements. We can find a system of transversal lines of about 400 Å periodicity in the longitudinal section of striated muscle; each transversal line crosses the fibril continuously, perpendicular to its longitudinal axis both in the I and the A bands. The same system of transversal lines has been demonstrated in our pictures of unstained, steamed material and the latter has been found to correspond to the system of transversal lines in the pictures of stained material. Considering the fact that the contrast of the steamed sections is chiefly supplied by shadowing, it is probable on the basis of the above mentioned findings that longitudinal elements ("filaments") are held together by periodic transversal elements both in the I and A bands. Finally, we should like to point out that the results obtained by the method of antibody treatment should be appreciated with criticism as the experimental proofs of this method — system of transversal lines developing under the effect of an antibody treatment — can be demonstrated also in normal, untreated muscle.

The author thanks Prof. E. Ernst for raising the question.

References

Draper, M. H., Hodge, A. J. (1949) Nature *163* 576
Draper, M. H., Hodge, A. J. (1950) Austr. J. Exp. Biol. Med. Sci. *28* 549
Ernst, E., Benedetzky, St. (1962) Acta Physiol. Acad. Sci. Hung. *22* 211
Ernst, E. et al. (1969) in press
Hall, C. E., Jakus, M. A., Schmitt, M. O. (1946) Biol. Bull. *90* 32
Huxley, H. E. (1951) Disc. Farad. Soc. *11* 148
Huxley, H. E. (1953) Biochim. Biophys. Acta *12* 387
Karnovsky, M. J. (1961) J. Biophys. Biochim. Cytol. *11* 729
Palade, G. E. (1952) J. Exp. Med. *95* 285
Pepe, F. A. (1967) J. Mol. Biol. *27* 203
Pepe, F. A. (1968) Int. Rev. Cytol. *24* 193
Pepe, F. A., Huxley, H. E. (1963) "Biochemistry of Muscle Contraction", p. 320. Little Brown, Boston, Massachusetts
Robertson, J. D. (1956) J. Biophys. Biochim. Cytol. *2* 369

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (4), pp. 395–402 (1969)

Structure of Water in Muscle

S. PÓCSIK

Biophysical Institute, Medical University, Pécs

(Received March 26, 1969)

The soundness of the assumption of an icelike structure of bound water was investigated concerning muscle water by freezing a part of the own water content of the muscle and performing density measurements after having cooled the muscle to −4 °C and −7 °C, resp. According to our measurements the density of cooled muscle and the density of cooled muscle water decrease as compared with the values before freezing because of the ice formed in the muscle; the decrease of the density is higher at −7 °C than at −4 °C. By freezing the gradually dried muscles the relative quantity of the frozen water decreases gradually together with the gradual decrease of the relative water content *i*. Furthermore, in the case of an equal relative water content the relative quantity of the frozen water is higher at lower than at higher temperature.

Our experimental results contradict the conception of an icelike structure of bound water because:

a) According to the present experiments — as it was expected — the density of muscle and the density of muscle water decrease on account of a part of the muscle water having assumed an icelike structure,

b) In our previous experiments the density of the rest of the muscle water increased with gradual drying of the muscle (Pócsik, 1967).

Introduction

The fact that the water content of the muscle is partly "bound" is more or less acknowledged nowadays. As regards the structure of bound water, the question is open. According to the frequently occurring iceberg hypothesis (e.g. Szent-Györgyi, 1957; Klotz, 1958, and others), the water surrounding proteins has an icelike structure.

Our previous experiments (Pócsik, 1967), corroborating earlier data (Ernst, 1963), are in favour of the existence of bound water in so far as the density of muscle water > 1; furthermore, by drying the muscle step by step, the density increases gradually, i.e. boundness shows an upward tendency. This cannot be reconciled with the iceberg hypothesis, therefore the question was further investigated considering the following fact: if, by freezing a given part of the own water content of the muscle, we form an icelike structure in a part of the muscle water content, we can expect a decrease in the density of muscle water (d_f) and in the density of muscle (D_f) as compared to the pre-freezing values (d, D); ($d_{ice} < d_{water}$).

Methods

The experiments consist of two main parts: 1. freezing, 2. determination of density.

Freezing: The forming of ice in muscle and muscle fibre, resp., has been investigated by several authors (Meryman, Platt, 1955; Wang et al., 1954; Rapatz, Luyet, 1959). Our experiments were performed on frog gastrocnemius. The preparation of muscle was done at room temperature. The mass of the prepared, fresh gastrocnemius was measured. Then it was dried to some extent in a vacuum desiccator over $CaCl_2$ at +2 °C; then it was frozen. The freezing was performed at -4 °C in some experiments and at -7 °C in others.[1]

Determination of density. The mass of the frozen muscle was measured in air, then in toluene at the temperature of freezing. (The density of toluene is 0.8894 g/cm^3 at – 4 °C and 0.8920 g/cm^3 at -7 °C.) The measurements were performed in a cold-room. Both the volume (V) and the density (D) of the muscle were determined from the data of measurements (Pócsik, 1967). The mass of the dry matter of the muscle was obtained after drying it further at +2 °C and finally at 105 °C. The time of drying increases as compared with that of the non-frozen muscles. The density of the dry matter was taken for 1.300 g/cm^3. The determination of the density of muscle water (Pócsik, 1967) was done on the basis of the equation

$$d = \frac{M - m}{V - v} \tag{1}$$

where M and m are the masses of the muscle and its dry matter, respectively; V and $v\left(=\frac{m}{1.300}\right)$ are their volumes.

Results and Discussion

1. Table 1 contains the data obtained on 20 muscles. The first ten muscles were frozen at – 4, and the ones from 11 to 20 at -7 °C. The number of the muscle is indicated in the first column; the mass of the fresh muscle (M'), the mass of the muscle measured in air after drying to a certain degree and after freezing (M), and that measured in toluene (M_t) are indicated in columns 2 to 4. The 5th column shows the mass of the wholly dried muscle, i.e. that of the dry matter (m), the 6th one shows the relative water content $\left(i = \frac{M - m}{m}\right)$ of the muscle dried to a certain degree (and frozen). Furthermore, in case of this relative water content i, the columns 7 to 10 show the density of muscles (D) and the density of muscle water (d) at 23 °C. (D_f) and (d_f) mean the density of the muscle and the muscle-water, resp., in case of frozen muscles (calculated on the basis of a work by Pócsik, 1967).[2]

[1] In the place of the water (ice) evaporated from the frozen muscle — chiefly in case of high relative water content — cavities formed in the muscle may destort the results of density determinations.

[2] In the $i = 4-0.31$ interval

$$D = \frac{i + 1}{0.9976\, i + 0.7318} \quad \text{and} \quad d = \frac{1}{0.9976 - \frac{0.0379}{i}}$$

Table 1

1	2	3	4	5	6	7	8	9	10
No.	M'	M	M_t	m (mg)	$i = \frac{M-m}{m}$	$D_f = \frac{M}{V}$	D	$d_f = \frac{M-m}{V-v}$	d
	mg					g/cm³			
1	740	664	82	149.2	3.45	1.015	1.066	0.954	1.014
2	723	636	81	147.3	3.32	1.019	1.068	0.957	1.014
3	784	583	86	162.8	2.58	1.043	1.083	0.969	1.017
4	817	566	87	165.5	2.42	1.051	1.087	0.974	1.018
5	873	599	93	178.7	2.35	1.053	1.089	0.974	1.019
6	782	538	84	162.4	2.31	1.054	1.090	0.974	1.019
7	970	658	104	202.2	2.25	1.056	1.092	0.976	1.020
8	961	628	103	197.9	2.17	1.064	1.094	0.982	1.020
9	977	531	96	187.9	1.83	1.086	1.107	0.996	1.024
10	904	443	93	189.7	1.34	1.126	1.131	1.023	1.032
11	706	677.6	72.1	134.4	4.04	0.998	1.058	0.944	1.012
12	871	682.6	88.0	173.0	2.95	1.024	1.075	0.955	1.015
13	863	676.7	88.7	174.7	2.87	1.027	1.076	0.957	1.016
14	717	502.6	71.7	143.6	2.50	1.040	1.085	0.963	1.018
15	672	444.6	66.1	133.7	2.34	1.048	1.089	0.967	1.019
16	794	563.5	85.9	176.2	2.20	1.052	1.093	0.969	1.020
17	807	479.6	80.7	170.6	1.81	1.072	1.107	0.978	1.024
18	806	454.4	79.8	171.2	1.65	1.082	1.114	0.983	1.026
19	785	381.5	75.0	167.2	1.28	1.110	1.135	0.997	1.033
20	795	348.7	76.8	170.3	1.05	1.144	1.152	1.027	1.040

2. The data of the 7th and 8th columns concern the densities of muscles dried to a certain degree, frozen (D_f) and non-frozen (D) and they show clearly that

$$D_f < D$$

in all cases. Furthermore, the data of the 9th and 10th columns — concerning the densities of the rest of the water content of muscles dried to a certain degree, frozen (d_f) and non-frozen (d) — similarly indicate that

$$d_f < d$$

in all cases, except the case of extreme drying

$$d_f < 1$$

That is to say, the fact that *the densities of muscle and muscle water, resp., do really decrease when a part of the water is frozen* — i.e. when it is in an icelike state — is justified. Namely, the lower the temperature of freezing, the greater the decrease of density (Fig. 1).

3. The question is raised: what quantity of water can be frozen off from the muscle by cooling, and what does the quantity of the frozen water (x) depend on. The volume of the water of the frozen muscle is composed of the volume of the

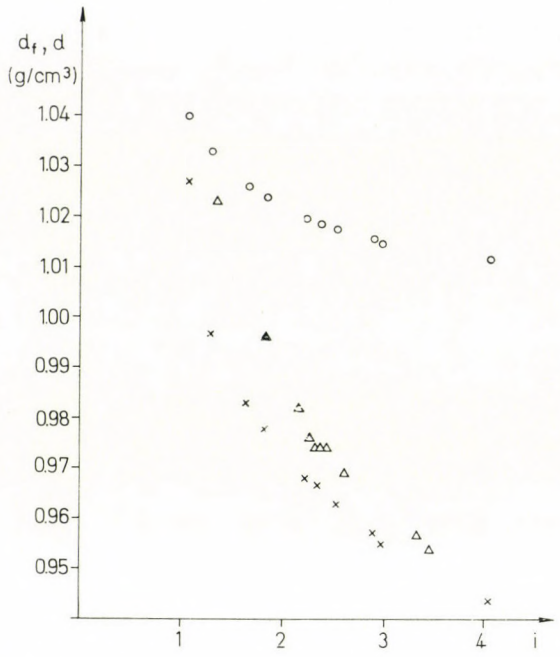


Fig. 1. Values of densities of muscle water (d_f, d) plotted against the relative water content (i) at temperatures of −7 °C (×), −4 °C (△) and 23 °C (○)

frozen water(ice) w' and the volume of the non-frozen water w. Considering this:

$$d_f = \frac{M - m}{w' + w} \tag{2}$$

is valid for the d_f values of the 9th volumn, where $w' = \frac{x}{d_{ice}}$ and $w = \frac{M - x - m}{d_{water}}$. The value d_{water} is the density of the non-frozen muscle water (this value is identified with the density of the muscle water of 23 °C which has the same relative water content — the 10th column of Table 1 — in the first approximation). Replacing the values w' and w, resp., and solving the equation for x we obtain:

$$x = (M - m)\frac{\frac{1}{d_f} + \frac{1}{d_{water}}}{\frac{1}{d_{ice}} - \frac{1}{d_{water}}} \tag{3}$$

In the first approximation ($x = x'$) e.g. with muscle No. 7:

$$x' = (658 - 202)\frac{\frac{1}{0.976} - \frac{1}{1.020}}{\frac{1}{0.917} + \frac{1}{1.020}} = 185 \text{ mg}$$

Table 2

1	2	3	4	5	6	7
No.	$M-m$ (mg)	$i=\frac{M-m}{m}$	x (mg)	$\frac{x}{M-m}\times 100$	$M-x-m$ (mg)	$\frac{M-x-m}{M-m}\times 100$
1	515	3.45	341	66	174	34
2	489	3.32	309	63	180	37
3	420	2.58	221	53	199	47
4	400	2.42	193	48	207	52
5	420	2.35	203	48	217	52
6	376	2.31	183	49	193	51
7	456	2.25	217	48	239	52
8	430	2.17	178	41	252	59
9	343	1.83	101	29	242	71
10	253	1.34	21	8	232	92
11	543.2	4.04	417	77	126	23
12	509.6	2.95	342	67	168	33
13	502.0	2.87	330	66	172	34
14	359.0	2.50	216	60	143	40
15	310.9	2.34	177	57	134	43
16	387.3	2.20	218	56	169	44
17	309.0	1.81	153	49	156	51
18	283.2	1.65	132	47	151	53
19	214.3	1.28	81	38	133	62
20	178.4	1.05	23	13	155	87

The calculation of the value x can be refined further on, considering the fact that the value d_{water} does not mean the density of the total water content, but that of the water content minus x mg ($M \quad x - m$). But it is indicated by the 10th column of Table 1 that the density of muscle water increases together with the decrease of the water content.[3] On the basis of our previous communication (Pócsik, 1967) the volume of the quantity of water can be calculated in case of a given water content. In the $i = 4 - 0.31$ interval:

$$w = 0.9976\,(M - x - m) - \frac{7.56}{200}\,m \tag{4}$$

Replacing this into (2):

$$x = 217 \text{ mg.}$$

These more correct x values obtained by a second approximation are contained in the 4th column of Table 2 for all values of the series of experiments. In addition, the following data are indicated in Table 2: the number of the muscle — in the 1st column, the water content $(M - m)$ and the relative water content $\left(i=\frac{M-m}{m}\right)$

[3] The difference in density belonging to the difference in temperatures of 23°C and −4 °C, −7 °C, resp., can also be considered.

of the muscle dried to a certain degree and frozen — in the 2nd and 3rd one. The 5th column gives the value of the quantity of frozen water expressed in per cent $\left(\frac{x}{M-m} \times 100\right)$ and finally the 6th and 7th columns give the quantity of the non-frozen water $(M-x-m)$ as well as its value expressed in per cent $\left(\frac{M-x-m}{M-m} \times 100\right)$.

4. If a part of the muscle water were bound to the substance of muscle by swelling (Ernst, 1963) its vapour pressure would be

$$R\ T\ 2.3\ \log \frac{p_0}{p_1} = \frac{ab}{(b+i)^2} \tag{5}$$

according to the empirical equation of Katz (1924), where R is the universal gas constant, T is the absolute temperature, p_0 is the vapour pressure of pure water, p_1 is that of the swollen system, i is the relative water content, a and b are constants.

Let us examine the validity of the Katz equation in case of the present experimental conditions; in case of freezing of a part of the muscle water (x) at temperature T the vapour pressure of the non-frozen part of water equals the vapour pressure of ice (p_1); p_0 is the vapour pressure of water of T temperature (supercooled water); $i = i_r = \frac{M-x-m}{m}$ is the relative water content concerning the non-frozen water, which can be determined on the basis of Tables 1 and 2. The constants a and b are determined on the basis of the experimental data of Ernst et al. (1963, p. 120). Thus

$$i_2 = \frac{22.0}{55.0-22.0} = 0.667 \qquad i_3 = \frac{8.4}{41.8-8.4} = 0.251$$

$$\frac{p_0}{p_1}_2 = \frac{15.3}{14.0} \qquad \frac{p_0}{p_1}_3 = \frac{16.5}{12.5}$$

$$T_2 = 291.0°\mathrm{K} \qquad T_3 = 292.2°\mathrm{K}$$

at the second and third stages. Replacing the data into (5):

$$b = 0.290$$

$$\frac{a}{k} = 0.122$$

where $k = 2.3\ \mathrm{R}\ 292.2$

Making use of the constants a, b (supposing that they are independent of the temperature)

$$R\ T\ 2.3\ \log \frac{p_{\text{water}}}{p_{\text{ice}}} = \frac{ab}{(b+i_r)^2} \tag{6}$$

where $T = -4\,°\mathrm{C} = 269.2\,°\mathrm{K}$

$p_{\text{water}} = 3.410$ mm Hg
$p_{\text{ice}} = 3.280$ mm Hg

i_r (the average of muscles 1 to 10 at $-4\,°\mathrm{C}$) $= 1.22 \pm 0.01$.
Replacing the data into equation (6)

$$4.55 \approx 4.53$$

Thus, with adaptation of the constants of the experimental data of Ernst et al., the Katz equation has proved to be valid with a good approximation in case of the present experimental conditions.

The data of Table 2 show that *by using gradually dried muscles (i.e. with the gradual decrease of* $i = \frac{M-m}{m}$*) for freezing, the relative quantity of the frozen water decreases gradually.* Furthermore, comparing the data obtained at -4 °C and -7 °C we can see that *in case of the same relative water content and at a lower temperature the relative quantity of the frozen water is higher.* By appropriate drying of the muscle we can attain that nothing of its water freezes at a given temperature. In this connection we refer to the high resistance of plants against freezing.

Though we can encounter even nowadays with statements like "There is no substantial evidence of 'bound' water . . ." (Zierler, 1968), the existence of bound water can be considered roughly accepted. As regards the structure of bound water, we are of the following opinion:

Since, on the one hand, according to our previous experiments, the density of the rest of the muscle water increased when drying the muscle and, on the other hand, according to the present experiments the density of the muscle and that of the muscle water, respectively, decrease when a part of the muscle water freezes, i.e. when it adopts an icelike structure, our experimental results contradict the conceptions considering this bound water to be of an icelike structure.

The author expresses his thanks to Prof. E. Ernst who called his attention to this question.

References

Ernst, E. (1963) Biophysics of the Striated Muscle. Akadémiai Kiadó, Budapest, pp 109–121
Frank, H. (1966) Federation Proc. *24* II. Suppl. 42 (Discussion on water solvent interaction)
Hechter, O. (1966) Federation Proc. *24* II. Suppl. 91
Katz, J. R. (1924) Ergebnisse der exakten Naturwissenschaften III. Verlag von Julius Springer, Berlin
Klotz, I. M. (1958) Science *128* 815
Meryman, H. T., Platt, W. T. (1955) Reports of U. S. Naval Med. Res. Institute, *13* 1
Pócsik, S. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 149
Rapatz, G., Luyet, B. (1959) Biodynamica *8* 121
Szent-Györgyi, A. (1957) Bioenergetics. Academic Press. New York
Wang, H., Auerbach, E., Bates, V., Doty, D. M., Kraybill, H. R. (1954) Food Research *19* 543
Zierler, K. L. (1968) in: Medical physiology. Vernon B. Mountcastle (editor), Saint Louis, p. 1161

Addendum

Investigating the role of water in biological systems we performed experiments mostly on muscle, the inhomogeneous structure of which has been emphasized by us especially in respect of its water content (Ernst, 1963). Notwithstanding, the latter was considered, when the results of the experiments were calculated, as of identical quality in its entirety (at a first approximation). Pócsik's papers start from the same consideration, not neglecting, of course, the data of today's literature.[4]

E. Ernst

[4] e.g. Frank (1966): "... each of these experimental technics (for the investigation of water structure) has its own traps and the interpretation of the raw data really calls for close examination." Or Hechter (1966): "... it seems fair to say that we have little or no definitive information about water structures inside the cell."

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (4), pp. 403—410 (1969)

Photobiology of Aging Bean Leaves in vivo

A. RAAFAT,* J. GAUSZ, L. SZALAY, I. HORVÁTH

Institute of Experimental Physics and Institute of Botany, Attila József University, Szeged

(Received March 26, 1969)

The peak position of the red band of the absorption spectrum of chlorophyll in the living primary bean leaves is practically the same for all ages, it is at 678—679 nm. The half band width of this band shows a maximum in the 15 days old plants and it decreases with the age. This leads to the assumption of a preferential loss of one of the two main chlorophyll-a components belonging to System I and System II during senescence. The concentration of the fluorescent chlorophyll species Chl-a 670 decreases gradually from the 15 to 29 days old plants, and the decomposition of this species becomes very fast in 34 days old plants. Though both the Chl-a 670 and the Chl-a 680 components are lost on aging, the amount of the non-fluorescent chlorophyll component Chl-a 680 seems to be relatively smaller than that of the fluorescent Chl-a 670 at the last stage of senescence. The time course of fluorescence exhibits a systematic change with age. The degree of polarization of fluorescence increases from $p = 0.193$ in the 10 days old plants to $p = 0.290$ in the 34 days old plants.

Introduction

Recently, the juvenility and senescence phenomena in plants have gained renewed interest. Results in this field may have important application in horticulture as in the problem of short life span of many fruits and ornamental trees and marketing of fruits and vegetables.

The main features of the aging process concerning the pigment system can be summarized as follows:

1. The senescence in tobacco leaves is accompanied by a reduction of the size of the chloroplast and by a change of its oblong shape to a spherical one as well as a drop of the amount of chlorophyll to 50 per cent per unit leaf surface. Further yellowing causes irreversible changes, i.e. further reduction of plastid volume. Finally, the disintegration of granathylakoids occurs and the plastid content becomes electron optically homogeneous and the chlorophyll content drops below 50 per cent (Ljubesic, 1967).

2. The aging process in tomato plant is associated with changes in pigment accumulation, meanwhile the chlorophyll-b content remains practically unchanged (Vitkovskaya and Shan Gina, 1958).

3. The absorption spectra of diatom cultures show clear changes on aging. The older cultures have the pigment P 707 not present in young cultures. Further-

* On leave from the Department of Agricultural Botany, Faculty of Agriculture, Ain Shams University, Cairo, U. A. R.

more, the amount of pigment P 680 shows a relative increase with the age when compared with pigment P 670 (French, 1967).

4. The chloroplast suspension of leaves of increasing age shows decreasing capacity for the photolysis of water (Clendenning, Gorham, 1950; Miller, 1960).

5. Plants reaching the photosynthetic maturity begin to decrease the photosynthetic activity at a rate higher than the rate of the decrease of the total chlorophyll content per leaf area (Sesták, 1963; Sesták, Vaclavik, 1963).

The present study was planned to obtain detailed information on the absorption spectrum, fluorescence spectrum, polarization of fluorescence and the time course of fluorescence of bean leaves *in vivo* during the process of aging.

Materials and Methods

Culturing. Bean plants (Phaseolus vulgaris L.) were grown in pots in a thermostat at 25 to 36 °C under daylight fluorescent lamps producing an illumination of 2000 lux for 16 hours daily. Sand culture technique was applied using Priyanisnikov's nutrient solution. Details of the method have been described elsewhere (Szalai, Frenyó, 1962).

Sampling. Representative samples of primary leaves were taken randomly when the plants were 10, 15, 22, 29 and 34 days old. In another experiment the sampling was arranged in such a way as to perform all the measurements in one day. Thus, to some extent we could avoid some usual changes in the apparatus, i.e. changes in the sensitivity of the photomultiplier, in the intensity of the exciting light source, etc., during 2—3 weeks. In the case of all samples we used five replicates and the results we give are the mean values obtained from the five replicates. Values showing more than 20 per cent deviation from the mean value were rejected.

Absorption spectrum. The absorption spectra of leaves *in vivo* were measured at room temperature with a Unicam SP 800 recording spectrophotometer provided with a sample holder position for highly scattering samples. For the correction of the residual scattering effect the apparent optical density at 800 nm was subtracted from the optical densities recorded throughout the whole spectrum.

Measurement of fluorescence. The exciting light source was a high pressure mercury lamp; the light from this lamp was approximately monochromatized by a metal interference filter at 436 nm. The fluorescence was measured with a Zeiss monochromator (type: SPM 2) and a photomultiplier (type: EMI 9558 A). The signals were amplified and traced on a digital voltmeter. The intensity of the illumination was about 10^{16} photon/cm^2 sec determined by measuring the energy from the light source with a calorimeter and estimating the energy in the region of the transmission of the filter. The degree of polarization of fluorescence of leaves at 690 nm was determined by using two polaroids in suitable positions in order to obtain p according to the definition: $p = \frac{(I_1 - I_2)}{(I_1 + I_2)}$, where I_1 and I_2 are the intensities of fluorescence with electric vector parallel and perpendicular to the electric vector of the exciting linear polarized light, respectively. The details of the experimental equipment are given elsewhere (Vize, 1969).

Results and Discussion

The results of the absorption and emission measurements are shown in Fig. 1, where the absorption spectra are already corrected for scattering and the emission spectra are corrected for the sensitivity of the photomultiplier. Some of the quantitative results are summarized in Table 1. The peak position is practically the same for all ages: 678—679 nm. This finding is in accordance with the results

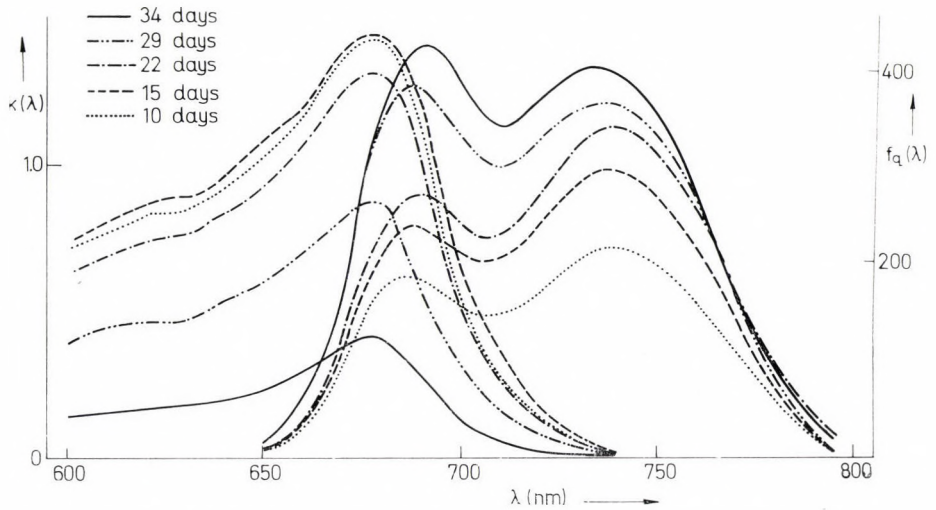


Fig. 1. Red absorption band corrected for scattering and fluorescence spectra corrected for the sensitivity of the detector at different ages of bean leaves

Table 1

Age, days	Peak position, nm	Half band width, nm	Max. opt. dens., $k(\lambda)_{max}$	Areas of fl. spectr. $I_{(\lambda')}$	$k(\lambda)_{max} * I(\lambda')$
10	678.0	37.0	1.44	40.6	58.5
15	679.0	40.0	1.45	42.5	61.5
22	678.0	39.0	1.32	44.0	58.1
29	678.0	34.0	0.86	61.4	52.8
34	678.5	34.0	0.40	55.5	22.2

obtained by Godnev and Efremova (1958). The absorption bands in Fig. 2 have been adjusted to the maxima, therefore, the shapes may be easily compared directly. The half band width shows a maximum in 15 days old plant; this value is lower both in younger and older plants; it varies between 34 and 40 nm. Since the half width of the red band in chlorophyll-a solutions *in vitro* is much less, it varies between 16 and 26 nm for a series of about 40 solvents (Seely, Jensen, 1965), the high value of the half width *in vivo* may be attributed to the presence of chlorophylla in different environment with slightly different red bands superimposed on

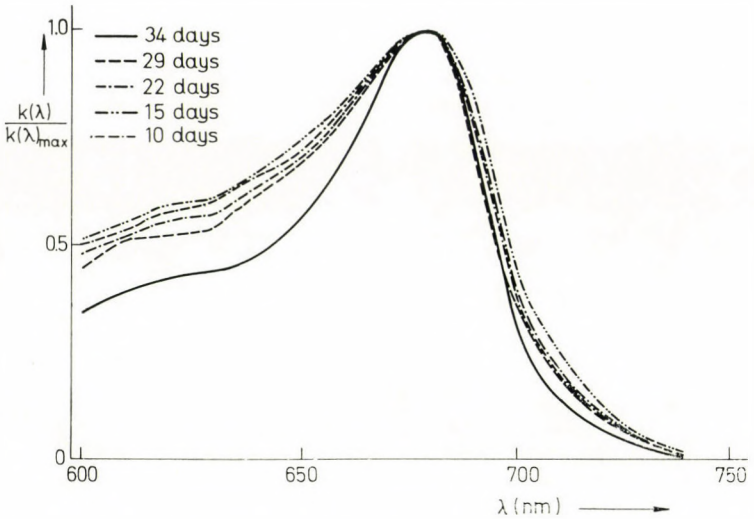


Fig. 2. The distribution of the relative optical density $k(\lambda)/k(\lambda)_{max}$ in the red band of bean leaves of different ages

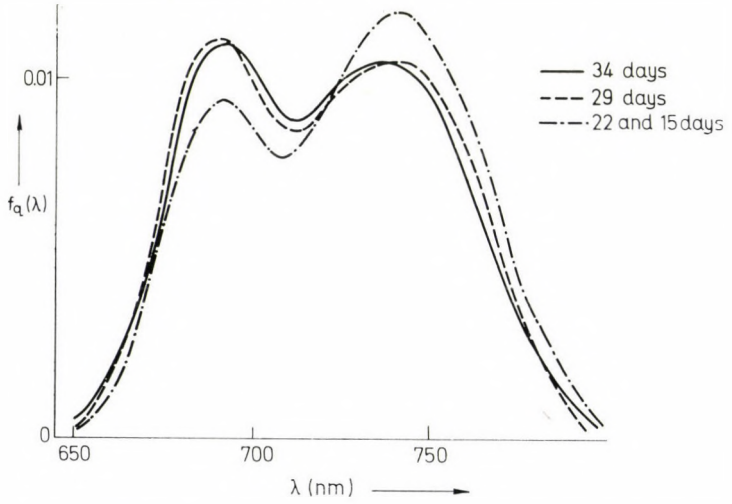


Fig. 3. Normalized fluorescence spectra of bean leaves of different ages [the condition of the normalization is $\int f_q(\lambda)d\lambda = 1$]

each other. If, as usual, we differentiate between two main chlorophyll-a components, belonging to System I and System II of photosynthesis, the changes of the half band width with the age can be attributed to a preferential loss of one of these components during senescence. If both components were decomposing at the same rate on aging, the relative spectral distribution of the absorbed energy in the red band should not change. Since, however, the half width is decreasing with the age, one of the two main components should be preferentially lost in older plants.

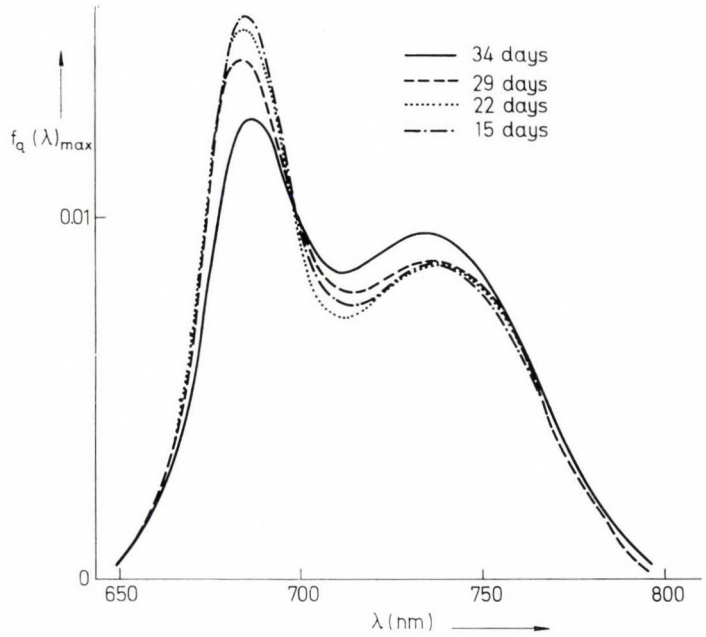


Fig. 4. Normalized fluorescence spectra of bean leaves of different ages corrected for reab sorption

An analysis of the the fluorescence spectra gives more information about the situation. Fig. 3 shows the normalized fluorescence spectra [the condition of the normalization is $\int_0^\infty f(\lambda')d\lambda' = I(\lambda')$] with two peaks at about 690 and 740 nm. In Fig. 3 the short wave band seems to increase with the age, while the long wave band shows an opposite trend. Since, however, the short wave band of the fluorescence and the red band of the absorption (Fig. 1) strongly overlap, the fluorescence spectrum may be strongly distorted on account of the reabsorption of fluorescence in the overlap region of the spectra. Therefore, the fluorescence spectra corrected for reabsorption should be compared.

The fluorescence spectra corrected for reabsorption and normalized in the same way as those in Fig. 3 are shown in Fig. 4. In Fig. 4 the corrected spectra can be seen with two peaks, at 685 and 740 nm. These two peaks are commonly found in chlorophyll-a emission spectra, and in *in vitro* systems the short wave band is always much higher than the long wave band. In our spectra the difference in the heights of the two peaks is less. This probably may be attributed to an inner reabsorption effect within the single cell not considered in the applied correction for reabsorption. In Fig. 4 there seems to be no systematic change of the fluorescence spectra with the age: the deviation from the mean value at the peak positions does not exceed 5 to 6 per cent. If we accept that these spectra do not differ to such an extent as to have been originated from different fluorescent species, we can attribute these spectra to the fluorescent species of chlorophyll-a (Chl-a 670). The con-

centration of this species is not known as a function of age, and there is no reliable way to determine it unless, to some extent, by a separation of the systems into chloroplast fractions. However, we can give an estimate of the amount of this fluorescent species from a comparison of the total absorption and the intensity of the emission. The intensity of the emission at wavelength λ' is $I(\lambda') = \text{const.} \frac{k_1(\lambda)}{k_1(\lambda) + k_2(\lambda)}$, where $k_1(\lambda)$ and $k_2(\lambda)$ denote the optical densities of the fluorescent and (practically) non-fluorescent Chl-a species at the exciting wavelength λ and the constant includes the quantum yield of fluorescence, the intensity of excitation, the reflection coefficient, the refractive index and a factor depending on the geometry of the measurement. The above relationship satisfactorily holds when instead of $I(\lambda')$ the integrated areas $\int_0^\infty f(\lambda')d\lambda'$ are introduced and the optical densities at the maximum of the red band $k(\lambda)_{max}$ are substituted for $k_1(\lambda) + k_2(\lambda) = k(\lambda)$. Since, furthermore, $k_1(\lambda) = \text{const. } c$, namely, the optical density of the fluorescent species of Chl-a is proportional to the concentration (c) of this species, the above equation can be rewritten as follows: $I(\lambda') \equiv \int_0^\infty f(\lambda')d\lambda' = \text{const.} \frac{c}{k(\lambda)_{max}}$. Therefore, the concentration of the fluorescent species of Chl-a is $c = \text{const. } k(\lambda)_{max} I(\lambda')$. Using the values of $k(\lambda)_{max}$ and $I(\lambda')$ given in the third and fourth columns of Table 1, the concentrations in arbitrary units are obtained with this formula as the products of these values introduced in the fifth column of Table 1.

Table 2

Age, days	Concentration of the non-fluorescent species	Concentration of the fluorescent species
10	48.5	52.0
15	50.0	50.0
22	47.0	44.0
29	42.5	17.0
34	18.0	9.6

If we assume that the two main chlorophyll-a components are of equal concentration in the mature state of the plant (e.g. at an age of 15 days), we can estimate the relative concentration of the non-fluorescent chlorophyll-a species in plants of different age from the 3rd and 5th columns of Table 1. Namely, in this case the contribution of the optical densities of the two components to the total maximum optical density, $k(\lambda)_{max}$, is almost equal (see Cederstrand et al., 1966). Table 2 shows these concentrations in per cent of the total amount of chlorophyll-a.

The comparison of these concentrations shows that, in plants of 15, 22 and 29 days' age, the main fluorescent species of chlorophyll-a (Chl-a 670) gradually decomposes. At the last stage, however, the decomposition of this species is very fast from a concentration of about 52.8 to 22.2, in Table 1 (or from 42.5 per cent to

18.0 per cent, in Table 2). The same behaviour applies to the non-fluorescent species but the decomposition of this component begins at an earlier stage from a concentration of 44.0 per cent (22 days, Table 2). The latter result is in disagreement with the finding of French (1967) obtained with diatom cultures.

In another experiment the intensity of fluorescence was measured as a function of time, i.e. the time course of fluorescence in bean leaves kept in the dark for 24 hours before the fluorescence was excited with a light of 436 nm. The fluorescence was observed at the short wave maximum of about 690 nm roughly every second. The results are shown in Fig. 5. Here the emphasis is on the slope of the curves, therefore, the relative intensities are plotted against the time in minutes. The

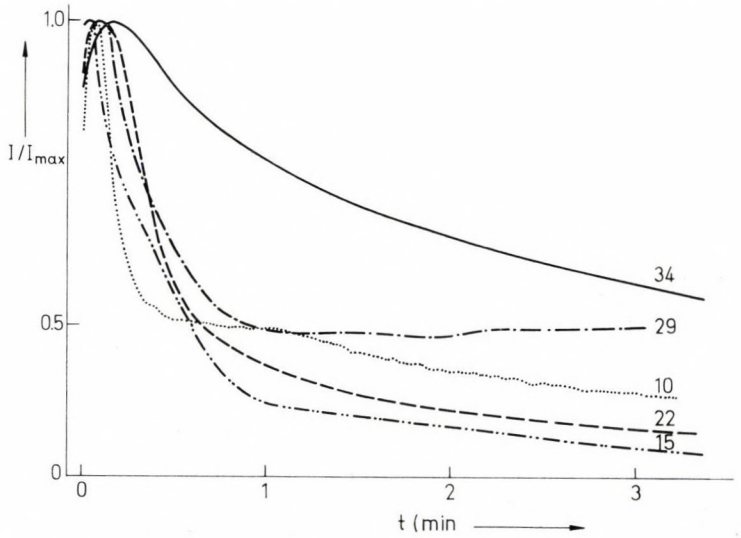


Fig. 5. Time course of fluorescence of bean leaves of different ages

time course of fluorescence shows the general appearance found in different plants. There is, however, a systematic change in the slope of the curves with the age. If, as usual, we assume that the build up and the decay of the curves are in correlation with the photosynthetic activity and with the accumulation and transport (or decomposition) of some quenching or sensitizing substances produced in the course of the photosynthetic process, the time course curves in Fig. 5 can be generally understood. Namely, in senescent leaves both photosynthetic activity and the changes of fluorescence with time are slow, while in other leaves both phenomena occur more rapidly. An attempt for a more detailed quantitative explanation of this phenomenon is planned.

There are some preliminary data on the polarization of fluorescence in plants of different age. This phenomenon is an extremely complex one. Under the same experimental conditions the degree of polarization of fluorescence increased from $p = 0.193$ to $p = 0.290$ with the age, namely, from 10 days old plants to 34 days old plants. Since the amount of the pigment is decreasing with the age, the

energy migration among the pigment particles is less effective in older leaves. Therefore, the depolarization of fluorescence is smaller. These results may indicate a lesser degree of orientation of pigment molecules as well as unorganized structure of the pigment complex in the chloroplasts on approaching the senescence. In this connection should be mentioned the finding of Ljubesic (1967) that further yellowing (senescence) leads finally to granathylakoid disintegration and to an electron-optically homogeneous plastid content. Further, since according to Sironval and Englert-Dujardin (1963) the total cytochrome content in the chloroplast is decreasing by a factor of 3 during the aging of leaf, the increase of the degree of polarization of the fluorescence may well be due partly to the smaller migration of energy by cytochromes in older leaves.

Thanks are due to Dr. L. Vize for his kind help in the polarization measurements.

References

Cederstrand, C. N., Rabinowitch, E., Govindjee (1966) Biochim. Biophys. Acta *126* 1
Clendenning, K. A., Gorham, P. R. (1950) Can. J. Res. C. *28* 114
French, C. S. (1967) Arch. Mikrobiol. *59* 93
Godnev, T. N., Efremova, R. V. (1968) Inzhinerno-Fizicheskii Zhur *1* 91
Ljubesic, N. (1967) European Photobiology Symposium, Hvar, Yugoslavia
Miller, J. H. (1960) Am. J. Bot. *47* 532
Seely, G. R., Jensen, R. G. (1964) Spectrochimica Acta *21* 1837
Sesták, Z. (1963) Photochem. and Photobiol. (Chl. Metabol. Sym.). Pergamon Press Ltd., London *2* 101
Sesták, Z., Vaclavik, J. (1963) Symposium on water stress in plants. Prague, Czechoslovakia
Sironval, C., Englert-Dujardin, E. (1963) Physiol. Veg. *1* (2) 191
Szalai, I., Frenyó, V. (1962) Növényélettani kísérletek. Tankönyvkiadó, Budapest
Vitkovskaya, V. V., Shan Gina, Z. I. (1958) Zap Leningradsk Sel' Skokhoz Inst. *13* 90
Vize, L. (1969) Acta Phys. et Chem. Szeged *15* 27

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (4), pp. 411—414 (1969)

Effect of X-Irradiation on the Mitotic Cycle of in vivo Cultured Lymphoma Ascites Cells

Study of cell kinetics by double isotope labelling

L. VARGA, V. VÁRTERÉSZ

"Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest

(Received February 27, 1969)

The authors studied postirradiation changes in cell kinetics on ascites lymphoma cells cultured *in vivo*. Prior to irradiation the cells were incubated first with ^{14}C- then with ^{3}H-labelled thymidine. On the basis of the incorporation of precursors given at different time intervals, the population could be divided into 4 groups. The G_1-stage cells constituted the inactive group. Cells in the first half of phase S (ES) were labelled but with ^{3}H, those in the second phase (LS), with both isotopes. The G_2 stage cells constituted the ^{14}C-labelled group. The cells of the four groups were differentiated by autoradiography.

Following X-irradiation with a dose of 250 R, as soon as mitosis inhibition was released, the entering of the single cell groups into the M phase was studied by the labelled mitosis method. According to the results obtained, in the first postirradiation mitotic cycle at a given time, the cells of various stages enter the mitotic phase mixed. When compared with the control, the group-sequence also disappears.

Introduction

Earlier intensive studies have developed the nowadays generally accepted view that dividing mammalian cells display different sensitivities to ionizing radiations. The degree of radiosensitivity mainly depends on the phase of the mitotic cycle in which the cell is at the time of irradiation. A great number of contradictions can be found in the literature on the radiosensitivity sequence of the various phases of the mitotic cycle. Contradictions are met with even for such cases when radiosensitivity has been studied on the same test object, under identical conditions and by the same methods. These contradictions may be attributed to several factors. In our opinion they may be explained also by the fact that, in the majority of experiments, the length of the postirradiation mitotic cycle and also the sequence of its single phases were taken for identical with those in the nonirradiated controls (Brewen, 1965; Dewey and Humphrey, 1962; Watanabe and Okada, 1966).

The studies in this paper were designed to reveal the postirradiation changes in the kinetics of ascites lymphoma cells. More precisely, to reveal, whether, after the release of mitotic inhibition, the cells in the various phases enter, and pass through the first postirradiation mitotic cycle in the same way as they do in the nonirradiated control group.

Materials and Methods

Németh – Kellner ascites lymphoma cells were used (Németh and Kellner, 1960). The minimum mitotic cycle of the cells was determined by the labelled mitosis method in a previous experiment (Varga and Várterész, 1968). Eight hours were obtained for G_1, 11 hours for S, 4 hours for G_2 and 22 to 23 hours for the entire mitotic cycle. In the experiments reported 5 μCi of ^{14}C-thymidine (Tdr) were injected intraperitoneally to each mouse 6 days after the injection of the ascites cells, at time 0 of the experiment. Four hours later these animals were given 20 μCi ^{3}H Tdr intraperitoneally. By ^{14}C and ^{3}H Tdr given successively with a shift in time, the asynchronous cell population was divided into 4 groups. One cell group incorporated only ^{14}C Tdr (cells in phase G_2), the second group incorporated both isotopes (2nd half of phase S), the third cell group was only labelled with ^{3}H Tdr (beginning of phase S), while the cells in group 4 (phase G_1) failed to incorporate any of the labelled precursors. These cells constituted the inactive group.

In the 25th minute of the 5th hour of the experiment one group of the animals was irradiated with 250 R under the following physical conditions: 180 kV, 10 mA, 0.5 mm Cu, dose rate: 28.7 R/min. The other group served as nonirradiated control.

Immediately before the injection of ^{3}H Tdr and at intervals of 3 to 4 hours after irradiation, smears were prepared and fixed in a 1 : 3 mixture of acetic acid and methanol. The dividing cells of the four different groups occurring in the smears were differentiated by double autoradiography. Fifty M-phase cells were always considered when evaluating the smears and the incidence rate of the 4 cell types was determined on this basis.

Results and Discussion

The results are shown in Figs 1 and 2. The time elapsed from ^{14}C Tdr administration is indicated on the X-axis of the coordinate system. The mitotic frequencies belonging to the four cell groups are given on the Y-axis in relative units. The Z-coordinate represents the mitotic cycle of the cells with the component parts. In the nonirradiated group (Fig. 1) the ^{14}C-labelled cells in phase G_2 divided earliest, next the cells deriving from the end and thereafter the cells from the beginning of the S phase. The inactive cells in phase G_1 divided after the active cells. In the 25th to 28th hours of the experiment the cells in phase G_2 entered the M phase again. The sequence of the frequency curves so obtained corresponds to the reverse sequence of the phases and is in proportion with their lengths.

In the irradiated group (Fig. 2) the ^{14}C labelled cells of phase G_2 just reached phase M at the time of irradiation. Irradiation induced a mitosis inhibition lasting 10 hours. After the restarting of the mitotic process, mitoses were observed in a mixed cell group, the majority of which consisted of cells from the second half of phase S. However, the ratio of the groups varied fastly. In the 21st hour of the experiment the various groups occurred in an equal proportion. Soon thereafter the doubly labelled cells became predominant again. The appearance of the various

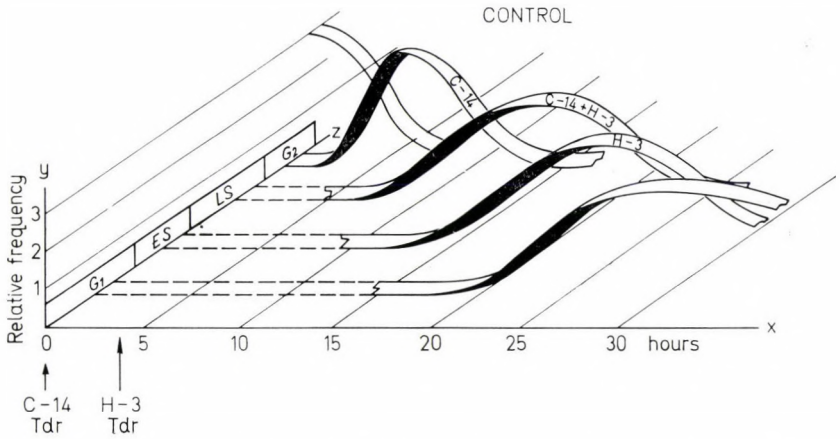


Fig. 1. Appearance of the four groups of nonirradiated ascites lymphoma cells in the M phase, as a function of time. The asynchronous cell population was divided into groups by ^{14}C Tdr and ^{3}H Tdr, given with a difference of 4 hours. The G_2-stage cells were labelled only with ^{14}C, the late S (LS) cells with ^{14}C and ^{3}H, the early S (ES) cells only with ^{3}H. Cells in phase G_1 constituted the nonlabelled group

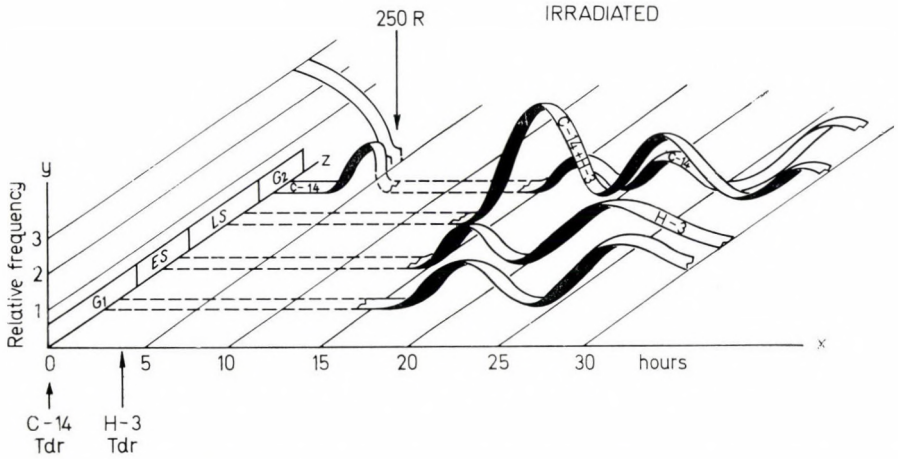


Fig. 2. Appearance of the four cell groups in the M phase after irradiation with 250 R and the release of mitotic inhibition. For key see Fig. 1

cell groups is more demonstrative in Fig. 3. The horizontal columns represent the half value widths of the incidence curve.

Using this way of plotting, the difference between the mitotic kinetics of the control and irradiated ascites cells is even more striking. Owing to the variation among the individual cells of the population, the limit between the cell groups of an identical stage becomes indistinct even in the control population. However, in a given period of time the cells from two groups succeeding each other may be well

differentiated. The transition (zone of mixing) from one group to the other is shorter between the G_2 and the late S group and is longer between the late S and early S, and between the early S and G_1 groups, resp. The mentioned sequence of the groups cannot be recognized at all in the irradiated cells. After the release of mitotic inhibition, practically in the first postirradiation mitotic cycle, cells originating from 2 to 3 various sites of the generation cycle did divide. Moreover, the simultaneous division of all the 4 cell groups could be observed in the smears in the 20th to 22nd hour of the experimental series. This means that as soon as the irradiation-induced mitosis inhibition is released, cells staying in the various phases at the time of irradiation will be superimposed with the restart of mitoses. Thus, cells from the

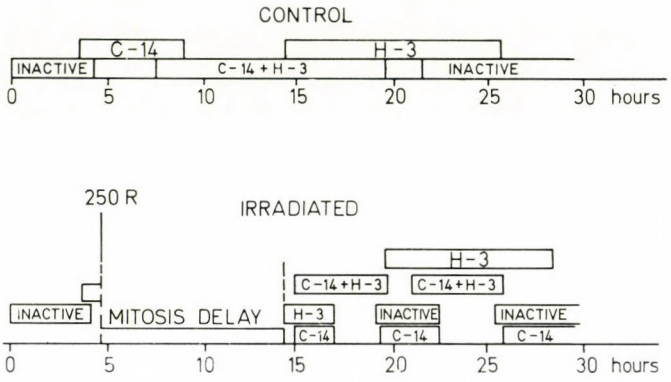


Fig. 3. Half value widths of the frequency curves indicated in Figs 1 and 2 in the form of a horizontal column and their sequence in the nonirradiated control (above), and in the ascites cells irradiated with 250 R in the 25th minute of the 5th hour of the experiment (below)

second half of the S phase, even those from its first half, indeed, catch up with the cells of phase G_2. What is more, they divide with an even higher frequency than do the latter ones.

The unlike radiosensitivities of the single phases may obviously play a part in the development of this phenomenon. One may also assume that irradiation accelerates the passage of the cells through the various phases. Finally, since the cells were irradiated in a host animal, one cannot exclude the possibility that the radiation reaction of the host may also modify the behaviour of the ascites cells.

References

Brewen, J. G. (1965) Int. J. Radiat. Biol. *9* 391
Dewey, W. C., Humphrey, R. M. (1962) Radiat. Res. *16* 503
Németh L., Kellner, B. (1960) Naturwissenschaften *47* 544
Varga, L., Várterész, V. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 217
Watanabe, I., Okada, S. (1966) Radiat. Res. *27* 290

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (4), pp. 415–419 (1969)

Uptake of Streptomycin by Escherichia coli B*

M. Szőgyi, Gy. Tamás, I. Tarján

Biophysical Institute, Medical University, Budapest

(Received February 22, 1969)

The uptake of streptomycin by E. coli B was studied in relation to the incubation time in case of different concentrations of streptomycin. On the curves obtained from our experiments three periods can be distinguished. In the first period lasting several minutes the quantity of streptomycin taken up increases relatively quickly, then it hardly changes for approximately half an hour (plateau period), and in the third period, it increases again. From the analysis of the curves we concluded that the antibiotic molecules were first bound on the cell surface and they penetrated into the interior of the bacteria only later.

As an explanation for the plateau formation we supposed that between the binding places on the surfaces of bacteria (molecular ranges) and the antibiotic molecules a process (adsorption-desorption) took place resulting in a state of equilibrium. Interpreting the kinetics, we determined the constant of equilibrium of the interaction between cell surface and antibiotics at three various temperatures. Then the penetration constant of streptomycin was calculated on the basis of the third period of the curves.

Introduction

The uptake of streptomycin by bacteria under different experimental conditions has been investigated by a number of scientists. Studying the streptomycin uptake of Staphylococcus, Berkmann, Henry, Housewright, Henry (1948) found that, under the given conditions, the bacteria took up 1 to 3 per cent of the streptomycin contents of the medium. Similar experiments with E. coli were made by Anand, Davis (1960); Hancock (1960); Anand, Davis, Armitage (1960) and Dubin, Hancock, Davis (1963). These authors traced the time course of the process and obtained curves consisting of two and three periods, respectively.

On the basis of the works of the above mentioned authors we have made further examinations concerning partial processes of the streptomycin uptake by E. coli B, with special regard to the physicochemical aspects of these processes.

Methods

We used E. coli B strain for our experiments. The bacteria in the logarithmic phase of multiplication were centrifuged and incubated in 5 ml of the synthetic medium M9 (1 g NH_4Cl, 0.13 g $MgSO_4$, 3 g KH_2PO_4, 6 g Na_2HPO_4, 4 g glucose in 1000 ml of distilled water) with a given concentration of streptomycin (20 to 200 μg/ml) for the required time. At the end of the incubation the number of living

* Delivered at the Vth Congress of the Hungarian Biophysical Society, Szeged August, 1968.

cells was determined by the plate dilution method. We poured the suspension of bacteria on a G5 filter and filtered the medium containing antibiotics. The cells remaining on the filter were washed with sterile water and suspended in 0.3 ml of water (about 10^9 bacteria). The determination of antibiotics taken up by the bacteria was carried out by the agar diffusion method (Szőgyi, 1967; Ševčik, 1963) using the above mentioned suspension in 0.1 ml quantities. In every case we made a standard series of dilution, whose inhibition zones were approximately identical with the inhibition zones produced by the streptomycin taken up by the bacteria.

Results

The set of curves presented in Fig. 1 shows a typical experimental result. The average quantity of streptomycin taken up by bacteria is plotted against the time in case of various streptomycin concentrations. On each experimental curve three

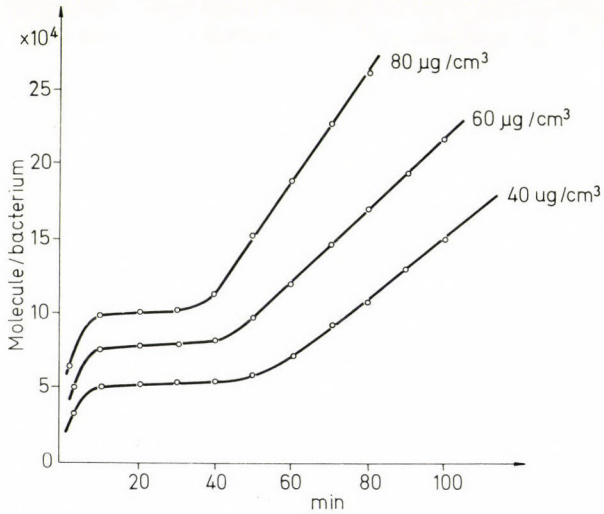


Fig. 1. The change in time of the quantity of streptomycin taken up by one bacterium in case of various streptomycin concentrations of 40, 60 and 80 μg/ml (t = 20 °C)

periods can be distinguished. The quantity of the streptomycin uptake increases quickly in the first period of several minutes, then it hardly changes for about half an hour (plateau period). In the third period the streptomycin uptake increases again.

Fig. 2 shows the quantity of streptomycin taken up by one bacterium in the plateau period at a temperature of 20 °C (after incubation for 15 minutes) in case of various concentrations of streptomycin. Fig. 2 shows that with the alteration of the external streptomycin concentration (20 to 200 μg/ml) the quantity of the streptomycin uptake changes according to a curve directed to saturation.

We also examined the dependence of the streptomycin uptake on the temperature in the plateau period at 5, 20 and 37 °C, respectively (Fig. 3). In case of a given streptomycin concentration, the uptake decreases with the increase of the temperature.

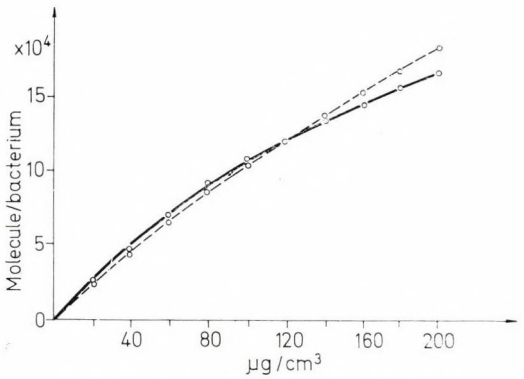


Fig. 2. The change of the quantity of streptomycin taken up by one bacterium in the plateau period at a temperature of 20 °C in case of various streptomycin concentrations. The individual points were derived from the mean values of 10 measurements each; the errors of the mean values are below ± 2 per cent

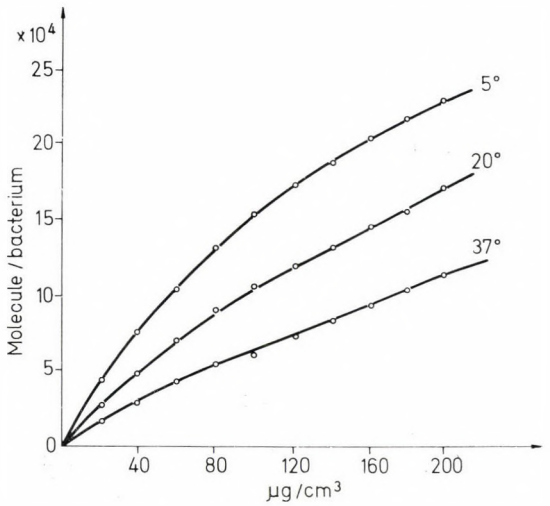


Fig. 3. The change of the quantity of streptomycin taken up by one bacterium in the plateau period in case of various streptomycin concentrations, at 5, 20 and 37 °C

Discussion

1. In interpreting our results we started with the following considerations. Let us suppose that, on the surface of a bacterium, there is a definite number of places or molecular ranges to each of which one streptomycin molecule is bound. The binding is of transient character; the bound molecule can break off from the cell surface. Binding and disjunction, as processes of opposite meaning, result in a state of equilibrium, similarly to the adsorption and desorption. This state of equilibrium is characterized by the approximately horizontal part of the curves in

Fig. 1 (plateau). A further significant change in the streptomycin uptake takes place when the streptomycin molecules are bound not only on the surface of a bacterium but they are able to penetrate into its interior too. The third period of the curves gives information about the speed of the penetration of streptomycin molecules into the interior of the bacterium.

2. a) Let us make a comparison between the above mentioned considerations and the results of the experiments. Let N be the number of possible binding sites on the surface of one bacterium while N_S is the number of places occupied by the streptomycin molecules in case of equilibrium. At a given temperature and in case of a given milieu, the value of N only depends on the antecedents of the bacteria (strain, stadium of proliferation) while the value N_S depends also on the concentration of streptomycin (C_S) as shown in Fig. 2. The relationship between N, N_S and C_S can be described by the well-known laws of adsorption which, in a given case, assume the following form:

$$N_S = \frac{C_S N}{\kappa + C_S}$$

where κ is the equilibrium constant under the given conditions (temperature, milieu). In Fig. 2 the curve calculated from the above equation is drawn by a broken line, while the continuous lines show the results of experiments. Considering the errors of the mean values of the experimental results, the calculated curve matches well the curve found in the experiment. A deviation only appears with higher streptomycin concentrations (160 to 200 μg/ml) which can be explained by secondary effects (membrane damage). The above mentioned relation provides an opportunity to determine in a given case the number of the possible binding sites (N). The value of N was approximately 6.10^5 which, in its order of magnitude, corresponds well to the values estimated by others in different ways (Engelberg, Artmann, 1962). From our measurements we calculated the equilibrium constant which was at a temperature of 20 °C $(3.6 \pm 0.04)\ 10^{-4}$ g/ml.

b) According to Fig. 3 the uptake of streptomycin in the plateau period decreases with increasing temperature. The calculated constants of equilibrium are $(2.2 \pm 0.03)\ 10^{-4}$ g/ml at 5 °C, and $(5.8 \pm 0.06)\ 10^{-4}$ g/ml at 37 °C. The pattern of the set of curves (isotherms) taken up at various temperatures allows to conclude at a characteristic adsorption phenomenon which supports our assumptions. By means of the isotherms we also determined the heat of adsorption of the streptomycin binding which corresponded to 4800 cal/mol, or to an energy of about 0.2 eV for each molecule.

c) The third period in Fig. 1 characterizes the penetration of streptomycin into the interior of the bacteria. After a certain period of time the penetration becomes considerable (plateau period). The binding of streptomycin initiates namely further processes on the bacterium surface and as a result of these processes, it is only after a definite period of time that some of the bound molecules can penetrate into the interior of the bacteria. In this connection we refer to the fact that, in the opinion of some authors (Anand, Davis, 1960; Anand et al., 1960; Davies et al., 1964; Hancock, 1960; Plotz, Davis, 1962), the streptomycin gives rise to damages

on the membrane of the sensitive bacteria and it is only in consequence of this fact that the molecules of antibiotics are able to penetrate into the cell. The cell membrane of resistant strains does not suffer damages under the influence of streptomycin, so the third period of the uptake fails to develop (Engelberg, Artmann, 1964; Tamás, Szőgyi, 1966).

According to our experiments the penetration time constant related to streptomycin of the bacterium membrane (Tamás, Szőgyi, 1966) is

$$k = 0.009/\text{min}$$

The value of the time constant does not depend on the temperature.

The authors thank Miss Julia Bitai for her participating in the experiments.

References

Anand, N., Davis, B. D. (1960) Nature *185* 22
Anand, N., Davis, B. D., Armitage, A. K. (1960) Nature *185* 23
Berkmann, S., Henry, R. J., Housewrigth, R. D., Henry, J. (1938) Proc. Soc. exp. biol. Med. *48* 65
Davies, J., Gilbert, W., Gorini, L. (1964) Proc. Nat. Acad. Sci. *51* 883
Dubin, D. T., Hancock, R., Davis, B. D. (1963) Biochim. Biophys. Acta *74* 476
Engelberg, H., Artmann, M. (1962) Biochim. Biophys. Acta *54* 533
Engelberg, H., Artmann, M. (1964) Biochim. Biophys. Acta *80* 256
Hancock, R. (1960) J. Gen. Mikrobiol. *23* 179
Plotz, R., Davis, B. D. (1962) J. Bact. *83* 802
Roth, H., Amos, H., Davis, B. D. (1960) Biochim. Biophys. Acta *37* 398
Ševčik, V. (1963) Antibiotica aus Actinomyceten. VEB Gustav Fischer Verlag, Jena
Szőgyi, M. (1967) Dissertation for doctor's degree in pharmacy
Tamás, Gy., Szőgyi, M. (1966) Kísérletes Orvostudomány *18* 185

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (4), pp. 421–427 (1969)

A Simple Method for the Autoradiography of Soluble Isotopes

Autoradiography of Soluble Materials

J. BRASCH, I. FEHÉR

"Frédéric Joliot-Curie" National Research Institute for Radiology and Radiohygiene, Budapest

(Received March 26, 1969)

A simple, easily reproducible method has been developed for the autoradiography of soluble radioactive isotopes.

1. The frozen organ is sectioned in kryostat,
2. Dehydrated at −10°C for 3 to 5 days.
3. The slides warmed up to room temperature are fixed in the vapour of concentrated formol for 5 minutes.
4. The slides are coated with stripping film, using the sandwich method.
5. The day before development the slides are coated with 1 per cent chrome gelatin.
6. Following development and fixation the slides are washed in 1 per cent NaCl solution for 30 minutes and stained with haematoxylin-eosin by the usual way.

Introduction

The use of radioactive materials has become almost indispensable in up-to-date medical and biological research. Also, it is often necessary to trace the way of the labelled material within the organism, to be able to locate it in a certain tissue or cell structure.

When performing such works, the basic requirement is that the labelled material should not leave its original site in the course of histological preparation and autoradiography. This condition may be easily realized in those cases when the labelled material is known to be built-in into compounds insoluble in water or in any other solvent used during histological preparation. This ideal condition is satisfactorily realized in the case of such labelled compounds that are built-in into biological macromolecules, because the isotopes built-in into proteins or nucleic acids are stabilized by the fixation denaturing the molecule.

However, a fair number of methodological problems arise if the labelled compound remains in soluble form. The conventional histological and autoradiographic methods cannot be resorted any more, since in the course of histological preparation the labelled molecule migrates into the inactive sites and will give misleading results. Maybe, partly or totally, it gets extracted from the tissue or disappears entirely. These problems are often met with when e.g. the localization of labelled drugs should be investigated by autoradiography.

A method satisfactory from every aspect for the autoradiography of soluble matters has not yet been developed.

As mentioned by Rogers (1967), the standard methods for the autoradiography of insoluble isotopes have developed during the past 10 years. On the other hand, many new, technically complicated and hardly reproducible methods have been suggested for the autoradiography of soluble compounds during this same period (Ullberg, 1958; Pellerin, 1961; Babel, Bonventre, 1963; Aitken, 1968, for whole body autoradiography; Wilske, Ross, 1965; Fitzgerald, 1961, for the autoradiography of microscopic slides).

The common feature in these methods is that frozen slides are exposed to autoradiography. It is well known that frozen sections never are technically as perfect as embedded preparations. However, for the time being, no other method is known for stabilizing soluble isotopes. Accordingly, frozen sections were used also in the present experiments. A method, more simple than those used hitherto, has been developed in our laboratory for the autoradiography of soluble isotopes. This method has been successfully used in our laboratory for some time, mainly to study the distribution of labelled drugs.

Method

Fixing and sectioning. The organ to be studied is removed from the isotope-treated animal immediately after killing and is fixed by freezing to the microtome block precooled to −30°C. Fixing is performed as follows: the microtome block is surrounded by dry-ice and the organ, on a piece of filter paper, is placed on the top of the block. All these operations should be completed as quickly as possible and one should take care of the temperature of the section to remain below the freezing point as long as dehydration is completed.

Three to five μ thick sections are then prepared in the cryostat at − 15 to 18°C. The sections are mounted to slides cooled to − 5 to 10°C and previously coated with 1 per cent chrome gelatin.

The next step is dehydration. Depending on the thickness of the slides, they are put into a refrigerator (−10°C) for 3 to 5 days. After dehydration the slides are quickly warmed up to room temperature in a hot air stream (this method avoids the fogging of the slide). A commercial hair-drier is used for this operation. For fixation the slides are put into a box containing a Petri dish with a piece of cotton previously soaked in concentrated formol. They are left in the box for 5 minutes.

Autoradiography. The film (Kodak AR 10) is removed from its glass support by floating on water. Next, the stripped film is draped over a clean slide with its *emulsion layer upwards* and let dry for 10 minutes. Then the slide with the section is pressed against the slide with the stripping film, in a sandwich-like way, so that the emulsion layer and the section should both contact and cover each other. The two slides are firmly pressed together to drive the air bubbles out. The free edges of the stripping film are gently smoothed by a brush onto the back side of the slide with the section and the slide carrying the film is then carefully removed. As a next step, the slides are put into a light-proof box. Some hygroscopic substance should be put into the box. Freshly dehydrated silica gel is used in our laboratory.

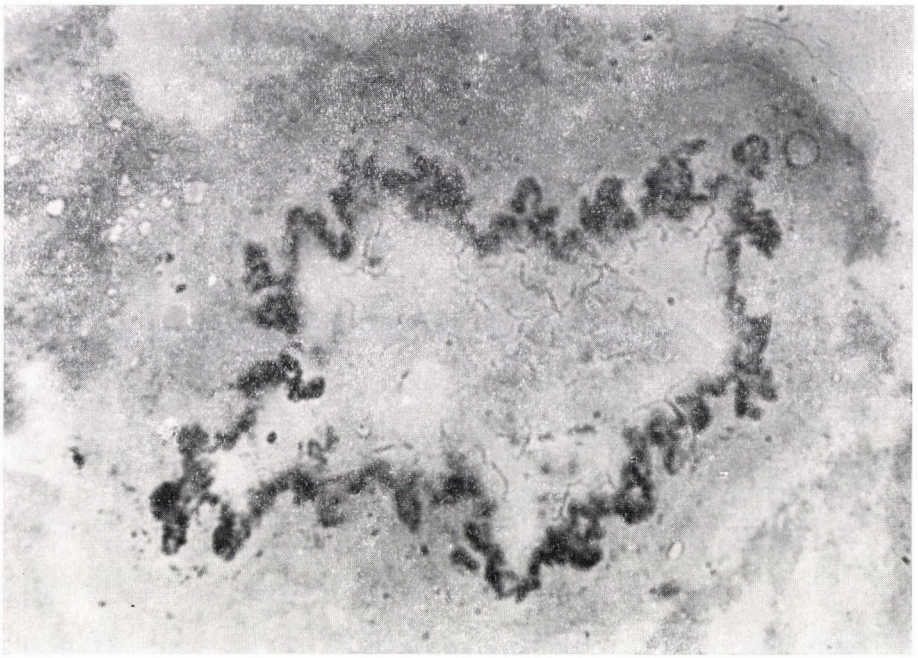


Fig. 1a. Distribution of 1 μCi/g ^{14}C-histamine in the mucous membrane of the bronchial tubes in the 5th minute after intravenous injection. × 120; H. E.

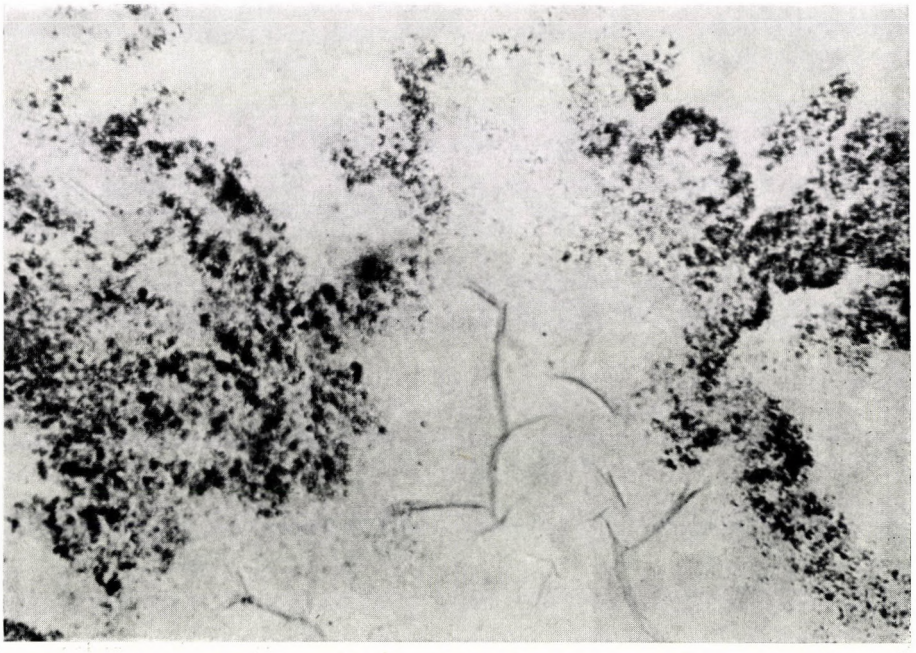


Fig. 1b. Magnified detail of Fig. 1a. × 400

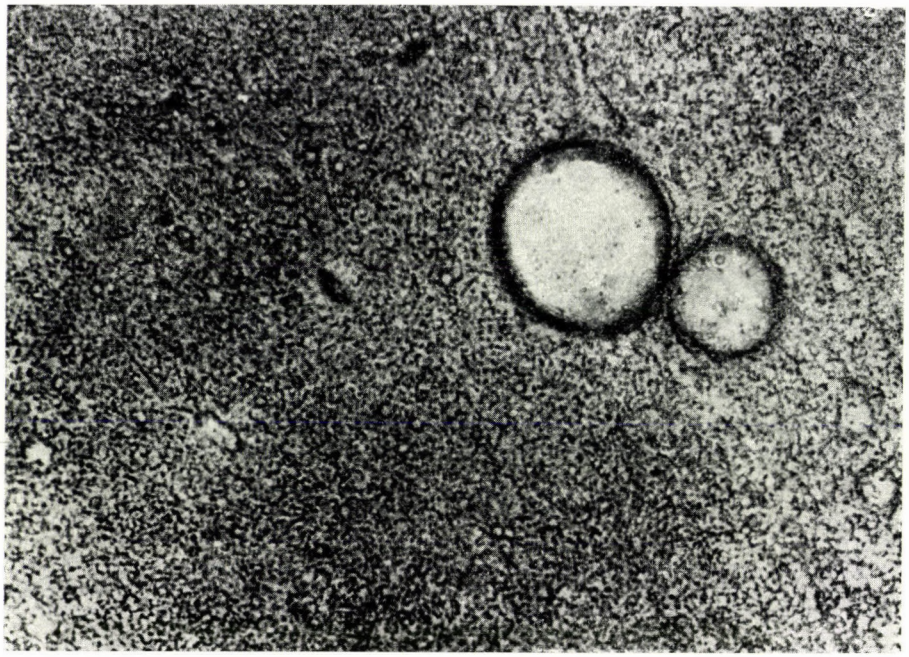


Fig. 2. Distribution of the ^{3}H-labelled sedative in the blood vessels of the pedunculus cerebri in the 60th minute after the intravenous injection of 2 μCi/g. × 800; H. E.

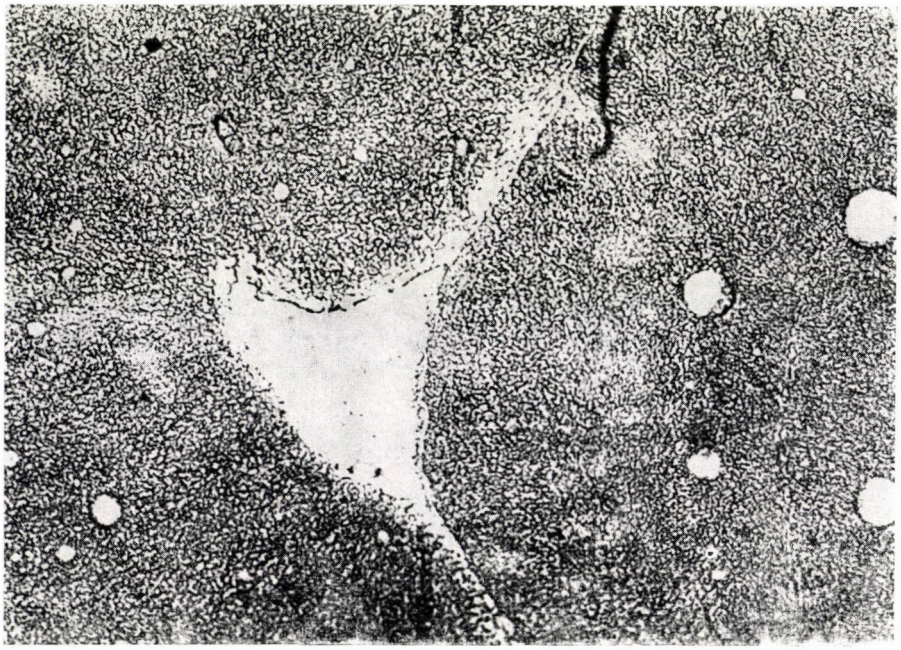


Fig. 3. Distribution of 2 μCi/g intravenously injected, ^{3}H-labelled sedative in the region of the fourth ventricle. × 900; H. E.

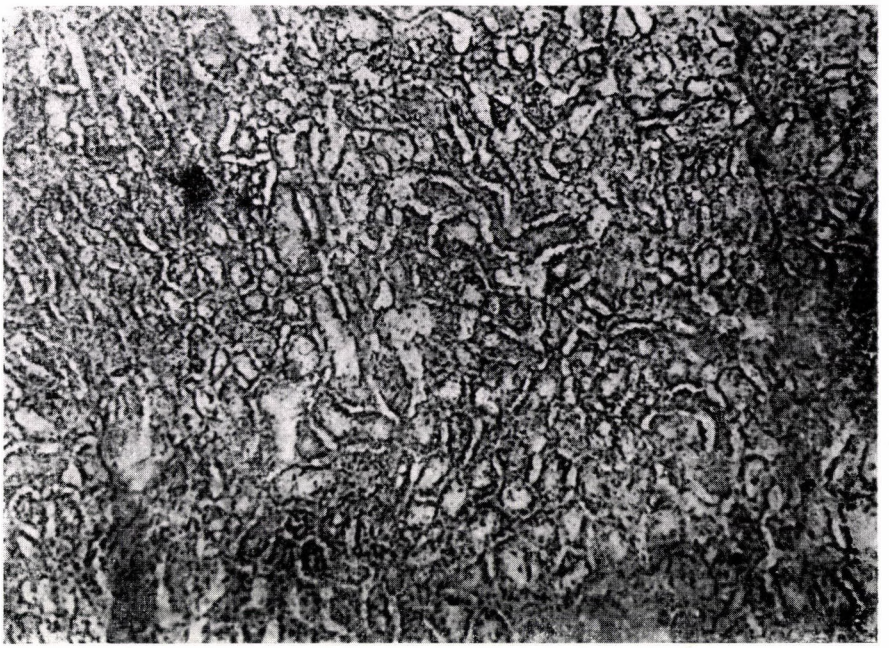


Fig. 4a. Distribution of ^{3}H-labelled sedative in the kidneys in the 6th hour after the intravenous injection of ^{3}H-labelled sedative. × 200; H. E.

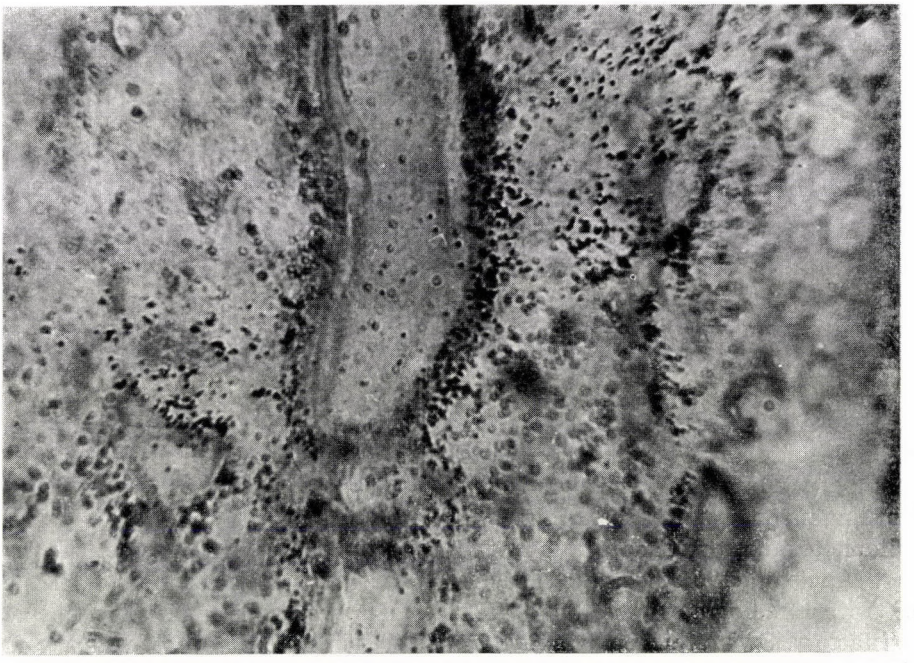


Fig. 4b. Magnified detail of Fig. 4a showing activity in the epithelium of the tubulus. × 1000; H. E.

Exposure lasts for a definite time (established by trials) at 4°C. One day before development the slides are again coated with 1 per cent chrome gelatin. The slides are developed in Kodak's D 19 developer for 5 to 20 minutes.

Staining. The developed and fixed slides are washed in 1 per cent NaCl solution for 30 minutes and rinsed in distilled water for 1 minute. The conventional histological methods (staining and mounting) may be used in the following to process the preparation.

Discussion

The quality of the sections cut in the cryostat is rather strongly influenced by dehydration. Any too long dehydration is harmful to the quality of the section, for a part of the thin sections pulverizes. The observance of the time proposed by us for dehydration is, therefore, of importance. Neither did we find satisfactory the method adopted by Stumpf and Roth (1964), i.e. freeze-drying in vacuum. Owing to the too rapid drying, some parts of the section happen to detach from the slide when applying this method.

The application of the radiosensitive emulsion or stripping film is one of the technically most difficult problems of soluble autoradiography. The data in the literature suggest a number of methods that avoid the wetting of the preparation (Miller et al., 1965; Stumpf, Roth, 1966). These methods have the common drawback that the application of the emulsion or film has to be performed mostly at a low temperature by which the film becomes rigid. This may result in its poor adhesion or its moving away. Fitzgerald et al. (1961) suggested the draping of dry stripping film over a cold slide. In this case the fog arising when the slide is warming up may be sufficient for the adhesion of the film, but very often it is sufficient also for loosening of the labelling.

Appleton's (1964) method is a rather wide-spread one. According to him, the frozen section has to be spread over a slide coated with emulsion. The technical difficulty of this method is that both cutting and mounting have to be performed in dark or by poor red light. Very often the section floats away from the slide.

The methods of Roberts et al. (1964) and of Stumpf and Roth (1964) gave very good results. They mounted the section on a Teflon slide and pressed it firmly against the emulsion during exposure. The "dangerous corner" of this method is that the section often moves away when removing the Teflon slide.

The dissolution or spreading of labelling may be prevented by impregnating the slide (Aitken et al., 1968). This method has the drawback that whenever isotopes emitting soft beta rays are used (e.g. in the case of the rather often used tritiated compounds), part or the beta-particles will be absorbed. Accordingly, either the labelling index of the exposure time will have to be raised. This method is only suitable for the rough localization of highly active isotopes.

Appleton (1964) fixed the slides in 4 per cent formol after the development of the autoradiograms. We found the autoradiography of unfixed sections dissatisfactory. Owing to chemography, fresh tissues in a close contact with the nuclear emulsion give a higher background than do fixed tissues. A further disadvantage of this method is that fixation and succeeding washings increase the probabil-

ity of floating or moving away of the film. We obtained better results with fixing the sections prior to mounting the film. The danger of the stripping film's moving away during the developing and staining procedures may practically be prevented by a chrome gelatin treatment before development. The use of NaCl solution instead of distilled water for washing serves the same purpose.

Figures 1 to 4 show photographs of slides prepared according to the above method. They demonstrate the advantages and disadvantages of the method. The activity is well localized; the labelled material has not diffused into the surrounding tissues during the process. However, the fine cellular and intracellular structure is not clearly distinguishable, just as in every unfixed and frozen section.

The figures are selected sections of different mouse tissues. For detailed data see the legends of the photographs.

References

Aitken, W. M., Wright, E. (1968) Stain. Techn. *43* 1
Appleton, T. C. (1964) J. Roy. Microscop. Soc. *83* 277
Babel, H. C., Bonventre, P. F. (1963) J. Lab. Clin. Med. *61* 324
Fitzgerald, P. J. (1961) Lab. Invest. *10* 846
Fitzgerald, P. J., Ord, M. G., Sticken, L. A. (1963) Nature *189* 55
Miller, O. L., Stone, G. E., Prescott, M. D. (1965) J. Cell Biol. *23* 654
Pellerin, P. (1961) Path. Biol. *9* 233
Roberts, M. L., Ciofalo, L., Martin, L. (1964) Stain. Techn. *39* 47
Rogers, A. W. (1967) Techniques of Autoradiography, p. 224. Elsevier Publ., Amsterdam
Stumpf, W. E., Roth, L. J. (1964) Stain. Techn. *39* 219
Stumpf, W. E., Roth, L. J. (1966) J. Histochem. Cytochem. *14* 274
Ullberg, S. (1958) Proc. 2nd Int. Conf. on Peaceful Uses of Atomic Energy *27* 248
Wilske, K. R., Ross, R. (1965) J. Histochem. Cytochem. *13* 38

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (4), pp. 429–436 (1969)

Action Potentials Induced by Photoelectrons*

T. LAKATOS

Biophysical Institute, Medical University, Pécs

(Received June 16, 1969)

Photodynamic experiments are reported on nerve cord of the crayfish *Astacus astacus* and of the sea crab *Squilla mantis*, as well as on frog sciatic nerve, and squid stellar nerve. The sensitized and illuminated nerves produced action potentials. The results are considered to be a contribution to the electron theory of biological excitatory phenomena.

Introduction

A series of photodynamic experiments was carried out previously (cf. Lakatos, Kollár-Mórocz, 1969) which showed a connection to exist between the occurrence of biological excitation and the photodynamic effect. The ability of light to induce electrical activity was demonstrated in the sensitized nerves of *Cancer pagurus* (Auger, Fessard, 1933). In the present paper photodynamic experiments are reported on different subjects: frog sciatic nerve, the ventral cord of the crayfish *Astacus astacus*, that of the sea crab *Squilla mantis*, and the stellar nerve (including a giant axon) of the squid *Loligo vulgaris* were used in the course of the experiments.

Materials and Methods

Sciatic nerve of the frog Rana esculenta was used in the first series of experiments. The nerves were excised in the usual way and kept in Ringer solution. After their excitability had been checked by measuring the stimulus threshold, it was controlled whether they showed any spontaneous activity in dark or during illumination. This procedure was followed by keeping the nerves for two hours in a Ringer solution containing one of the sensitizers. The stained nerve was watched again whether it produced any electrical activity in dark or during illumination for 5 minutes.

The electrical stimulator was a transistorized square wave generator. The duration of pulses were 0.1, 0.5, or 1.0 msec, resp., with an amplitude adjustable with an accuracy of 0.01 V. A cathod ray oscilloscope was used to indicate the electrical activity; it had an A. C. input of 0.5 Mohm, a time constant of 3 sec and a sensitivity of 0.5 mV/cm.

* Part of the work reported here was made in the Brain Research Laboratory of the Marine Biological Station, Kotor, Yugoslavia.

Eosin (sodium tetrabromine fluorescein), neutral red and bengal rose dissolved in Ringer solution in different concentrations between 10^{-5} and 10^{-3} g/ml were used as sensitizer.

The illumination was produced by a tungsten filament bulb (Tungsram Type 64519 IMP T4). The light beam collected by a condenser lens 14 cm in diameter passed through a 1 per cent solution of $CuSO_4$ which filtered it from infrared radiation. The increase of temperature was not more than 1°C. The illumination intensity was 80 000 lux in every case, if not stated otherwise.

Extracellular electrodes were used for stimulation as well as for recording the action potentials. Four platinum wires were mounted in a plexiglass moist chamber covered with brass plate in order to keep the nerves and the electrodes in dark and to shield them electrically. A glass window covered by a removable metal cap allowed the illumination. The top side of the chamber was covered by a container in which ice was put for keeping the inside of the chamber below room temperature, at about 15°C.

The ventral cord of the crayfish Astacus astacus was dissected after the animal had been cooled down in a refrigerator; the dissected ventral cord was kept in Pringle's solution. Experiments on ventral cords were performed like those on sciatic nerves but the staining did not take more than 10 minutes. The sensitizer was always eosin dissolved in Pringle's solution in a concentration of 10^{-4} g/ml. The same experiment was performed with the ventral cord of the sea crab *Squilla mantis*, using in this case sea water instead of Pringle's solution.

Stellar nerve of the squid Loligo vulgaris was dissected from freshly captured animals. A stellar nerve consists of a giant axon surrounded by a sheath of connective tissue in the inside of which small nerve fibres are adhered very closely to the axon (e.g. Hodgkin, 1967).

The mantle muscle of the squid was kept in sea water during the dissection which was performed under a binocular dissecting microscope. The dissected nerve (3 to 6 cm long) kept in pure oxygenated sea water was active for a few hours. The sensitizer was eosin (Fluka AG eosin y) dissolved in pure sea water in a concentration of 10^{-4} g/ml; the staining took 10 minutes. The experiments were performed in the way described above. A GRASS stimulator and cathod ray oscilloscope were used in these experiments, the recorder was a "Nikhon" camera suitable for making single frame shots as well as continuous records.

Results

Frog sciatic nerves. None of the investigated nerves showed spontaneous activity either unstained in dark and illuminated, or stained in dark irrespective of the dye used. 20 out of 80 nerves stained with eosin produced electric activity during the illumination. 125 nerves were stained with neutral red; 9 of them spiked when illuminated, and 13 out of 108 nerves stained in bengal rose solution proved to be electrically active. Altogether 313 nerves were investigated, and action potentials were evoked by the visible light in 42 cases, when the nerves were stained. The spikes appeared 30 seconds after the beginning of the illumination. Single

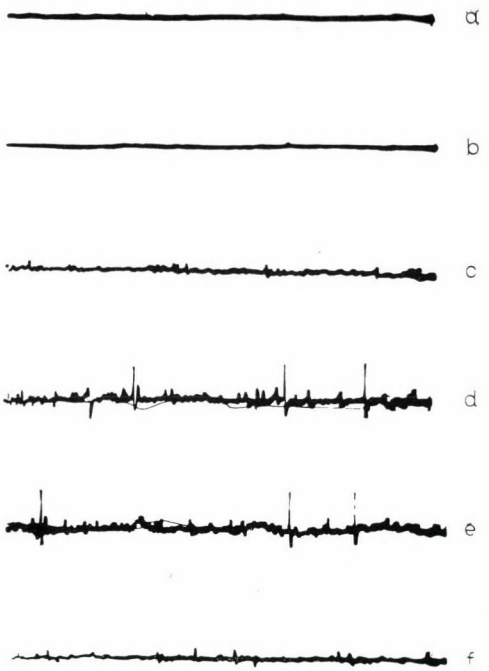


Fig. 1. Nerve cord of crayfish. *Unstained* a: in dark; b: illuminated. *Stained* c: in dark; d, e: illuminated; f: in dark again

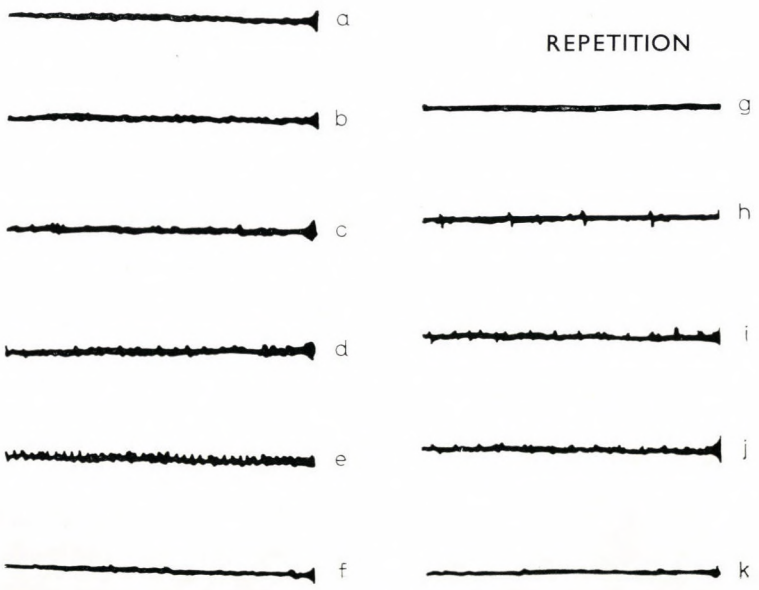


Fig. 2. Nerve cord of crayfish. *Unstained* a: in dark; b: illuminated. *Stained* c: in dark; d, e: illuminated; f, g: in dark again; h, i, j: illuminated again; k: in dark

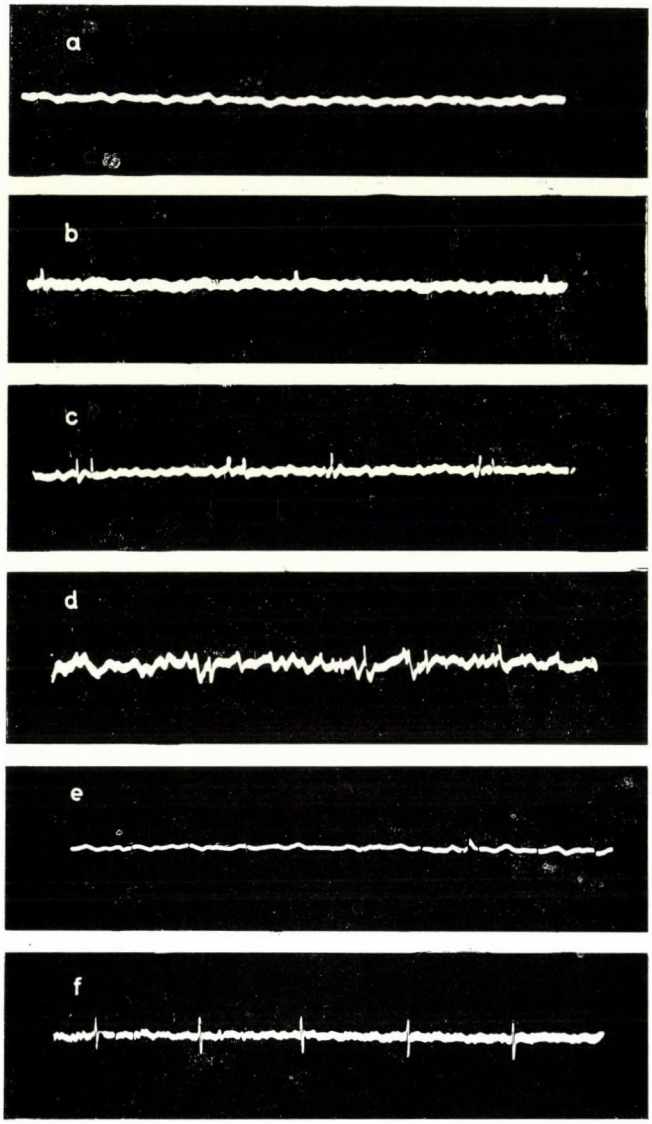


Fig. 3. *Squilla mantis* nerve cord. *All stained* a: in dark; b, c, d: illuminated; e: in dark again; f: illuminated again

spikes and short bursts of spikes were observable during the illumination and after switching off the light for a few minutes. The appearance of spiking was at random.

The most efficacious dye was eosin: considering all the 80 experiments performed in the presence of eosin at different concentrations the light brought about action potentials in 25 per cent of the experiments, but this rate was 36 per cent (17 out of 42 experiments) when the concentration was 10^{-3} g/ml.

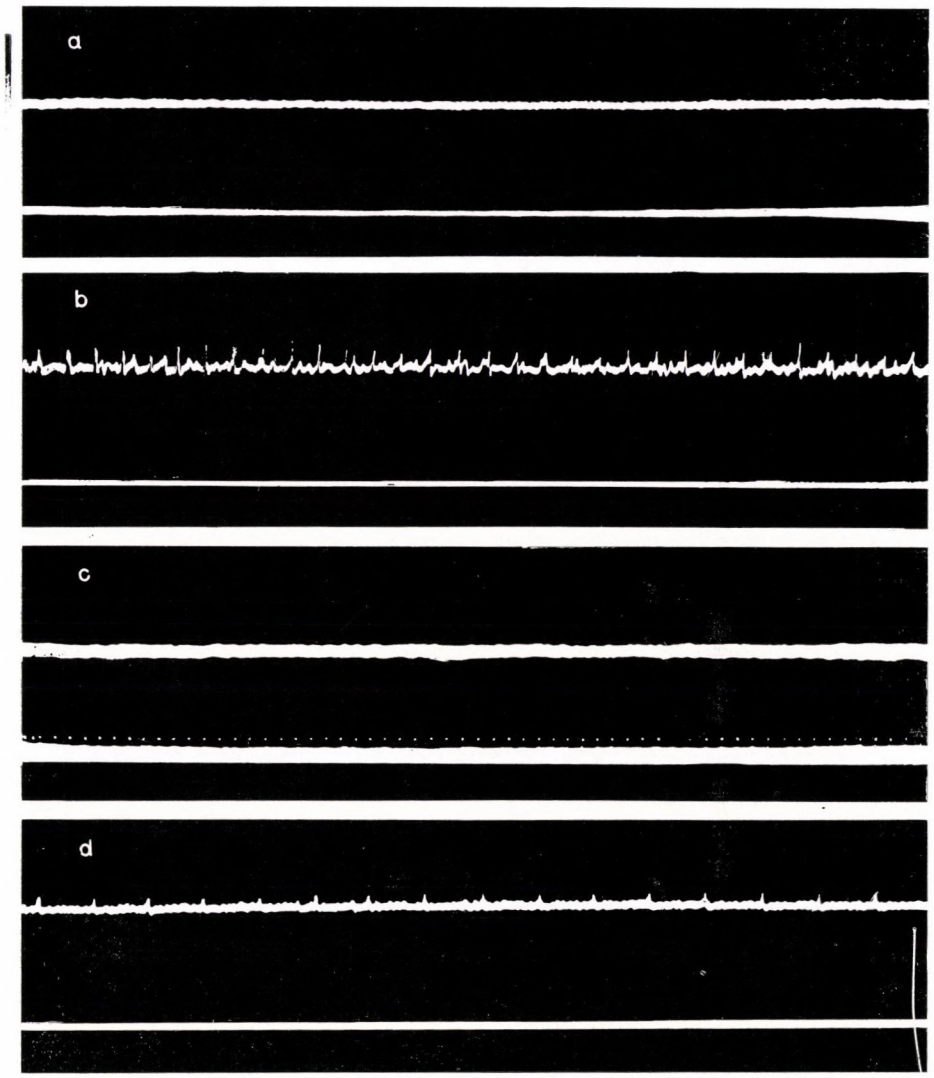


Fig. 4. *Loligo* stellar nerve. *All stained* a, c: in dark; b, d: illuminated

The ventral cord of crayfish was a much more sensible subject. The freshly dissected ventral cord showed spontaneous activity in several cases, but not always. The specimens showing no activity or only exhibiting a slight degree of activity were chosen for performing experiments. When the unstained nerve cord produced no action potentials in dark, it did not do so during illumination either (Figs. 1a, b), and even if the specimen showed a slight activity, it was not influenced by the light. Some activity was usually observed after the nerve cord had been stained but kept in dark (Fig. 1c). 5 to 10 seconds after switching on the light frequent

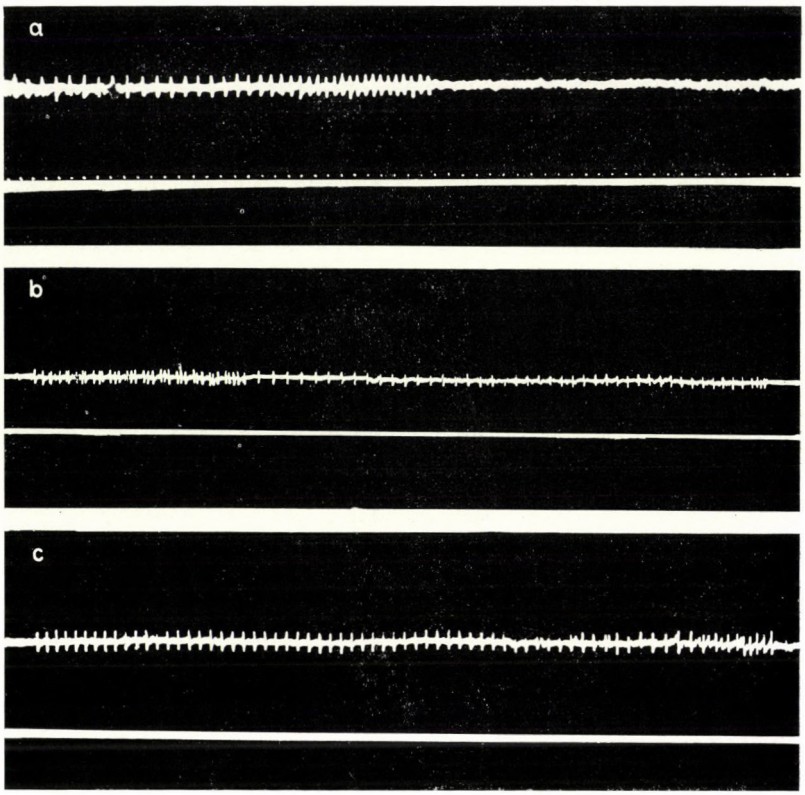


Fig. 5. *Loligo* stellar nerve. *All stained.* a: the end of a train of spikes, which lasted for 10 seconds after a 30 sec illumination. b and c: short train of spikes after switching off the illumination

spikes could be observed (Fig. 1d, e), sometimes the spiking was quite periodical (Fig. 2e—h). After switching off the light the activity gradually decreased and after a few minutes, no spontaneous activity was seen (Figs 1f, 2f, g). The photodynamic activity could be evoked by illuminating the nerve cord again (Fig. 2g—k). The same phenomenon was shown by the ventral cord of the sea crab *Squilla mantis* (Fig. 3).

The simplest conditions were met with in the case of the *stellar nerve (including a giant axon) of the squid* which, when unstained, never produced spontaneous activity either in dark or upon illumination. The stained axon was inactive in dark (Fig. 4a). About 20 seconds after starting illumination (70 000 lux in this case) a strong spiking began suddenly (Fig. 4b). After switching off the light the spiking did not come to an end at once, but it lasted a few minutes further, and, before the nerve became quiet, short periodical bursts of spikes followed each other at irregular intervals (Figs 5, 6). The quiet periods between the bursts became longer and longer and finally the spiking ceased completely. Then, a new illumination

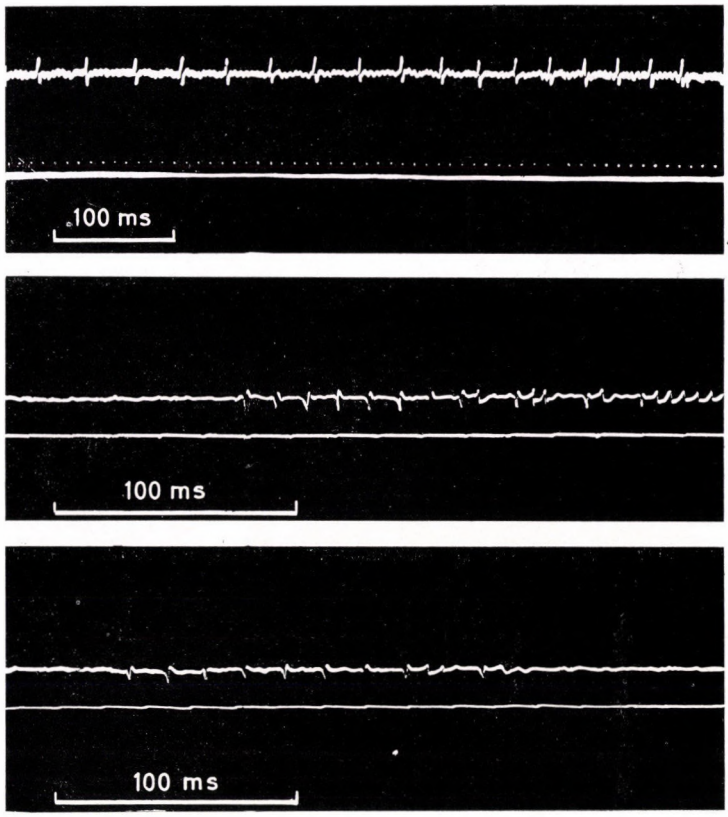


Fig. 6. *Loligo* stellar nerve. *All stained.* Short bursts of spikes after switching off the illumination, but before the nerve became perfectly quiet

could, again, provoke trains of action potential (sometimes strictly periodical) (Fig. 4d).

One and all the investigated 11 axons produced the same results: spontaneous activity never came about; staining or illumination failed to provoke action potentials separately, while the joint action of staining and illumination always resulted in train of spikes.

Discussion

All these experiments prove the ability of light to produce biological excitation.

Though the phenomenon is of statistical character in the case of frog sciatic nerves, there is no need to check the results by statistical test, because action potentials never occurred with unstained nerves either in dark or in strong light; stained nerves did not produce any activity in dark; action potentials were only evoked by the simultaneous action of stain and light.

When the sensitized stellar nerve of the squid was illuminated, bursts of spikes appeared, but the amplitude and frequency of the spikes were not constant.

The main component of a stellar nerve is a giant axon. There are, however, several small fibres in the sheath of connective tissue around it. Therefore the specimen did not show the clear response of a single fibre, but the amplitude and the frequency of spiking changed at random. Later in the course of experiments periodical trains of spikes were found with a constant amplitude, when the illumination lasted for a long time (Fig. 5a) or was repeated after the nerve had been kept in dark for a while (Fig. 4e). Very likely, the small fibres were exhausted in a shorter period of time than the giant axon itself, therefore these late periodic trains of spikes could be considered as the activity of one single giant axon.

The occurrence of excitation processes in excitable tissues has no solid explanation. Different theories have been constructed (cf. Ridge, Walker, 1963; Hodgkin, 1967), but all of them had a number of difficulties (Ernst, 1963; Ridge, Walker, 1963). The idea that electron processes take a prominent part in biological excitatory phenomena (Ernst, 1955, 1956) offered an opportunity to perform new experiments and to investigate the experimental results from a new point of view.

It has been expounded previously (e.g. Lakatos, Kollár-Mórocz, 1967) that the influence of light on the excitation of biological tissues supported the electron theory of excitation (Ernst, 1968). The experiments reported here immediately indicate that light is able to bring about biological excitation in sensitized nerves. *That means the photons excite electrons in the dye;* the energy of excitation migrates into the electron system of the molecules which the irritable tissue consists of, and this change in the state of nerve tissue is enough to evoke the excitation process, i.e. an action potential. More detailed experiments are required to make clear how the biological excitation is produced under the effect of excited electrons.

The author is indebted to Prof. Ernst for raising the problem as well as to Prof. Rakić and Ivanuš (Yugoslavia) for ensuring the facilities to work with squids. The study has been supported in part by the Marine Biological Station of Kotor, Yugoslavia.

References

Auger, D., Fessard, A. (1933) Ann. de Physiol. *9* 873
Ernst, E. (1955–56) Wiss. Z. Humboldt Univ. Berlin, Math.-Naturwiss. Reihe *5* 167
Эрнст, Э. (1956) Биофизика *1* 297
Ernst, E. (1963) Biophysics of the Striated Muscle. Akadémiai Kiadó, Budapest
Ernst, E. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 115
Hodgkin, A. L. (1967) The Conduction of the Nervous Impulse. C. C. Thomas Publisher, Springfield, Ill., USA
Lakatos, T., Kollár-Mórocz, A. (1967) Acta Biochim. Biophys. Acad. Sci. Hung. *2* 191
Lakatos, T., Kollár-Mórocz, A. (1969) Acta Biochim. Biophys. Acad. Sci. Hung. *4* 111
Ridge, R. A., Walker, R. J. (1963) Bioelectric Potentials. In: Kerkut, G. A.: Problems in Biology, I, p. 91–201

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (4), pp. 437–438 (1969)

Information Content of End-plate Potentials

(Short Communication)

G. BIRÓ

Biophysical Institute, Medical University, Pécs

(Received March 15, 1969)

Synaptic transmission is a field of biocybernetical examinations (Bullock, 1962; Segundo et al. 1966). The data on the probabilistic bases of the neuromuscular transmission permit of examining the neuromuscular transmission by means of information theory. The assumption that the motor end-plate has a coding function has been reported by Ernst (1961) and Biró (1968). The present short communication deals with the estimated information content represented by the miniature and the normal end-plate potentials.

According to del Castillo and Katz (1954) the normal end-plate potentials are built up of the miniature end-plate potentials. Their basic assumption is that, in the end-plate there is a large number (n) of units capable of generating a miniature end-plate potential and if the probability of such a generation is p, the average number (m) of units responding to a nerve impulse is given by

$$m = p \cdot n.$$

On experimental basis estimations have been published on the values of p and n. These values are about 0.14 and 700, respectively, for frog neuromuscular junction (Martin, 1966).

Adopting the information theory (e.g. Reza, 1961) for interpretation of the data mentioned above, the following considerations can be put forward:

1. If the number of units generating miniature end-plate potential is 700, then, theoretically, 700 different end-plate potentials can come into existence through summarization. Thus the maximum value of the average amount of information (I') associated with an end-plate potential is given by

$$I' = ld\, n = 9.5 \text{ bit}$$

2. Let E_1 and E_2 be the two responses referring to the events when a unit in the end-plate is generating a miniature end-plate potential or not. This situation is represented by the following matrix:

$$\begin{bmatrix} E \\ P \end{bmatrix} = \begin{bmatrix} E_1 & E_2 \\ p & 1-p \end{bmatrix}$$

The average amount of information (I'') associated with the response of a unit is given by

$$I'' = -p \cdot ld\, p - (1 - p) \cdot ld\, (1 - p) = 0.6 \text{ bit}$$

These data concerning the amount of information of the end-plate potential (I') and the miniature end-plate potential (I'') permit of concluding on the nature of the coding process taking place in the end-plate. The amount of information of 9.5 bit is the amount of information of the end-plate potential on condition that the end-plate potential develops from the miniature end-plate potentials by summarization. In this case the alterations in the order of the miniature end-plate potentials do not mean difference between the end-plate potentials. However, supposing the case in which the order of the miniature end-plate potentials would be also significant, the 700 units generating the miniature end-plate potentials could transmit an amount of information of 420 bit, and an amount of information of 9.5 bit would only need 16 units transmitting individually an amount of information of 0.6 bit.

This condition points to the possibility of a redundant coding process taking place in the motor end-plate. The code of high redundancy could be connected with the internal noise in the excitable channels (Rosenblith, 1961; Eijkman, Vendrik, 1963; Biró, Királyfalvi, 1966).

The former data concerning the information content can be considered to be just as "real" as the values of p and n. Nevertheless, the process of neuromuscular transmission investigated from the point of view of information theory requires still further examinations.

References

Biró, G. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 107
Biró, G., Királyfalvi, L. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 315
Bullock, T. H. (1962) in: Information Processing in the Nervous System. Gerard, R. W., Duyff, J. W. (editors), International Congress Series No. 49, Excerpta Medica Foundation, Amsterdam, p. 98
del Castillo, J., Katz, B. (1954) J. Physiol. *124* 560
Eijkman, E., Vendrik, A. J. H. (1963) Biophys. J. *3* 65
Ernst, E. (1961) Acta Physiol. Acad. Sci. Hung. *19* 51
Martin, A. R. (1966) Physiol. Rev. *46* 51
Reza, F. (1961) An Introduction to Information Theory. McGraw-Hill Book Co., Inc., New York
Rosenblith, W. A. (1961) in: Biophysical science — A study program. Oncley, J. C. (editor) New York, p. 485
Segundo, J. P., Perkel, D. H., Moore, G. P. (1966) Kibernetik *3* 67

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 4 (4), pp. 439–441 (1969)

Book Reviews

Physikalische Grundlagen der Medizin. Abhandlungen aus der Biophysik. Heft 7. Herausgegeben von Prof. Dr. W. Beier. VEB Georg Thieme, Leipzig, 1967. S. 205. (The Physical Bases of Medicine. Treatises from the Topic of Biophysics. Booklet 7. Editor: Prof. Dr. W. Beier. VEB Georg Thieme, Leipzig, 1967. 205 pages. In German.)

The booklet contains, in its whole extent, the main summarizing lectures (12) of the symposium held in the Leipzig Biophysical Institute about molecular biophysics as well as those of the sessions held by the Biophysical Society of GDR and the Czechoslovakian Biophysical Section at Leipzig from 18 to 21 Oct. 1965. In addition, it enumerates the authors and the titles of the 69 short lectures delivered. Finally, there are remarkable notes and a detailed program about the teaching and syllabus of biophysics, written by W. Beier and F. Pliquett.

The summarizing lectures deal with the following themes:

A. Wacker et al. (Frankfurt a. Main): "The radiochemical changes of nucleic acids and their biological effect." The paper gives a detailed treatment of the changes caused by X-rays, neutrons and ultraviolet rays as well as visible light. It states among others that the radiation-sensitivity of purine bases is lower than that of the pyrimidine bases. This, again, offers an opportunity for sequence analysis; furthermore it can give a chemical explanation for certain biological radiation-sensitivities which depend on guanine-cytosine content. The authors succeeded in demonstrating the molecular-biological effect of neutrons on genetic material and on protein synthesis.

S. N. Alexandrov (Leningrad): "On the genotypic control of the radiation-sensitivity of the organism." The genotype determines also the radiation-sensitivity. The concrete form and way of this determination are looked for. On the basis of experiments performed on mice and Drosophila it is supposed that the different radiation-sensitivity is connected with the different regenerating ability of the cells.

A. Kalaidjiew, L. Segal, J. Segal (Berlin): "Denaturation of proteins by acids and bases." The assumption that proteins can undergo a reversible denaturation in physiological pH region is supported by the results of nephelometric, redox titrimetric, electrophoretic and spectroscopic measurements. The phenomenon can be traced back to the lability of hydrogen bridges. This fact helps us in a more clear understanding of the close connection between the electrical component of excitation and the activity of enzymes, the mechanical work of contractile molecules and the active transport processes carried out by the transport proteins.

E. Ernst (Pécs): In his lecture entitled "Muscle-machine" the author deals with the quantitative interpretation on the basis of the sine function, of the simple and tetanic contractions of the striated muscle. Making use of the connections of shortening and tension he deals with the calculation of the theoretical maximum work (the mechanical efficiency of muscle-machine is, in right interpretation, nearly of one unit), and with the question of the "crystallization" based on the volume decrease, heat production, increase in birefringence and decrease of solubility caused by stretching. He pre-

sents his hypothesis according to which the energy of the stretchwork appears, through a mechanochemical connection, as a chemical energy in the hypertrophying muscle.

H. Pauly (Erlangen): "On the passive electric features of cytoplasmic membrane and cytoplasm." The author summarizes the electric features of membranes behaving as capacities (erythrocytes, tumour-cells, mitochondrium, natural and artificial myelin sheaths) and the data available concerning the electric conductivity of cytoplasm on the basis of several own and literary data.

W. Sandritter, G. Kiefer (Giessen): "Optical micro-methods in biology." The authors present those methods of quantitative histochemistry (microinterferometry, microphotometry) which can be applied without damaging the biological structure and give examples of their possible applications.

W. Grassme (Jena): "The position of the laser-technology and some applications of the laser." After presenting the kinds and features of lasers the author shortly deals also with the biological possibilities of their utilization. The small focal spot ($< 5\,\mu$) and the high power-density give opportunities for its application e.g. in microspectral-analysis, in the research of mutation, in ophthalmology, and perhaps, for therapeutic purposes too.

I. M. Gelfand et al. (Moscow): "Some questions of the investigation of movements." The work, fitting into the topic of the physiology of motions and containing chiefly theoretical generalizations, deals with the problems of regulation in the nervous system and the muscle groups coordinated by it, as well as with their modelling.

P. O. Makarov (Leningrad): "Adequatometry in neurodynamics." The author writes about the quantitative experimental examination of the irritation processes of human mechanoreceptors (cornea reflex) and chemoreceptors (smelling) for determining the connections between parameters like e.g. the intensity, duration and gradient of the stimulus.

H. Drischel (Leipzig): "Biocybernetics." It is a short theoretical summary of the history and problems of the branch of science indicated in the title.

W. Beier (Leipzig): "Metrical and relational aspects in theoretical biophysics." It is a work also touching upon epistemological problems; it emphasizes the dominating role of correlations, relational aspects in biology; it attempts to interpret the organism mathematically as a topological complex with the aid of the graph theory.

R. Laue (Leipzig): "On the metrics of biotopological models." The consistently relational outlook of the biological system, together with its advantages, has the drawback of missing the quantitative perceptibility. The author attempts to describe with linear differential equations the changes in time of the functions, as extensive quantities, of an open biological system characterized by a given graph. This method perhaps with an appropriate fusion of irreversible thermodynamics and information theory, seems to have perspectives.

F. VETŐ

Физика для врачей и диологов (Physics for Physicians and Biologists). In Russian. Editor I. Tarján. Publishing House of the Hungarian Academy of Sciences. Budapest, 1969. 600 pages.

This publication is the Russian translation of the second edition of a book of the same title, published in Hungarian in 1968. It consists of 10 chapters: mechanics, oscillations and waves, acoustics, thermodynamics, electrical engineering, optics, X-ray radiation, atomic physics and nuclear physics. Though the book has been written for satisfying the needs of biologists and physicians, it is a logically built, didactically excellently constructed and a complete one in itself. It is a useful manual for the biophysical training of medical students, but also beginners in biological research work as well as physicians can make a good use of it in obtaining and refreshing the physical knowledge needed in their work. The author supposes the readers to be familiar with the basic physical notions and connections. The chapters are completed with several clearly arranged and therefore

practically very well utilizable figures, tables, schemes and photos.

The 10th chapter contains the conversion tables of force-, energy-, and pressure units, the most important material constants of solid and liquid materials as well as gases, the most frequently used physical constants and a summarizing table of the characteristic data of the more important radioactive isotopes.

The appendix summarizes the elements of the most important methods of the mathematical apparatus indispensably necessary for the modern biological research and view (differential and integral calculus and calculus of probabilities).

The short list of references enumerates 42 review articles from the field of physics, physicalchemistry, chemistry, biophysics, biochemistry, physiology and mathematics.

An excellent index completes the book making its use very easy. The typography is also very good.

A. NIEDETZKY

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója — Műszaki Szerkesztő: Farkas Sándor
A kézirat nyomdába érkezett: 1969. IX. 9. — Terjedelem: 9 (A/5) ív, 41 ábra

69.68276 Akadémiai Nyomda, Budapest Felelős vezető: Bernát György

Hoppe-Seyler's Zeitschrift für

Physiologische Chemie

Editors in Chief

A. Butenandt . K. Thomas . G. Weitzel

Subscription Rates

For one volume (12 parts) DM 360,—

Vol. 350 No. 5 May 1969

CONTENTS

Walter de Gruyter & Co · Berlin 30

ERRATA

Volume 4

p. 77. In the 3rd line of Table 3 the correct number is 0.146 ± 0.030

p. 107. The correct text of the sentence beginning in the 4th line is as follows:

"In our experiments on muscles below 19 °C a positive sign of the warm end was found, a fact indicating that the TEMF was produced by negative carriers, while the change in the sign of the differential thermoelectric power at 23 °C (and the negativity of the warm end above this temperature in a few cases) indicated that either positive carriers were dominating over this temperature range or the mobility of positive carriers was greater than that of the negative carriers."

p. 366. In the 10th line read 0.9 instead of 1.1.

p. 430. The correct name of the camera mentioned on this page was "PC 1B NIHON KOHDEN KOGYO CO TOKYO".

Reviews of the Hungarian Academy of Sciences are obtainable at the following addresses:

ALBANIA

Ndermarja Shtetnore e Botimeve
Tirana

AUSTRALIA

A. Keesing
Box 4886, GPO
Sydney

AUSTRIA

Globus Buchvertrieb
Salzgries 16
Wien I

BELGIUM

Office International de Librairie
30, Avenue Marnix
Bruxelles 5
Du Monde Entier
5, Place St. Jean
Bruxelles

BULGARIA

Raznoiznos
1, Tzar Assen
Sofia

CANADA

Pannonia Books
2, Spadina Road
Toronto 4, Ont.

CHINA

Waiwen Shudian
Peking
P. O. B. 88

CZECHOSLOVAKIA

Artia
Ve Směčkách 30
Praha 2
Poštovná Novinová Služba
Dovoz tisku
Vinohradská 46
Praha 2
Mad'arská Kultura
Václavské nám. 2
Praha I
Poštová Novinová Služba
Dovoz tlače
Leningradská 14
Bratislava

DENMARK

Ejnar Munksgaard
Nörregade 6
Copenhagen

FINLAND

Akateeminen Kirjakauppa
Keskuskatu 2
Helsinki

FRANCE

Office International de Documentation et Librairie
48, rue Gay Lussac
Paris 5

GERMAN DEMOCRATIC REPUBLIC

Deutscher Buch-Export und Import
Leninstraße 16
Leipzig 701

Zeitungsvertriebsamt
Fruchtstrasse 3—4
1004 Berlin

GERMAN FEDERAL REPUBLIC

Kunst und Wissen
Erich Bieber
Postfach 46
7 Stuttgart S.

GREAT BRITAIN

Collet's Holdings Ltd.
Dennington Estate
London Rd.
Wellingborough. Northants.
Robert Maxwell and Co. Ltd.
Waynflete Bldg. The Plain
Oxford

HOLLAND

Swetz and Zeitlinger
Keizersgracht 471—487
Amsterdam C.
Martinus Nijhof
Lange Voorhout 9
The Hague

INDIA

Current Technical Literature
Co. Private Ltd.
India House OPP
GPO Post Box 1374
Bombay I

ITALY

Santo Vanasia
Via M. Macchi 71
Milano
Libreria Commissionaria Sansoni
Via La Marmora 45
Firenze

JAPAN

Nauka Ltd.
92, Ikebukur O-Higashi 1-chome
Toshima-ku
Tokyo
Maruzen and Co. Ltd.
P. O. Box 605
Tokyo-Central
Far Eastern Booksellers
Kanda P. O. Box 72
Tokyo

KOREA

Chulpanmul
Phenjan

NORWAY

Johan Grundt Tanum
Karl Johansgatan 43
Oslo

POLAND

Ruch
ul. Wronia 23
Warszawa

ROUMANIA

Cartimex
Str. Aristide Briand 14—18
Bucureşti

SOVIET UNION

Mezhdunarodnaya Kniga
Moscow G—200

SWEDEN

Almquist and Wiksell
Gamla Brogatan 26
Stockholm

USA

Stechert Hafner Inc.
31, East 10th Street
New York, N. Y. 10003
Walter J. Johnson
111, Fifth Avenue
New York, N. Y. 10003

VIETNAM

Xunhasaba
19, Tran Quoc Toan
Hanoi

YUGOSLAVIA

Forum
Vojvode Mišića broj 1
Novi Sad
Jugoslovenska Knjiga
Terazije 27
Beograd

39,— Ft

Contents

22. XII. 1969 Index: 26.030