VOLUME 1
NUMBER 1
1966

Acta biochimica et biophysica

ACADEMIAE SCIENTIARUM HUNGARICAE

AKADÉMIAI KIADÓ, BUDAPEST

Acta Biochimica et Biophysica

Academiae Scientiarum Hungaricae

Szerkeszti:

STRAUB F. BRUNÓ és ERNST JENŐ

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

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Acta Biochimica et Biophysica

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Volume 1

Akadémiai Kiadó, Budapest

Foreword

The rapid increase in volume of research in the biomedical field demands constant readjustment and places a heavy responsibility on editors. The Acta Physiologica Academiae Scientiarum Hungaricae has in its past fifteen years published the results of biomedical research work of Hungarian laboratories covering a wide range of fields including not only physiology, as indicated in its title, but also pathophysiology, pharmacology, biochemistry and biophysics as well. Throughout the world there is a tendency of scientific journals to narrow down their field. Accordingly the Hungarian Academy of Sciences has agreed to publish a new journal: *Acta Biochimica et Biophysica Academiae Scientiarum Hungaricae* which is scheduled to appear in one volume (four issues, about 400 to 500 pages) per annum.

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The Editors hope that the separation of the fields according to the above publishing policy will facilitate the retrieval of information and serve the interest of both authors and readers.

The Editors

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 5—12 (1966)

Studies on D-Glyceraldehyde-3-Phosphate Dehydrogenase

XXIII. The Effect of Ag Ions on the Binding of Zn

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(Received October 20, 1965)

Blocking of the SH groups of D-glyceraldehyde-3-phosphate dehydrogenase with Ag ions does not make the Zn bound to the protein exchangeable.

If GAPD treated with PCMB and reactivated with cysteine is reacted with Ag ions protein becomes to bind Zn firmly.

The changes in the steric structure of the active center may not be parallel with that of the whole molecule.

In a previous work (Keleti, 1964) we have demonstrated that Zn cannot be exchanged in native GAPD* isolated from mammalian muscle. After a modification of the steric structure by blocking some of the SH groups of the protein with PCMB the tightly bound Zn became exchangeable.

One of us (Boross, 1965) has shown that Ag forms a stable ternary complex with $GAPD(NAD)_3$ and that this complex can be crystallized. In this case Ag is bound to the SH groups of the protein.

The present paper deals with the exchangeability of Zn in GAPD following the treatment of the enzyme's SH groups with Ag ions.

Materials and Methods

Swine muscle GAPD recrystallized four times was used (Elődi and Szörényi, 1956). The molecular weight of the protein was taken as 140 000 (Elődi, 1958). The protein content was determined spectrophotometrically by the absorption at 280 mμ.

PCMB was a commercial preparation of 94 per cent purity as calculated from the molar extinction coefficient of the solution (Boyer, 1954). All other chemicals — cysteine, mercaptoethanol, $AgNO_3$, etc. — were commercial preparations of reagent grade.

* *Abbreviations*: GAP=D-glyceraldehyde-3-phosphate; GAPD=D-glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase, phosphorylating, EC. 1. 2. 1. 12); NAD=nicotinamide-adenine-dinucleotide; PCMB=p-(chloro)mercuribenzoate.

^{65}Zn was in form of $Zn(NO_3)_2$ solution, specific activity 502 mC/g Zn. ^{110}Ag isotope was applied in the form of $AgNO_3$ solution, specific activity 90 mC/g Ag.

Gel-filtrations were made on Sephadex G-50 medium (Pharmacia) columns (6 to 8 g). They were washed and equilibrated with the solvent used for the protein (distilled water adjusted to pH 8.5 with NH_4OH or Cl^- free 0.1 M glycine buffer, pH 8.5). The applied protein solutions were about 3 ml.

Enzymic activity was measured by Warburg's optical test as described earlier (Keleti and Batke, 1965), using GAP (Szewczuk et al., 1961), NAD (Boehringer preparation of 85 per cent purity) and phosphate as substrates.

The tryptic digestibility of the protein was measured by the method of Szabolcsi (Szabolcsi et al. 1959) in 0.1 M glycine buffer, pH 8.5 at 20°C, using $0.42 - 8.75 \times 10^{-5}$ $[TU]^{Hb}_{ml}$ trypsin concentration and 5 mg/ml GAPD as substrate. Aliquots were taken in every 10 minutes, deproteinized with TCA of 6.6 per cent final concentration, centrifuged and the rate of digestion was determined by the increase in optical density of the supernatant at 280 mμ in the first 10 minutes. Trypsin was a three times recrystallized Trypure Novo preparation, incubated for 24 hours in a 0.2 per cent HCl solution at 37°C in order to inactivate chymotryptic contaminations. Proteolytic activity was determined by Anson's test.

The formation of mercaptide linkages was followed by the spectrophotometric test (Boyer, 1954). The formation of Ag_3-GAPD-NAD_3 complex was measured spectrophotometrically (Boross, 1965). The formation of the complexes containing more than 3 equivalents of Ag ion per mole of GAPD was controlled using ^{110}Ag isotope as described earlier (Boross and Keleti, 1965).

GAPD containing ^{65}Zn isotope incorporated in vitro was prepared as described earlier (Keleti, 1964).

A Hilger UVISPEK spectrophotometer was used.

The radioactivity was measured with dried samples in infinite thin layer.

Results

Native GAPD and GAPD containing 3 to 15 equivalents of bound Ag ion was incubated for 16—18 hours at 4°C in a medium containing ^{65}Zn isotope. After incubation the protein was treated with 200 equivalents of cysteine and 30 equivalents of NAD, gel-filtered and the amount of ^{65}Zn bound was determined by measuring the radioactivity of the protein fractions. The results are summarized in Table 1.

The data in Table 1 A. show that following mercaptidation of the SH groups with Ag ions Zn in GAPD is not exchanged with ^{65}Zn in the medium. The treatment with Ag (from 0 to 12 equivalents) and the gel-filtration do not affect the binding of Zn since the samples can be enzymically reactivated after the removal of Ag ions.

Table 1

Effect of Ag on the exchangeability of Zn in GAPD

3 ml of 24—32 mg/ml GAPD or 10—18 mg/ml GAPD-$^{65}Zn_3$ solution in distilled water adjusted to pH 8.5 or in 0.1 M glycine buffer, pH 8.5 was treated with 10^{-2}M $AgNO_3$ and with 0.2 ml 1.071 mg/ml $^{65}Zn(NO_3)_2$ or $Zn(NO_3)_2$ specific activity 186 μC/ml solution and incubated for 14 hours at 4°C. After incubation the mixtures were treated with 200 eq. cysteine or mercaptoethanole and 30 eq. NAD and gel-filtered on a Sephadex G-50 medium column (6—8g). The protein content of the fractions was determined spectrophotometrically, the Zn-content by radioactivity

	mole-equivalents of Ag bound to GAPD					
	n = 0	3	6	9	12	15
A. Eq. of ^{65}Zn bound to GAPD-Ag_n after incubation in a medium containing ^{65}Zn.	0.3	0.2	0.3	0.1	0.25	0.1
B_1 Eq. of ^{65}Zn bound to Ag_n-GAPD-$^{65}Zn_3$ after incubation in a medium containing nonradioactive Zn	2.7	0.3	0.25	—	—	—
B_2 Eq. of ^{65}Zn bound to Ag_n-GAPD-$^{65}Zn_3$ after incubation in a medium containing ^{65}Zn	—	—	0.1	—	—	0.15
B_3 Eq. of ^{65}Zn bound to Ag_n-GAPD-$^{65}Zn_3$ after incubation in a medium containing no Zn	2.65	0.2	—	—	—	—

PCMB loosens the conformation of GAPD (Szabolcsi, 1958; Szabolcsi et al., 1959; 1960; Szabolcsi and Biszku, 1961).

In a previous work we have shown that Zn can be exchanged in GAPD following PCMB treatment of the enzyme (Keleti, 1964). Removal of PCMB results in a practically fully active GAPD with labelled Zn incorporated into the protein (GAPD-$^{65}Zn_3$). In the following series of experiments the effect of Ag ions on GAPD-$^{65}Zn_3$ was tested.

The data in Table 1 B_1, B_2, B_3 show that when GAPD was pretreated with PCMB, blocking of the SH groups with Ag ions rendered the protein unable to bind firmly Zn. In agreement with our previous findings (Keleti and Telegdi, 1959; Keleti et al., 1962), these GAPD samples depleted of Zn are enzymically inactive and very unstable.

The elution profiles of the gel-filtrations are shown in Fig. 1.

It can be seen that blocking of the SH groups with Ag ions promotes the dissociation of firmly bound Zn in GAPD pretreated with PCMB.

These results suggest that the conformational changes induced by the first PCMB treatment are not fully reversible in spite of the fact that enzymic activity is practically completely recovered (Keleti, 1964; 1965). Therefore the reversibility of the structural changes induced by PCMB treatment was examined by measuring the tryptic digestibility of GAPD (Fig. 2).

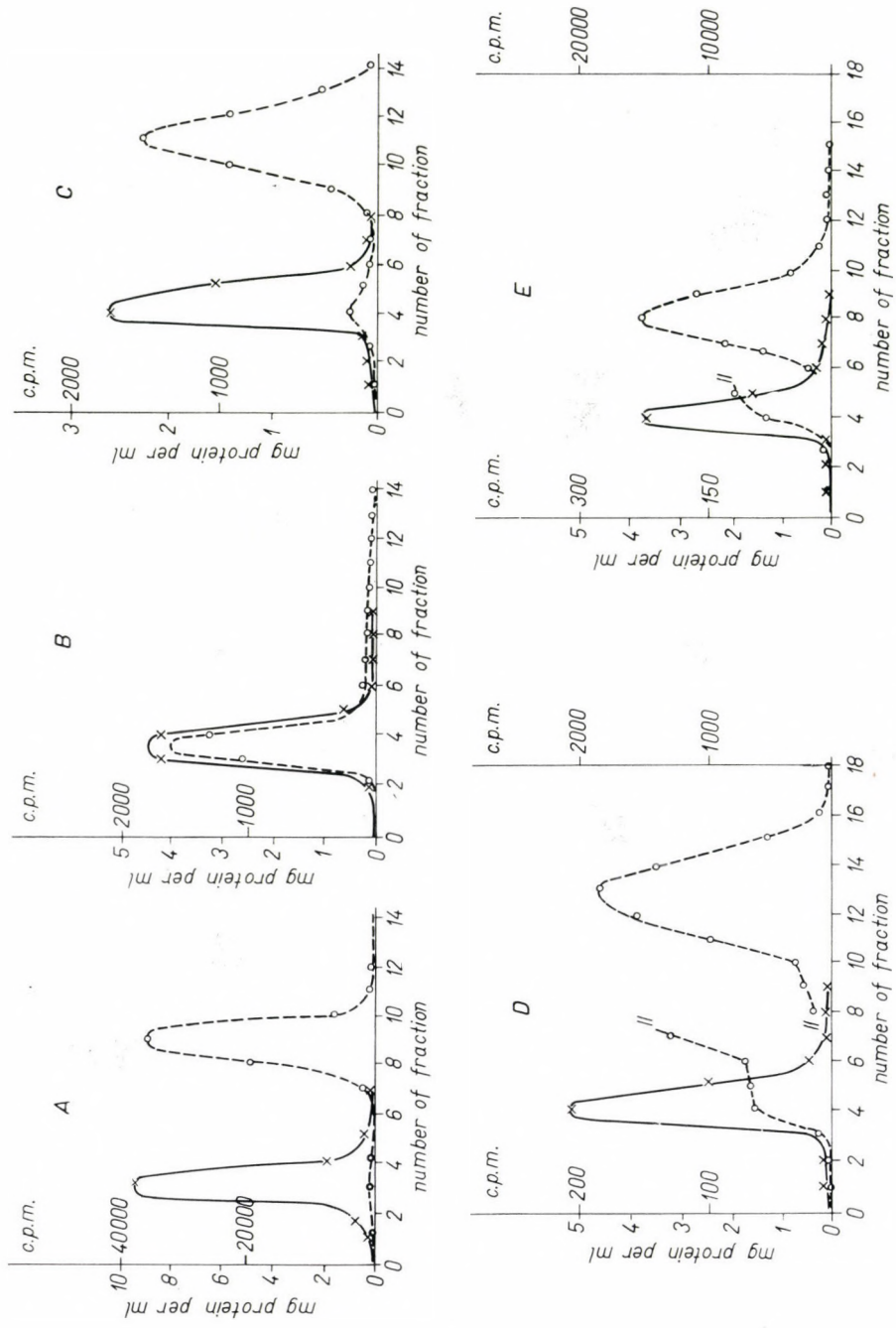

Fig. 1

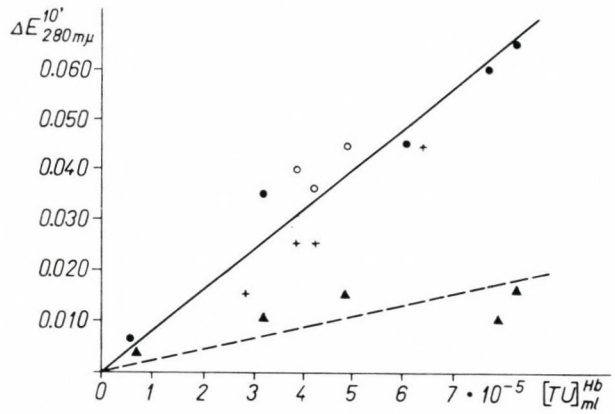

Fig. 2. The digestibility of PCMB-blocked and restored GAPD

Samples of gel-filtered GAPD, 5 mg/ml, were digested with 0.42—8.74 × 10^{-5} $[TU]^{Hb}_{ml}$ trypsin in 0.1 M glycine buffer, pH 8.5, 20°C for 1 to 3 hours. — The rates of digestion are plotted as a function of trypsin concentration. The digestibility of native GAPD under these conditions was practically zero. — The enzymic activity of the samples was determined before digestion GAPD-$(PCMB)_7$ shows an enzymic activity of 5 per cent of the original after 1 hour of incubation. After 16 hours of incubation 53 per cent is recovered according to a disproportionation mechanism which results in native GAPD and fully blocked GAPD (Szabolcsi et al., 1960). The enzymic activity of these samples may be restored with 200 eq. of cysteine or mercaptoethanole + 30 eq. of NAD up to about 90 per cent. — ● : GAPD incubated for 1 hour with 7 eq. PCMB, — ○ : GAPD incubated for 16 hours with 7 eq. PCMB, ▲ : GAPD incubated for 1 hour with 7 eq. PCMB and reactivated with cysteine + NAD, + : GAPD incubated for 16 hours with 7 eq. PCMB and reactivated with cysteine and NAD.

Fig. 1. Gel-filtration experiments with GAPD after treatment with Ag ion
Solid line, protein content as measured by the absorbancy at 280 mμ. Dotted line, radioactivity in counts per min. — The samples were incubated at 4°C, for 12—16 hours in 0.1 M glycine buffer, pH 8.5, then treated with 200 equivalents of cysteine or mercaptoethanole and 30 eq. of NAD and gel-filtered. — A: GAPD + 6 eq. Ag ions, incubated in a medium containing ^{65}Zn. To 60 mg protein 2.6 μmoles $AgNO_3$ and 1 min. later 0.236 mg, 6.32 μC $^{65}Zn(NO_3)_2$ were added. — B: GAPD-$^{65}Zn_3$, 3 ml of 26.1 mg/ml protein solution was gel-filtered. — C: GAPD-$^{65}Zn_3$ + 3 eq. Ag ions. To 23.77 mg protein 0.5 μmoles $AgNO_3$ were added. D: GAPD-$^{65}Zn_3$ + 9 eq. Ag ions, incubated in a medium containing non radioactive Zn. To 60 mg protein 3.9 μmoles $AgNO_3$ and 1 min. later 0.236 mg $Zn(NO_3)_2$ were added. The radioactivity in the low molecular weight fractions is represented on the right ordinate. — E: GAPD-$^{65}Zn_3$ + 7 eq. Ag ions, incubated in a medium containing ^{65}Zn. To 17.2 mg protein 0.9 μmoles $AgNO_3$ and 1 min. later 0.059 mg, 1.58 μC ^{65}Zn $(NO_3)_2$ were added. The radioactivity in the low molecular weight fractions is represented on the right ordinate

Samples of GAPD treated with 7 mole equivalents of PCMB were incubated for 1 to 16 hours and then submitted to the action of trypsin. Before digestion GAPD activity was controlled as well as reactivation with mercaptans.

When the incubation of the protein with PCMB did not exceed one hour, the structural changes and the enzymic inhibition induced were reversible.

When the incubation was longer than one hour the structural changes seemed to be partially irreversible, the enzymic activity was, however, practically restored.

Long incubation of the protein with Ag ions also induced partially irreversible structural changes in the protein.

Discussion

It is known that the Zn of GAPD isolated from mammalian muscle is not exchangeable if the native protein is incubated in a medium containing ^{65}Zn. If, however, some of the SH groups of the protein are blocked with PCMB, Zn becomes exchangeable. After treatment of the blocked enzyme with cysteine, i.e. removal of PCMB, an enzymically fully active GAPD, containing Zn isotope may be obtained (Keleti, 1964).

In contrast to the effect of PCMB, if the SH groups of the enzyme are partially or fully blocked with Ag ions no binding of ^{65}Zn is detectable suggesting that bound Zn does not exchange under these conditions. The gel-filtration profiles support this assumption since the column used separated completely the Zn isotope from the protein. Ag treatment does not cause depletion of the original Zn content, because the enzyme samples recovered after gel-filtrations are practically fully reactivable. These experiments rule out also the assumption that Ag may replace Zn in the protein molecule, as we have shown previously that GAPD depleted of its Zn complement is irreversibly denatured (Keleti et al., 1962).

It is interesting to note that Ag ions exhibit different effects on the structure of GAPD. According to Boross (1965) blocking of 3 SH groups with Ag ions gives rise to a crystallizable Ag_3-GAPD-NAD_3 complex. The enzymic activity of this complex may be completely restored with cysteine, the complex contains the coenzyme firmly bound to the enzyme and tryptic digestibility of the complex does not differ from that of the native enzyme (Boross, 1965). During the incubation period used no disproportionation of Ag on the enzyme takes place (Boross and Keleti, 1965). Therefore it may be supposed that Zn is not exchanged because the steric structure of GAPD is not altered by 3 equivalents of Ag ion.

On the other hand blocking of further SH groups by Ag renders the protein unstable and its tryptic digestibility is increased (Boross, 1965). However, this alteration in the steric structure of GAPD does not render the firmly bound Zn exchangeable, suggesting that Ag ions alter the conformation of GAPD at an other site or in a different way than PCMB.

We have obtained different results when GAPD-$^{65}Zn_3$ was tested. In this case after the blocking of the SH groups with Ag the gel-filtration profiles showed

a continuous dissociation of Zn isotope from the enzyme. It may be assumed that if Ag is added to GAPD-$^{65}Zn_3$, the steric structure of which has been altered by the previous PCMB treatment, new conformational changes are being induced or those induced by PCMB are stabilized and therefore Zn becomes dissociable.

It seems that a second PCMB treatment has a different effect. If PCMB is added to GAPD-$^{65}Zn_3$, Zn remains exchangeable and firmly bound after the cysteine treatment. After the second removal of PCMB the enzymic activity of the samples is about 65 per cent of the original.

The data of Fig. 2 show that after prolonged incubation the alterations in the steric structure of GAPD induced by PCMB seem to be partially irreversible. The changes of the steric structure of the active center, however, may not be parallel with that of the whole molecule. After treating the PCMB-blocked protein with cysteine or mercaptoethanole the enzymic activity is completely restored while the digestibility of the protein remains much greater than that of the native enzyme.

Acknowledgements

We are deeply indebted to Mrs. M. Szegvári for the valuable technical assistance.

References

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
Elődi, P. (1958) Acta Physiol. Acad. Sci. Hung. *13* 199
Elődi, P., Szörényi, E. T. (1956) Acta Physiol. Acad. Sci. Hung. *9* 339
Keleti, T. (1964) Biochim. Biophys. Acta *89* 422
Keleti, T. (1965) Acta Biol. Med. Germanica Suppl. III. p. 245
Keleti, T., Batke, J. (1965) Acta Physiol. Acad. Sci. Hung. *28* 195
Keleti, T., Györgyi, S., Telegdi, M., Zaluska, H. (1962) Acta Physiol. Acad. Sci. Hung. *22* 11
Keleti, T., Telegdi, M. (1959) Acta Physiol. Acad. Sci. Hung. *15* 281
Szabolcsi, G. (1958) Acta Physiol. Acad. Sci. Hung. *13* 213
Szabolcsi, G., Biszku, E. (1961) Biochim. Biophys. Acta *48* 335
Szabolcsi, G., Biszku, E., Sajgó, M. (1960) Acta Physiol. Acad. Sci. Hung. *17* 183
Szabolcsi, G., Biszku, E., Szörényi, E. T. (1959) Biochim. Biophys. Acta *35* 237
Szewczuk, A., Wolny, E., Wolny, M., Baranowski, T. (1961) Acta Biochim. Polon. *8* 201

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 13—28 (1966)

Studies on Proteins and Protein Complexes of Muscle by means of Proteolysis

I. Influence of Ionic Milieu on the Proteolysis of Myosin

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The time course of tryptic digestion of myosin depends remarkably on the ionic environment. The digestion rate as studied by the pH-stat method is highest in 0.02 M KCl. If the concentration of KCl is raised up to 1 M proteolysis is strongly suppressed. We obtain a similar inhibition by adding $CaCl_2$ or $MgCl_2$ to the myosin suspension in 0.02 M KCl; — above 0.01 M concentration of alkaline earth metals the inhibition is not raised any further.

Light meromyosin behaves under similar conditions much in the same way as myosin does, but the extent of inhibition is even more expressed.

The digestion of heavy meromyosin is suppressed by KCl to a lesser degree than in the case of myosin but it is not suppressed by $CaCl_2$.

Heavy meromyosin, if it is free of trypsin inhibitor impurities, is not less digestible by trypsin than myosin or light meromyosin is.

Under circumstances representing three extremes of the ionic milieu studied, the pH-stat records were analysed according to Mihályi and Harrington (1959). On the basis of this analysis it can be tentatively suggested that the changes observed are due to a conformational change of the protein molecules brought about by cation binding. It is suggested that the inhibition by KCl is caused in first line by a change of this kind in the heavy meromyosin part of the myosin molecule, while the influence of Ca is due to the (perhaps complete) depression of the splitting of the "random region" in the protein.

As a result of several reports we have a rather detailed picture of the architecture of the myosin molecule (Woods et al., 1963). In the development of the present concept on the structure proposed, the data obtained from studies on the proteolysis of myosin have an outstanding share. In spite of the wide scope of these investigations the proteolytic fragmentation of myosin has been observed so far only under relatively restricted conditions i.e. with myosin dissolved in saline of high ionic strength.

We have observed occasionally that myosin at low ionic strength i.e. in form of gel suspension was digested considerably faster than when dissolved. Following up this line we have found that the concentration and kind of cations present have a great influence on the course of tryptic proteolysis of myosin. Since the results of kinetic studies on the process (Mihályi and Harrington, 1959) greatly contrib-

Abbreviations: DFP=diisopropylfluorophosphate, HMM=heavy meromyosin, LMM=light meromyosin, TCA=trichloroacetic acid.

uted to the development of Harrington's myosin model (Woods et al., 1963) we thought that these phenomena deserve a thorough study. In this paper our results on the influence of cations on the kinetics of digestion will be presented.

Materials and Methods

Myosin was prepared according to Portzehl et al. (1950). For the experiments in the pH-stat it was precipitated by dialysis against 0.02 M KCl and was kept at 0°C as a suspension until used.

Meromyosins were prepared according to A.G. Szent-Györgyi (1953), with the modification that digestion was stopped by the addition of a concentrated, neutralized solution of DFP in isopropanol to a final concentration of 0.01 M. Such a high concentration of DFP will stop the action of trypsin in two-three min., a delay negligible in preparative work. (This procedure was suggested in a personal communication by Dr. Gruda, Warsaw.)

In heavy meromyosin prepared in this way some traces of DFP were detected even after dialyzing it overnight to remove ammonium sulphate. The presence of DFP showed itself by a slow continuous drift in the pH-stat caused by liberation of hydrofluoric acid. When heavy meromyosin was dialyzed against a borate buffer of pH 8.2 the drift was no longer observed. We adopted this dialysis followed by dialysis against 0.02 M KCl as a routine to prepare heavy meromyosine for the experiments in the pH-stat.

The trypsin and chymotrypsin used were lyophilized preparations of the firm *Kőbányai Gyógyszergyár*. The former had an $E^{cm}_{mg/ml} = 1.60$. Taking the factor given for trypsin by Laskowski (1955), this would mean a 90 per cent purity. In Kunitz's test however, calculating trypsin concentration by using the factor given by Laskowski (1955), we obtained activity which was only 72 per cent of that of Kunitz's standard trypsin. In this paper trypsin concentrations are always designating weight concentrations of the batch of enzyme characterized here.

In order to avoid the effect of traces of chymotrypsin impurities eventually present, stock solutions of trypsin in 0.003 N HCl were incubated at 37°C for 16 hours. They were kept further in a refrigerator at 4°C and were freshly prepared every 5 days.

pH-stat measurements were carried out at 22°C and at a pH 8.2 (except for experiment of Fig. 4), with a Radiometer TTT 1, + SBU/SBR 2 assembly. Usually 60 mg of protein were digested in 10 ml volume with a final trypsin concentration of 50 μg/ml. Other additions are specified in the legends of the different experiments*. For titration 0.03 N NaOH was used. Before the addition of trypsin the

* In the experiments of Figs 1—3 digestion was carried out in the presence of 0.02 M borate buffer. The presence of buffer results in a depression of the titration values at the beginning of the experiment, the extent of which depends on the buffering capacity of the system and this error is eliminated asymptotically, theoretically at infinite time (Jacobsen et al. 1957). It should be noted that this effect was in our actual system very small and of no consequence in those experiments which were evaluated only qualitatively.

reaction mixture was adjusted to the desired pH the constancy of which was observed for 20—40 min by rinsing continuously the gase phase with N_2.

The reaction was started by the addition of 1 ml of 0.05 per cent trypsin, dissolved in 0.0004 n HCl. Proportional band setting of the apparatus was kept at 0.1 throughout the recording.

Viscosimetry was carried out in an Ostwald type viscometer, with an outflow time of about 1 min at 0°C.

For the mathematical evaluation the values, representing one minute intervals of the pH-stat records, were plotted on a graph paper and the points were connected with the best smooth curve. The curve obtained was digitalized in two minute intervals and this numerical function was differentiated by a conventional Taylor-series formula (Lánczos, 1957), using an Ural I computer. The derivative of the function was plotted on a semilogarithmic graph paper and was treated as described by Mihályi and Harrington (1959). It should be mentioned that the curve representing the fast reaction, obtained after subtraction of the slow process from the overall reaction could in many cases hardly be regarded as an exponential function, especially in cases where the fast process reached relatively rapid completion. This makes the value of k_1 rather arbitrary. The plateau representing A_1 was nevertheless always sufficiently apparent.

For the conversion of base consumed to peptide bonds split the pK values given by Mihályi and Harrington (1959) were used.

Results

The tryptic digestion of myosin as observed by the pH-stat procedure, is substantially faster in 0.02 M KCl when myosin is a suspension than in 0.5 M KCl under identical conditions. If to the myosin suspension, besides the low concentration of KCl, $CaCl_2$ or $MgCl_2$ is added, the proteolysis is substantially depressed alike to high KCl concentrations. These facts are displayed on the pH-stat records of Figs 1 and 2, which show the effect of the concentration of KCl and $CaCl_2$ on the time course of proteolysis. Maximum effect with Ca ions is obtained at about 0.01 M, while with KCl the inhibition goes on up to 1 M. The effect of $CaCl_2$ is not specific, as $MgCl_2$ has a very similar effect (Fig. 3 curves 5 and 6). The effect of alkaline and alkaline earth ions is not additive: if to the myosin dissolved in 0.5 M KCl 0.01 M of the Ca or Mg salts is added the shape of the curves is but insignificantly altered. (Compare curve 2 with curves 3 and 4 in Fig. 3.)

Study of proteolysis in a heterogeneous system may seem to be a rather questionable kind of experiment. Therefore before describing our detailed results it seems necessary to state that according to our experiments the pH-stat curves obtained with myosin suspensions are quite well reproducible. They do not depend on the "life-history" of the stock myosin suspension used, whether precipitated by dialysis or by dilution, kept in precipitated state for some days at 0°C, etc. Some data supporting this point are given in "IEG 4 memorandum No 43" (Biró, Bálint, 1965).

The observed differences in the rate of proteolysis are not directly connected with the dissolved or precipitated state of myosin. If the aggregated state of myosin would seriously interfere with the access of the enzyme, inhibition not activation

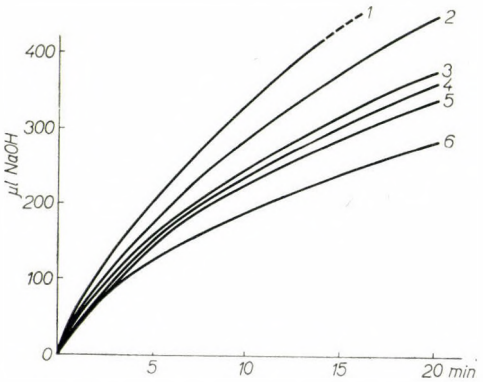

Fig. 1. Digestion of myosin by trypsin in 0.02 M borax-borate buffer at different KCl concentrations. pH-stat records. For experimental details see methods. Additions: 1 — 0.02 M KCl; 2 — 0.05 M KCl; 3—0.08 M KCl; 4 — 0.17 M KCl; 5 — 0.32M KCl; 6 — 0.52 M KCl

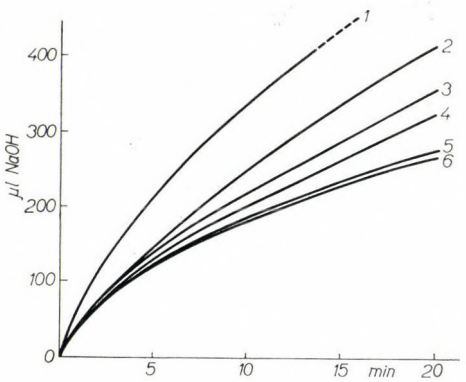

Fig. 2. Digestion of myosin by trypsin in 0.02 M borax-borate buffer + 0.02 M KCl in the presence of different concentrations of Ca. — For experimental details see methods. Additions: 1 — none; 2 — 0.002; 3 — 0.003; 4 — 0.004; 5 — 0.007; 6 — 0.010 M $CaCl_2$

should be observed in precipitated state. That the state of aggregation is by no means the principal factor is shown by the experiment in Fig. 4: at pH 9.2 where myosin is dissolved even in 0.02 M KCl the depressing effect of high KCl concentration is observed nontheless. A further argument in this respect is offered by Fig. 1, too: in spite of the fact that the limit of dissolution of myosin is certainly

below 0.25 M the inhibition caused by high concentrations of KCl goes on up to 1.00 M, i.e. through a concentration range where myosin is dissolved.

We have shown in separate experiments that the proton liberation observed in the pH-stat is in fact the result of proteolysis and not of a change in the state of

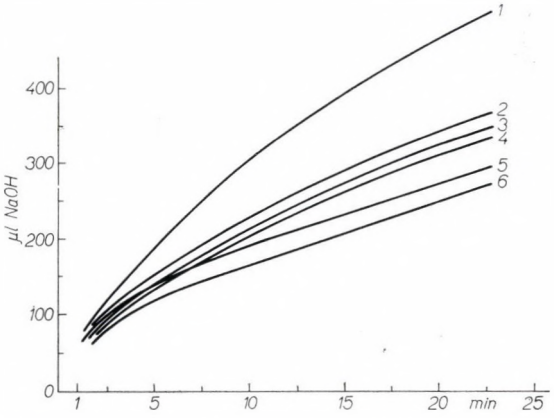

Fig. 3. Digestion of myosin by trypsin in 0.02 M borax-borate buffer + 0.02 M KCl and the additions as follows: 1 — none; 2 — 0.5 M KCl; 3 — 0.5 M KCl + 0.01 M $MgCl_2$; 4 — 0.5 M KCl + 0.01 M $CaCl_2$; 5 — 0.01 M $MgCl_2$; 6 — 0.01 M $CaCl_2$. For other experimental details see: Methods

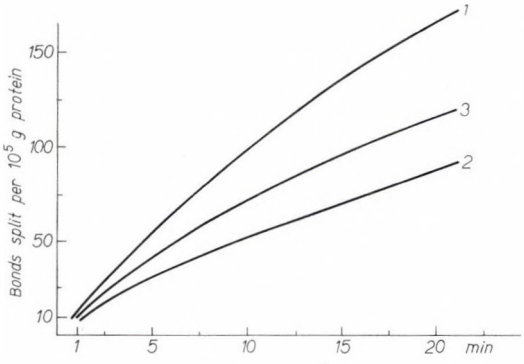

Fig. 4. Digestion of myosin at pH 9.2. 1: 0.02 M KCl; — 2: 0.02 M KCl + 0.01 M $CaCl_2$; — 3: 0.52 M KCl. Other details as in Fig. 1

ionization of the protein. In Table I proteolysis under various experimental conditions is compared with pH-stat measurements and with the u.v. absorption of the nonprotein fractions formed, respectively. It may be seen that the relative extent of proteolysis obtained with the two methods is in a rather good agreement. The differences observed in the pH-stat reflect in fact the differences in the actual rate of proteolysis.

Table I

Comparison of the extent of proteolysis of myosin estimated by base consumption and by the ultraviolet absorption of nonprotein fraction liberated

Digestion of myosin as described in "Methods", in 0.02 M KCl, 0.02 M borax-borate buffer plus the additions as given in the table. — After 20 mins of proteolysis an aliquot was transfered to an equal vol. of 10% TCA, and the extinction of the filtrate was measured at 280 μm

NaOH consumed and u. v. absorbing material liberated are expressed in the percentage of values found when digested in borate-KCl, with no other addition

Additions to the borate-KCl medium	Per cent of uv abs. substances	Per cent of base consumed
None	100.0	100.0
0.002 M $CaCl_2$	78.5	76.0
0.003 M $CaCl_2$	67.5	72.5
0.004 M $CaCl_2$	62.0	60.1
0.007 M $CaCl_2$	52.0	53.0
0.010 M $CaCl_2$	51.5	51.5
0.03 M KCl	85.5	82.9
0.06 M KCl	72.0	71.5
0.15 M KCl	69.5	60.0
0.30 M KCl	64.5	47.2
0.50 M KCl	55.0	52.5

As Mihályi and Harrington deduced important conclusions regarding the secondary structures of myosin from the kinetics of digestion, our first assumption on the phenomena described above was that the secondary structure of myosin is changed by the changed ionic environment and that the higher rate of proteolysis observed in 0.02 M KCl is due to a certain degree of "unfolding" of the structure. Therefore we analyzed the pH-stat records in a similar way as Mihályi and Harrington (1959) had done. To this end we registered the process up to 90 min. in three different salines representing extremes of the salt concentrations concerned. Besides myosin the proteolysis of LMM and of HMM was observed under conditions identical with those used for myosin.

The pH-stat records of these experiments are shown in Figs 5, 6 and 7. Before the description of the results obtained by the mathematical analysis of these records we have to point out some qualitative features of the digestion process under different conditions.

It may be seen that the tryptic digestion of LMM as compared to that in 0.02 M KCl is inhibited by elevated concentrations of KCl or $CaCl_2$ much like in the case of myosin but the inhibition is even more expressed. In contrast, $CaCl_2$ does not influence substantially the digestion of HMM. KCl is inhibitory here, too, but to a lesser degree than in the case of myosin or LMM digestion.

According to Fig. 7 HMM is not less digestible than the two other proteins concerned. This result is in sharp contradiction to all the results reported hereto-

fore on the digestion of HMM. It seemed to us advisable therefore to corroborate these findings by other methods. Proteolysis of HMM was followed like in the work of Mueller and Perry (1961) by viscosimetry. As it can be seen in Fig. 8 with a trypsin to HMM ratio about 30 times lower than that used by Mueller and Perry (1961, 1962) we obtained time curves much similar to theirs.

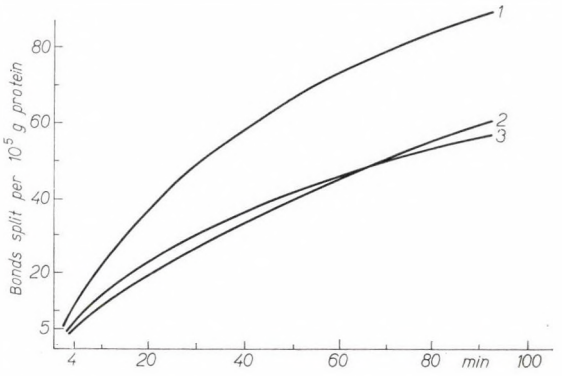

Fig. 5. Digestion of myosin in different ionic media. pH-stat records

1 — 0.02 M KCl; 2 — 0.02 M KCl + 0.01 M $CaCl_2$; 3 — 0.52 M KCl.
For other experimental details see: Methods

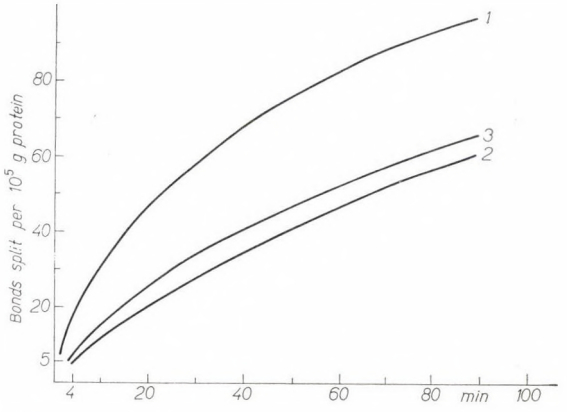

Fig. 6. Digestion of L-meromyosin. All details as in Fig. 4

As far as the quantitative aspects of the problem are concerned the analysis of our curves obtained in the pH-stat was carried out essentially according to Mihályi and Harrington (1959). As it is known this analysis is based on the assumption that the time course of proteolysis can be described as the sum of two first order processes, one being substantially faster than the other, the difference in the

rate constant being of one order of magnitude. The two processes reflect as it is assumed, the splitting of peptide bonds in relatively unorganized "random" regions and in regions with more rigid, ordered secondary structure, respectively. The former is, like "uncoiled", denatured proteins in general more easily attacked

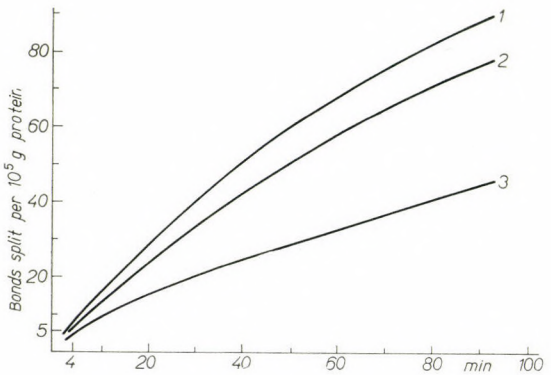

Fig. 7. Digestion of H-meromyosin. All details as in Fig. 5

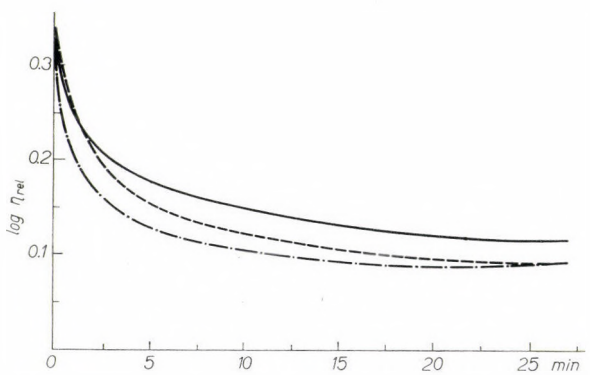

Fig. 8. Digestion of H-meromyosin followed by viscosimetry. HMM 6.9 mg/ml, trypsin 23 μg/ml, 0.02 M borate-NaOH buffer, pH 8.2.
·— —· 0.02 M KCl, — — — — — 0.02 M KCl + 0.01 M $CaCl_2$, ——— 0.52 M KCl

by proteolytic enzymes. The number of peptide bonds susceptible to the fast reaction (A_1) is relatively small but the splitting of these (or part of these) bonds results in the dissociation of the myosin molecule into the meromyosins. The peptide bonds attacked in the slow process are located in regions of the parent molecule representing the meromyosins. In spite of the splitting of a low proportion (as related to the total accessible amount) of these bonds secondary forces maintain the overall structure of the meromyosins.

The conclusions on the ion effects can be summarized on the basis of the data in Table II as follows: 1) In the case of myosin the inhibition observed in 0.52 M KCl (as compared to proteolysis in 0.02 M) is in the first line the result of a decrease of both the rate constant (k_2) of, and the number of bonds accessible (A_2) in the slow reaction. 2) The inhibition caused by Ca reflects itself in depression of the rate constant of the slow process as well as in a decrease to an insignificant value of the number of bonds accessible in the fast process. 3) In the case of LMM we obtain a picture much resembling the one obtained for myosin with the exception that the number of bonds split in the slow process (A_2) is practically not depressed by 0.5 M KCl. 4) In the case of HMM, the A_1 values are very low. (A_1 may be significant perhaps only in the case of 0.02 M KCl.) It is interesting to note that in 0.52 M KCl a lowering of the number of bonds affected in the slow process is observed in this case too, but to a lesser degree than with myosin.

The results presented in Table II could be interpreted in general by an assumed change in the conformation of the myosin molecule. As a result some bonds become less accessible which is reflected in the changes of the "A" values. The fact, however, that big changes of the rate constants are observed as well, makes the conclusion somewhat uncertain. A further point which makes the conclusions uncertain is the fact that in the case of HMM the deduced total number of bonds accessible is in all three cases considerably higher than 100 per cent. This finding raises some doubt as to the validity of the extrapolations involved in the mathematical procedure. Considering these difficulties it seemed to us advisable to design cruder but more direct experiments to support our assumption.

In one type of experiments carried out to this end myosin was digested for six hours and with the double amount of trypsin used in the pH-stat experiments. It is to be noted that Mihályi and Harrington (1959) reported on similar controls in their paper. The amount of protein precipitable by TCA after six hours of digestion (Table III) was the highest in 0.52 M KCl, lowest in 0.02 M and in between the two values when Ca was present. This is in good agreement with the predictions of the results in Table II for the three cases (see the $A_1 + A_2$ values in Table II). There is in fact such a good agreement that one is tempted to add the percentages of the protein remaining undigested in the experiment of Table III to the percentage value of bonds digestible as given in Table II. In this way we arrive to 104, 95 and 107 per cent for 0.02 M, 0.52 M KCl and for $CaCl_2$ respectively. It is somewhat doubtful whether these values can be taken as significant, but a mere qualitative agreement may be encouraging as far as the conclusions drawn from digestion kinetics are concerned.

As an other line of approach the digestion of denatured proteins was compared to the digestibility of native ones. Denaturation was effected by following exactly the procedure for "depolymerization" of LMM by urea treatment as described by Szent-Györgyi and Borbiró (1956). These authors have found that under their conditions of urea treatment LMM dissociates into polypeptides with an approximate molecular weight of 4000—5000.

Table II

Summary of the kinetic analysis of titration data

The pH-stat records represented in Figs 5, 6 and 7. were analysed as described in the text (see also "methods"). Number of bonds accessible for the fast and slow process respectively (A_1 resp. A_2) expressed as bonds per molecule using the molecular weights as indicated in the table (Young et al. 1964). For theoretical value of peptide bonds attackable, the sum of arginine and lysine per molecule was calculated on the basis of the analyses of Lowey and Cohen (1962)

Rate constants expressed in units of $100 \times \text{min}^{-1}$, calculated with natural logarithms

	A_1	A_2	A_1+A_2	$\frac{A_1 \times 100}{A_1+A_2}$	A_1+A_2 perc. theoret.	k_1	k_2
Myosin, MW = 600,000, arg + lys per mole = 810							
Mihályi and Harrington (1959)*	87	378	465	19	57.5	16.0	1.20
Mihályi and Harrington's experiment reproduced*	115	378	493	23	60.8	12.4	1.15
0.02 M KCl	79	625	704	11	87.0	11.5	1.5
0.52 M KCl	74	442	516	14	64.0	8.5	1.0
0.02 M KCl + 0.01 $CaCl_2$	24	661	685	3.5	84.5	25.8	0.8
LMM, MW = 160,000, arg + lys per mole = 234							
0.02 M KCl	29	156	185	16	79.0	16.6	1.9
0.52 M KCl	21	162	183	12	78.0	7.5	0.8
0.02 M KCl + 0.01 $CaCl_2$	6.4	155	161	4	69	10.6	0.9
HMM, MW = 360,000, arg + lys per mole = 431							
0.02 M KCl	27	522	549	5	128	10.1	0.9
0.52 M KCl	14	440	454	3	105	18.4	0.4
0.02 M KCl + 0.01 M $CaCl_2$	14	525	539	3	125	8.6	0.8

* In these experiments digestion was carried out in 0.5 M KCl, pH 8.3 and at 25°C with 48 mg myosin in 6 ml trypsin (Worthington) concentration 0.0755 mg/ml.

Table III

The amount of undigested protein after 6 hours of digestion of myosin under different conditions

60 mg myosin in 10 ml was digested with 100 μg/ml trypsin, in the presence of 0.04 M borax NaOH buffer of pH 8.2 and the additions shown in the table. — Digestion was carried out with continuous shaking at room temperature (22°C). — Digestion was stopped by the addition of 13% TCA (final concentration). The precipitated proteins were centrifuged and estimated

Conditions of digestion	Undigested protein	
	mg	per cent of total
0.02 M KCl	7.4	12.4
0.52 M KCl	18.6	31.0
0.02 M KCl + 0.01 M $CaCl_2$	13.7	22.9

There is some uncertainty as to the desorganizing effect of this urea treatment on HMM and on myosin (Szent-Györgyi and Borbíró, 1956, cf. also Hotta and Kojima, 1964). Anyhow, our urea treated preparations lost any trace of enzyme activity.

According to the experiment shown in Table IV in the cases where proteolysis in the native state is relatively depressed, digestibility is more than doubled by denaturation. The relatively high digestibility of the native proteins in 0.02 M KCl on the other hand is but slightly increased by denaturation or is even depressed (in the case of LMM). Only the behaviour of HMM in the presence of Ca does

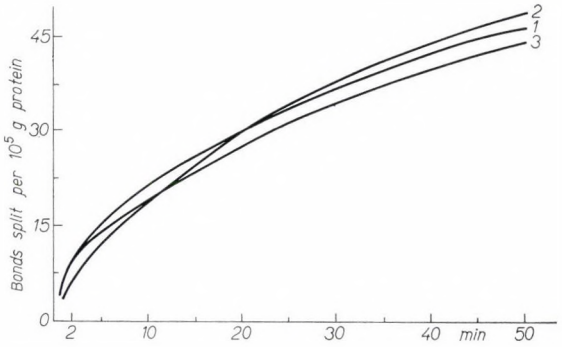

Fig. 9. Digestion of myosin by chymotrypsin. pH-stat records. All details as in the experiment in Fig. 1 except that 400 μg/ml chymotrypsin was used

not fit in this picture: in spite of there being no depression of proteolysis of the native protein as related to digestion without Ca, denaturation causes a substantial increase in splitting.

The findings on the denatured proteins seem to support the assumption that the increased digestibility in 0.02 M KCl is caused by a loosened up structure

Table IV

Digestion of native and denatured myosins

For the conditions of denaturation see text. 30 mg of proteins in 10 ml were digested in the pH-stat with 25 μg/ml trypsin, at pH 8.2 and 22°. From the pH-stat records the number of bonds split/10^5 g during 20 minutes was calculated

Conditions of digestion	myosin		LMM		HMM	
	native	denat.	native	denat.	native	denat.
0.02 M KCl	38	60	46	38	25	32
0.52 M KCl	24.6	60	20	46	16	35
0.02 M KCl + 0.01 M $CaCl_2$	21	74	25	57	25	47

of the proteins under these conditions, and that this conformation facilitates the access of trypsin to the susceptible peptide bonds. But it should be pointed out that the magnitude of the effect of denaturation is due partly to the fact that Ca does *increase* proteolysis in the case of denatured proteins.

This uncertainties induced us to do another very plausible kind of experiment. We recorded the proteolysis of myosin by chymotrypsin in the three ionic milieux concerned. As it is seen in Fig. 9, with this proteolytic enzyme there was no substantial difference. This experiment suggests that the influence of cations on the tryptic digestion of myosin might be explained by more specific causes than an overall conformational change. If the accessibility for the enzyme plays the primary role it is difficult to explain that the alleged "opening up" of the molecule makes the access easier for trypsin than for chymotrypsin. In this connection it is interesting to point to the results of Fischer et al. (1961). According to these authors the reversible change of digestibility of amylases caused by the removal of structurally important Ca ions can be observed equally well both with trypsin and chymotrypsin.

Discussion

The results presented by different authors on the proteolysis of proteins and on the influence of ions on this process represent a set of extremely divergent facts.

Several authors have found an *activating* effect of Ca on tryptic activity (Gorini, 1951; Bier and Nord, 1951; Duke et al., 1952). This was explained mainly by the inhibitory action of this ion on the autolysis of the enzyme. On the digestion of salmin and of chymotrypsinogen on the other hand Ca has *no effect* (Green and Neurath, 1963). According to Gorini and Audrain (1952) the digestion of native and denatured serum albumin is *inhibited* by bivalent metals. In the case of lysozyme as substrate the activity of trypsin is inhibited specifically by Mn^{++} ions due to the stabilization of the undigestible native form of lysozyme (Gorini et al. 1952). The dramatic increase in the digestibility (by trypsin and by chymotrypsin) of amylases upon removal of structurally essential Ca ions (Fischer et al., 1961) has a similar cause.

Extensive studies on the influence of alkali and alkaline earth metal ions on the tryptic and chymotryptic digestion of serum albumin and lactoglobulin in the native and denatured state were conducted by Yon (1957, 1958, 1958a, 1960). She observed an inhibition by alkali metal and alkaline earth ions. The mechanism of this influence was quite different with native and denatured proteins. The effect of Ca was explained by this author by the Ca-binding of lactoglobulin.

Through all the divergencies of facts and speculations two points of view are emerging: The cation binding on one hand and the change or stabilization of conformation on the other hand are considered in first line when an explanation of the observations is sought for. We will try to discuss our results along these two general lines.

Regarding the significant capacity of myosin and meromyosins to bind alkali metals and alkaline earth metals (Nanninga, 1955; Lewis and Saroff, 1957; Nanninga, 1957; Mühlrad et al., 1962) it seems plausible to assigne the observed effects to cation binding. The curves of Fig. 3 are in a good qualitative agreement with this view. They show that in the presence of 0.5 M KCl the effect of Ca or Mg does not prevail as it can be expected on the basis of the fact that alkali metal ions compete with the alkaline earth's (Nanninga, 1957; Mühlrad et al., 1962). It is difficult to understand, however, the facts described in the present communication merely on the basis of cation binding. There is practically no difference between the two kinds of meromyosins with respect to alkaline earth metal binding (Nanninga, 1957). The influence of these ions on proteolysis is on the other hand markedly different for the two.

In order to establish a quantitative relationship between our findings and the rather well known data of ion binding, an analysis of the kinetics of proteolysis in clear molecular terms would be needed, but this seems to be a very difficult task. We think that the rate constants arrived at by assuming two synchronous first order processes are not satisfactory in this respect. (See our "IEG4 memorandum No 43." for further discussion of this point.) Even in the case of synthetic substrates the time course of splitting by proteolytic enzymes cannot be represented by a simple first order law (Elkins-Kaufman and Neurath, 1948) Nevertheless our results summarized in Table II do fit quite well in the picture drawn by Mihályi and Harrington (1959): The number of bonds per molecule attacked by the fast process is considerably less for LMM than for myosin and is very low for HMM, i.e. a great part of the "random region" is digested during the formation of meromyosins.

Our experiments performed in 0.5 M KCl support the view that in myosin a significant part of the bonds susceptible is not accessible to trypsin. The number of bonds split increases if digestion is carried out in 0.02 M KCl. The increase occurs in the slow process and it may be supposed that it takes place mostly in the HMM part of myosin.

In contrast to the effect of K ions the effect of Ca seems to be localized in the "random part". When myosin or LMM are digested in the presence of Ca the number of bonds accessible in the fast process is decreased to an unsignificant level. Analysis of the fragments formed during digestion in presence of Ca suggests that the myosin molecule is severed in this case at an other region than when the traditional meromyosins are formed. This result is again in agreement with Harrington's myosin model which assumes that the digestion of the "random region" leads to the formation of the meromyosins and in the presence of Ca this process is minimized.

We feel that because the easy digestion of HMM found in our experiments is in sharp contradiction to all the results reported heretofore, it needs some clarification. In the experiments of Mueller and Perry (1961, 1962) the protein: trypsin ratio was unusually high, in the experiments of Nankina et al. (1964) digestion

for several hours at 28°C was needed to achieve the digestion of HMM. The results of Young et al. (1964) on the kinetic analysis of digestion show the same. These latter authors have found that the poor digestibility of HMM was due to the presence of trypsin inhibitor which accumulated during the isolation procedure in this fraction. After separation of a small protein impurity by gel-filtration an increased digestibility was found. Nevertheless, even after this treatment their HMM preparation remained more resistant to trypsin than LMM or myosin. The trypsin to protein ratio was relatively high also in their experiments, i.e. they worked with the same trypsin to protein *molar* ratio as Mihályi and Harrington (1959) used with myosin, nevertheless the amount of bonds susceptible to the action of trypsin, as deduced by mathematical analysis from the pH-stat records was much less than with myosin or LMM. We think the discrepancy of our experiences with all the results reported in literature stems from the fact that all the cited authors used soybean trypsin inhibitor for the blocking of proteolysis during the preparation of the meromyosins, while we used DFP, which hydrolyzes during the later course of preparation (see: Methods). It seems thus highly probable that the HMM preparation of the authors mentioned contained an unknown amount of trypsin inhibitor even in the case of Young's et al. (1964) HMM purified by gel-filtration. We infer from our findings that HMM cannot be regarded as a part of the myosin molecule which is extremely resistant to trypsin action.

Acknowledgements

We are indebted to Dr. A. Mühlrad for valuable ideas which contributed to our off hand debates going on continuously in our laboratory on actual work.

In the mathematical treatment of our results Mr. L. Varga of the Central Physical Research Institute of the Hungarian Academy of Sciences, Budapest gave us an extremely valuable help which we aknowledge here sincerely.

References

Bier, M. and Nord, F. F. (1951) Arch. Biochem. Biophys. *33* 320

Biró, N. A. and Bálint, M. (1965) "IEG—4" memorandum, No. 43

Duke, J. A., Bier, M. and Nord, F. F. (1952) Arch. Biochem. Biophys. *40* 424

Elkins-Kaufman, E. and Neurath, H. (1948) J. Biol. Chem. *175* 893

Fischer, E. H. and Stein, E. A. (1961) in Boyer, P. D., Lardy, H. and Myrbäck, K. (editors): The Enzymes, Academic Press, New York, Vol. 4, pp. 326—330.

Gorini, L. (1951) Biochim. Biophys. Acta *7* 318

Gorini, L. and Audrain, K. (1952) Biochim. Biophys. Acta *9* 180

Gorini, L., Félix, F. and Fromogat, C. (1952) Biochim. Biophys. Acta *12* 283

Hotta, K. and Kojima, S. (1964) Biochemistry *3* 1431

Jacobsen, C. F., Léonis, J., Linderström-Lang, K. and Ottesen, M. (1957) in D. Glick (editor): Methods of Biochemical Analysis, Vol 4. Interscience Publishers, New York.

Lánczos, C. (1957) Applied Analysis, Isaac Pittman and Sons, London, p. 321

Laskowski, M. (1955) in Colowick, S. P. and Kaplan, N. O. (editors) Methods in Enzymology, Vol. 2. Academic Press, New York.

Lewis, M. S. and Saroff, H. A. (1957) J. Am. Chem. Soc. *79* 2112
Lowey, S. and Cohen, C. (1962) J. Mol. Biol. *4* 292
Mihályi, E. and Harrington, W. F. (1959) Biochim. Biophys. Acta *36* 447
Mueller, H. and Perry, S. V. (1961) Biochim. Biophys. Acta *50* 509
Mueller, H. and Perry, S. V. (1962) Biochem. J. *85* 431
Mühlrad, A., Biró, N. A. and Vértes, K. (1962) Acta Physiol. Acad. Sci. Hung. *21* 16
Nankina, V. P., Kofman, E. B., Tsherniak, B. J. and Kalamkarova, M. V. (1964) Biokhimija *29* 424
Nanninga, L. B. (1955) Arch. Biochem. Biophys. *56* 334, 349
Nanninga, L. B. (1957) Arch. Biochem. Biophys. *70* 346
Portzehl, H., Schramm, G. and Weber, H. H. (1950) Z. Naturforschung *50* 61
Szent-Györgyi, A. G. (1953) Arch. Biochem. Biophys. *42* 305
Szent-Györgyi, A. G. and Borbiró, M. (1956) Arch. Biochem. Biophys. *60* 180
Woods, E. F., Himmelfarb, S. and Harrington, W. F. (1963) J. Biol. Chem. *238* 2374
Yon, J. (1957) J. Chim. Phys. *52* 452
Yon, J. (1958) Biochim. Biophys. Acta *27* 111
Yon, J. (1958a) Bull. Soc. Chim. Biol. *40* 379
Yon, J. (1960) Bull. Soc. Chim. Biol. *42* 1263
Young, D. M., Himmelfarb, S. and Harrington, W. F. (1964) J. Biol. Chem. *230* 2822

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 29—38 (1966)

Nucleic Acids

VII. Separation of Ribo-oligonucleotides by DEAE-Cellulose Chromatography*

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(Received November 15, 1965)

A pancreatic ribonuclease digest of rabbit liver ribonucleic acid was chromatographed on DEAE-cellulose with NH_4HCO_3 elution. The salt-concentration was increased stepwise by 0.07—0.08 M. We confirmed Staehelin's results in separating the mono-, di-, and trinucleotides, even sequence-isomers. In addition, the composition of five, tetra- and one pentanucleotide was established, out of which the well-separated ApApApCp has not been demonstrated in yeast RNA. In additional three tetranucleotides the terminal purine member was determined (Gp/ApGp/Cp, ApGpGpCp, and Gp/ApGp/Up), and thus in case of ApGpGpCp the complete sequence was obtained. Products of the same nucleotide composition have been detected in yeast s-RNA-s. The fifth tetranucleotide, (ApGpGp)Up, was eluted already together with the pentanucleotide (ApApGpGp)Cp. From the theoretically possible digestion products we have found the four dinucleotides, the eight trinucleotides and five out of the 16 tetranucleotides. Among the mononucleotides, a considerable amount of pseudo-uridylic acid was found. Similar data on rabbit liver RNA are missing in the literature. Penta- and higher oligonucleotides could not be sufficiently resolved, they overlapped and showed mixed elution patterns.

For the separation of enzymic digest of nucleic acids Dowex ion exchange resins have formerly been used (cf. Tankó, 1959b). The disadvantage of these methods was that they involved the use of non-volatile eluents, which made the isolation of the fractions very difficult and resulted in overlaps even after the mononucleotides.

The methods for the separation of oligonucleotides have greatly developed in the last years. This development was due to the necessity of investigating the base-sequence of nucleotides. Using DEAE-cellulose chromatography, Staehelin et al. (1959) have introduced volatile eluents, which facilitated considerably the desalting of the isolated products. Tomlinson and Tener (1962, 1963) carried out the elution in the presence of 7 M urea and succeeded in this way in the separation of larger oligonucleotides according to their chain length. By the use of the above methods Holley et al. (1964) have first determined the exact nucleotide-

* Supported by a special grant of Hungarian Research Council of Medicine.

Abbreviations: RNA, ribonucleic acid; s-RNA, soluble RNA; Gp, 3'-guanylic acid; Ap, 3'-adenylic acid; Cp, 3'-cytidylic acid; Up, 3'-uridylic acid; ψp, pseudouridylic acid; A, adenosine, pG, 5'-guanylic acid; pC, 5'-cytidylic acid; Pu, purine; Py, pyrimidine.

sequence of larger nucleotides, and later the complete sequence of alanine s-RNA (Holley et al. 1965).

The aim of our investigations was to separate and isolate smaller and larger oligonucleotides, in order to use them as model compounds, after the determination of their composition, for comparing various periodate-methods suitable for the stepwise degradation of nucleotides. To this end the pancreatic RNase digest of rabbit liver RNA was chromatographed on DEAE-cellulose according to the method of Staehelin et al. (1959, 1961). The separation was carried out in the absence of urea, because the dinucleotides and the sequence-isomers of trinucleotides can be separated only in this way without re-chromatography. Moreover, the aim of our experiments was to see to what extent the tetra- and even larger oligonucleotides can be resolved without the use of urea in the eluent and without rechromatography. Our results, a brief account of which has already been given (Berencsi, Zsindely, 1964, 1965), are presented in this paper.

Experimental

Sodium-RNA was prepared from rabbit liver with guanidine-hydrochloride (Zsindely et al. 1959). Analytical data: P, 8.5—8.7% dry weight; ε(P), 7600—7800; base-composition, G 34.2, A 18.4, C 29.3, U 18.1 mole percentage. Absorption increase after alkaline hydrolysis and RNase-digestion was 41.4 and 26 per cent, respectively (Zsindely, 1962). The preparations showed one single peak on sedimentation in the ultracentrifuge ($s^0_{20,w} = 9—10$) (Szabolcs, Zsindely, 1963).

Preparation of the digestion mixture. 50—500 mg sodium-RNA, at a concentration of 2 mg/ml, was incubated with crystalline RNase (Light or Reanal), at a substrate/ enzyme ratio of 100:1, in 0.1 M $NaHCO_3$, pH 7.6—7.8, at 37°C for 4 hours, and was stored at 0°C until used (4—8 hours). Prior to column chromatography the pH was adjusted to 8.6 with 0.1 N NH_4OH.

DEAE-cellulose (Whatman DE 50) was prepared according to Staehelin (1961a), and was stored as a suspension in 0.01 M NH_4HCO_3 (pH 8.6) at 0°C until used. About 110 g of adsorbent was used for a column of 2 × 130 cm in size. For concentration and desalting (Rushizky et al. 1962) 1 × 6 cm columns were employed.

Elution was carried out with NH_4HCO_3 at a flow rate of 10 ml per 15—20 minutes. After diesterase splitting 3 ml, otherwise 10 ml fractions were collected in a "Fractiomat" automatic fraction collector. The salt concentration was increased stepwise, i.e. no "varigrad" apparatus was used (Peterson, Sober, 1959), because on standing in this apparatus the pH of the eluent gradually increased above 9.0, which is undesirable. The total volume of the eluates varied between 2 and 16 litres with the different runs, while the time of separation varied between 2 and 15 days. The pH of the eluent was adjusted to 8.4—8.6 with cc. NH_4OH in a "Radelkisz" pH-meter. The ultraviolet absorption of fractions was measured in a Beckman spectrophotometer at 260 mμ.

For the isolation of substances a horizontal vacuum evaporator was used. On evaporation at 35—40°C most of the NH_4HCO_3 was decomposed, and the complete removal of the salt was achieved by several evaporations after dissolving the residue in water. The residue was dissolved in 0.1 N HCl and was allowed to stay at room temperature for 1—2 hours to achieve the splitting of the possible terminal cyclic phosphate. The product was then precipitated with 3—4 volumes of ethanol, and was kept over silica gel after drying. In certain cases, especially with substances containing only Ap and Up, in addition to ethanol 1—2 volumes of ether was also necessary to get a high yield. The yield was in average 80—90 per cent.

The identification of substances was based on ultraviolet spectra in acid and alkali, on optical density ratios and on chromatographic analysis following hydrolysis of the isolated products. The hydrolysis was made in 70% perchloric acid or N HCl, and the products were separated by descending paper chromatography in isopropanol-HCl-water (Wyatt, 1951). Quantitative estimation was performed in a Beckman spectrophotometer. (As to the details of analysis we refer to the survey by Tankó, 1959a.) The examination of tri- and tetranucleotides was supplemented also with an enzymic method. The terminal phosphate was split off by a purified (Ostrowsky, Tsugita, 1961) phosphomonoesterase prepared from prostata (Davidson, Fishman, 1959). The phosphomonoesterase treatment was carried out in 0.02 M acetate buffer (pH 5) with 0.1 ml of enzyme ($E_{280}^{1cm} = 0.4$) at 37°C for 6—12 hours. The product was treated with snake venom phosphodiesterase ("Light" crystalline preparation), in 0.02 M NH_4HCO_3 (pH 8.6) 0.01 M $MgCl_2$ with 0.05 mg of enzyme at 37°C for 4—8 hours. The degradation products were separated on a DEAE-cellulose column, 0.6 × 6—7 cm, with 0.01, 0.05, and 0.08 M NH_4HCO_3 as eluent.

With the exception of ψp which appeared among the mononucleotides, we were not interested in rare nucleotides. Therefore in the establishment of composition, either directly or by calculation, only the four well-known nucleotides were taken into account.

Results

First of all the stepwise increase in salt concentration resulting in a satisfactory separation was investigated. It was found that the products could be eluted at the following salt concentrations: cyclic mononucleotides 0.05 M, mononucleotides 0.08 M, dinucleotides 0.14—0.16 M, trinucleotides 0.24 M, tetranucleotides 0.35 M, certain penta- and hexanucleotides 0.4—0.5 M. Finally, the salt concentration was raised to 0.8 M. In this way 85 per cent of the ultraviolet-absorbing material of the digestion mixture could be recovered.

The separation was carried out after a non-exhaustive enzymic digestion in order to avoid the splitting of the polynucleotide chain at all possible sites, and thus to preserve some products with a higher chain length in the digestion mixture. A considerable portion of Py mononucleotides was isolated in the cyclic form

(Fig. 1, Cp:, Up:, ψp:). This shows that the enzymic reaction did not go to completion, as these compounds are well-known intermediate products of pancreatic RNase action (cf Tankó, 1959b). ψp, which was isolated almost completely in the cyclic form, amounted to about 3% of the total mononucleotide content (Table I). The four main dinucleotides (Fig. 1) could be well separated. The overlap between ApUp and GpCp is also negligible.

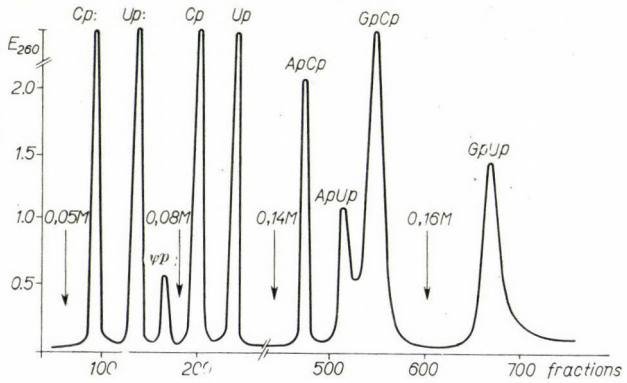

Fig. 1. Elution pattern of mono- and dinucleotides. Separation of a RNase-digest of 300 mg of rabbit liver RNA on DEAE-cellulose column (2 × 130 cm), with NH_4HCO_3 as eluent at a flow rate of 10 ml/15–20 minutes. Digestion was carried out at a substrate/enzyme ratio of 100/1, at 37°C for 4 hours, then the mixture was allowed to stand at 0°C for 8 hours before administration to the column. The numbers at the arrows indicate the change in salt concentration. Cp:, Up:, and ψp: are the corresponding cyclic mononucleotides

Table I

Results of analysis of mono- and dinucleotides separated and isolated from a RNase-digest of 300 mg of rabbit liver RNA on DEAE-cellulose column
(cf. Fig. 1)

Elution peak No.	Pu/Py	Per cent* of digestion mixture	Products of hydrolysis, 10^{-1} μmole				Isolated product
			G	A	C	U	
1.	—	1.485	—	—	1.06	—	cyclic Cp
2.	—	2.97	—	—	—	1. 14	cyclic Up
3.	—	0.585	—	—	—	0.547	cyclic ψp
4.	—	12.93	—	—	0.983	—	Cp
5.	—	4.22	—	—	—	0.796	Up
6.	1.05	3.36	—	1.05	1.06	—	ApCp
7.	1.03	1.63	—	0.671	—	0.650	ApUp
8.	0.98	8.25	0.590	0.04	0.604	0.042	GpCp
9.	1.01	3.78	0.547	—	—	0.554	GpUp

* Based on O. D. values at 260 mμ.

The elution sequence as well as the analytical data of the trinucleotides are shown in Fig. 2 and Table II. All the eight possible trinucleotides were isolated. Since RNase, owing to its specificity, splits only at the pyrimidines, the number of the possible products in the case of di-, tri-, and tetranucleotides is 4, 8, and 16, respectively. The separation of ApApUp, GpApCp, and ApGpCp is not sharp, while it is excellent with GpApUp and ApGpUp. As it could be expected on the basis of the nucleotide composition of RNA preparations (Gp and Cp dominance), among the di- and trinucleotides GpCp and GpGpCp occur in the largest amount (Tables I and II). Between GpGpCp and GpGpUp a peak appeared in the elution profile containing Gp, Ap, and Cp, which, based upon the Pu/Py ratio, could be

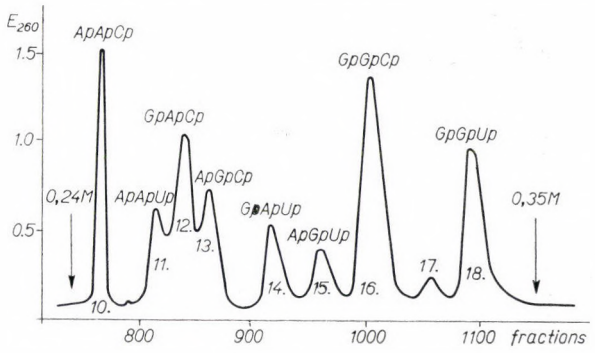

Fig. 2. Elution pattern of trinucleotides. Separation of a RNase-digest of 300 mg of rabbit liver RNA on DEAE-cellulose column (see Fig. 1). The numbers at the arrows indicate the change in salt concentration

regarded as a tetranucleotide. We could not identify it, however, since in addition, it contained a small amount of Up as well.

As it can be seen in Fig. 3, later on only peaks Nos 19, 20, 24, and 27 were well separated, in the last peak however, the shoulder indicates a mixture. In peaks Nos 21, 22, 23, and 25, 26 there is a considerable overlap. As it can be seen in Table II, the elution peak No. 19 proved to be ApApApCp, a tetranucleotide. Among the hydrolysis products of the material found in the well-separated peak No. 20, all four bases occurred with a Pu/Py ratio of 2.0, i.e. it should be a mixture of trinucleotides. However, this is not probable considering the position of this peak. It may be a mixture of special composition containing also rare nucleotides. They cannot be taken for trinucleoside-tetraphosphates either, since after monoesterase treatment 1/3 of the total phosphate content was liberated as inorganic phosphate. (In the above case it should be 1/2.) Elution peaks Nos 21, 22, and 23 contain tetranucleotides, the first two are sequence isomers with Cp terminals, the third is a mixture of products with Up terminals. In peak No 24 the Pu/Py ratio is 3.54, i.e. we have a mixture of about 1 : 1 ratio of tetra- and pentanucleotides. According to the data of base-analyses, if Cp is regarded as unity and 3 units of Gp

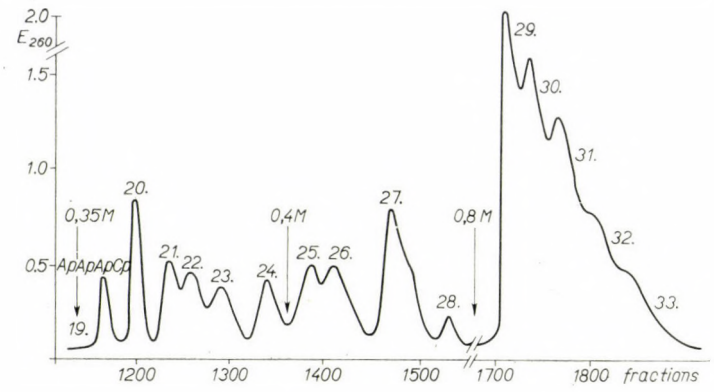

Fig. 3. Elution pattern of tetra-, penta-, and larger oligonucleotides. Separation of a RNase-digest of 300 mg of rabbit liver RNA on DEAE-cellulose column (see Figs 1 and 2). The numbers at the arrows indicate the change in salt concentration

Table II

Results of analysis of tri- and tetranucleotides separated and isolated from a RNase-digest of 300 mg of rabbit liver RNA on DEAE-cellulose column
(cf. Figs 1, 2, and 3)

Elution peak No.	Pu/Py	Per cent* of digestion mixture	Products of hydrolysis, 10^{-1} μmole				Isolated product
			G	A	C	U	
10.	1.91	2.22	0.030	0.223	0.112	0.024	ApApCp
11.	1.89	0.99	0.076	0.309	0.079	0.125	ApApUp
12.	2.05	1.72	0.266	0.332	0.257	0.034	GpApCp
13.	2.07	1.82	0.433	0.441	0.400	0.022	ApGpCp
14.	1.97	1.94	0.362	0.374	0.017	0.357	GpApUp
15.	2.08	1.35	0.456	0.443	—	0.432	ApGpUp
16.	1.97	5.36	0.421	0.01	0.210	0.009	GpGpCp
18.	1.95	3.40	0.653	0.018	0.023	0.321	GpGpUp
20.	2.03	1.89	0.334	0.330	0.200	0.127	?
17.	2.94	1.02	0.393	0.275	0.181	0.046	?
19.	3.01	0.60	0.016	0.522	0.171	0.008	ApApApCp
21.	2.92	0.73	0.432	0.230	0.216	0.011	Gp(ApGp)Cp
22.	3.03	1.42	0.552	0.285	0.190	0.085	ApGpGpCp
23.	3.10	0.66	0.620	0.304	0.089	0.209	Gp(ApGp)Up
24.	3.54	0.71	0.450	0.357	0.139	0.089	(ApGpGp)Up
							(ApApGpGp)Cp

* Based on O. D. values at 260 mμ.

would belong to it, then 0.033 Gp is left. This is not possible, because the amount of Up is 0.089. If 1 Gp belongs to 1 Cp, 0.311 Gp is left. This is not an integral multiple of the amount of Up either, therefore it has to be ruled out, too. Consequently, 2 Gp-s (0.278) belong to Cp. The remaining 0.172 is just the double of Up, therefore 2 Gp-s can be found beside the latter, too. The amount of Ap can be distributed between the two products without any amount left over, if 2 Ap-s belong to Cp (0.278), and 1 Ap to Up (0.08). On this basis it can be assumed that in peak No. 24 the tetranucleotide (ApGpGp)Up and pentanucleotide (ApApGpGp)Cp are present.

The analysis of tri- and tetranucleotides was supplemented with an enzymic method. The terminal phosphate was split off by prostata phosphomonoesterase (PME) and the product was treated with snake venom phosphodiesterase (SDE). The dephosphorylated substance was decomposed to its constituents among which the terminal Pu appeared as a nucleoside, while the others as 5′-nucleotides. The method is schematically represented with a tetranucleotide:

$$\text{ApGpGpCp} \xrightarrow{\text{PME}} \text{ApGpGpC} + \text{p} \xrightarrow{\text{SDE}} \text{A} + \text{pG} + \text{pG} + \text{pC}$$

The degradation products were separated on a small DEAE-cellulose column. The Pu nucleoside was eluted already at 0.01 M, while 5′-nucleotides only at 0.05—0.08 M salt concentration. Thus, with the trinucleotides the sequence is also given. (cf. GpApCp, ApGpCp, GpApUp, and ApGpUp sequence isomers in Table II.) With the tetranucleotides and larger oligomers the terminal members can be determined in this way, but the position of the inner ones remains unknown. Therefore, they are written in brackets. By using this method, among the tetranucleotides in peaks Nos 21 and 22 guanosine and adenosine appeared on diesterase action. Hence, we demonstrated the presence of sequence isomers which differ only in the 5′-Pu-member (Gp/ApGp/Cp and ApGpGpCp). In the case of substance No. 22, as the left terminal is Ap, the exact sequence is also given, while with peak No. 21 there are two further possibilities (GpApGpCp and GpGpApCp). With the substance in peak No. 23 also guanosine appeared as nucleoside in the digestion mixture, i.e. the product is Gp(ApGp)Up, but it may also be a mixture of sequence isomers.

Pentanucleotides were found in several elution peaks (Fig. 3 and Table III). (ApApGpGp)Cp which was found first is shown in Table II, because it was eluted in peak No. 24 together with a tetranucleotide. According to the data of base-analyses the pentanucleotides are mixtures, moreover a portion of them is eluted together with hexanucleotides. We failed to establish their base composition due partly to overlaps and partly to their mixed character. In the case of substance No. 25, e.g., it is certain that it is a mixture of pentanucleotides having Cp and Up as terminals, but since the amount of Cp and Up is about the same, and the amount of Gp and Ap is nearly an integral multiple ($3\times$ and $5\times$, respectively) of this value, there are a number of possible variations for the composition of the products. The same holds for substances in peaks Nos 26, 27 and 28. On

Table III

Results of analysis of penta-, hexa-, and larger oligonucleotides separated and isolated from a RNase-digest of 300 mg rabbit liver RNA on DEAE-cellulose column (cf. Fig. 3)

Elution peak No.	Pu/Py	Per cent* of digestion mixture	Products of hydrolysis, 10^{-1} μmole				Isolated product
			G	A	C	U	
25.	4.15	1.03	0.180	0.282	0.054	0.057	pentanucleotides
26.	4.11	1.08	0.754	0.520	0.092	0.217	,,
27.	4.28	3.15	0.283	0.216	0.078	0.039	penta- and hexanucleotides
28.	4.51	0.90	0.298	0.380	0.069	0.083	,,
29.—33.	4.6—5.9	11.4					penta-, hexa- and larger oligonucleotides

* Based on O. D. values at 260 mμ.

the basis of the Pu/Py ratio the latter are already mixtures of penta- and hexanucleotides. From the last peak which is eluted when the salt concentration is raised to 0.8 M and which consists of several substances, it can be seen that a considerable amount of products can still be isolated, which appear to be penta-, hexa- and larger oligonucleotides on the basis of their Pu/Py ratios. The separation here is not good. This shows that penta- and hexanucleotides cannot be separated even on a 130 cm long column, which is longer than usual.

Discussion

During the chromatography without varigrade of RNase digest of rabbit liver RNA, salt concentration was increased stepwise, by 0.07—0.08 M, to achieve the elution of the products containing one more nucleotide each. No reference of this kind can be found in the literature, though as judged from the figures a concentration increase of about 0.06 M is required for this effect if a continuously changing concentration gradient is applied (Staehelin, 1961a), in the presence of 7 M urea in sodium acetate, while about 0.05 M when a linear concentration gradient is applied (Tomlinson, Tener, 1962). Since the separation was performed after incomplete enzymic degradation, part of the Py-mononucleotides was obtained in the cyclic form. Staehelin (1961c) has found cyclic Pu-mononucleotides in the pancreatic RNase-digest of tobacco mosaic virus RNA, which he attributes to the action of contaminating tobacco leaf RNase. He does not mention cyclic pyrimidines, although in our opinion they do occur in small amounts in his anal-

ysis. Staehelin (1964) has demonstrated the presence of a large amount of dimethyl-GpCp and dimethyl-Gpψp in the digest of yeast RNA, which emerged from the column before the normal dinucleotides. The digest of rabbit liver RNA contains these components only in extremely small amounts, while it contains much more ψp as mononucleotide, consequently it must be inserted mainly between pyrimidines in the polynucleotide chain. The mono-, di-, and trinucleotides are well separated and are eluted in accordance with Staehelin (1961a). GpUp and ApApCp are also well separated, moreover, in the case of ApGpUp and GpApUp even the sequence isomers.

We examined whether the secondary binding forces of the larger oligomers differ enough as to permit their separation by this method, i.e. without the use of urea. Five out of the 16 possible tetranucleotides were found in the digest of rabbit liver RNA. ApApApCp was well separated, while the three others, though they overlapped, can certainly be resolved by the use of a longer column and by collecting smaller fractions. In case of the latter products the terminal Pu residue was also determined by means of diesterase [Gp(ApGp)Cp, ApGpGpCp, and Gp(ApGp)Up], and thus in one of them the complete sequence was established. (ApGpGp)Up formed a common peak with the pentanucleotide (ApApGpGp)Cp. Staehelin (1964) has found (ApGpGp)Up and (ApGpGp)Cp tetranucleotides in serine s-RNA, but no sequence data are presented. Holley et al. (1965a) have detected GpGpApCp, the sequence isomer of our product, and pGpGpGpCp in alanine s-RNA. Concerning the pancreatic RNase digest of rabbit liver RNA, McCully and Cantoni (1962) reported the separation of the di- and some trinucleotides only.

As in the further elution peaks penta- and hexanucleotides were eluted as mixtures and overlapped, the method in its present form is unsatisfactory for the resolution of these oligomers. Therefore, in order to isolate these larger products, too, after the separation of tetranucleotides the elution has to be continued in the presence of 7 M urea (Tomlinson, Tener, 1962, 1963). Since in the experiments of Holley et al. (1965a), separation carried out in the presence of 7 M urea proved to be successful also for the resolution of oligonucleotides having the same chain length this method may provide better results in our case, too.

For our purposes the results presented were satisfactory, since starting from 300 mg of sodium-RNA, we could isolate, in 5—25 mg amounts, the dinucleotides, the majority of trinucleotides and ApApApCp out of the tetranucleotides. Thus we could use these products as model compounds in other investigations.

Acknowledgements

We wish to thank Prof. B. Tankó for his continuous support and helpful advices in the compilation of this paper; Dr. G. Diószeghy, leader of the Department of Urology, Hajdú-Bihar County Hospital, for kindly supplying the material from prostata operations.

References

Berencsi, G., Zsindely, A. (1964) 6th Congress of Biochemistry, Tihany
Berencsi, G., Zsindely, A. (1965) 2nd Meeting of the Hungarian Biochemical Society, Budapest
Davidson, J. N., Fishman, W. H. (1959) J. Biol. Chem. *234* 526
Holley, R.W., Madison, J. T., Zamir, A. (1964) Biochem. Biophys. Res. Comm. *17* 389
Holley, R.W., Apgar, J., Everett, G. A., Madison J. T., Marquisee, M., Merill, S. H., Penswick, J. R., Zamir, A. (1965) Science *147* 1462
Holley,R. W., Everett, G. A., Madison, J. T., Zamir, A. (1965a) J. Biol. Chem. *240* 2122
McCully, K. S., Cantoni, G. L. (1962) J. Biol. Chem. *237* 3760
Ostrowsky, W., Tsugita, A. (1961) Arch. Biochem. Biophys. *94* 68
Peterson, E. A., Sober, H. A.(1959) Anal. Chem. *31* 857
Rushizky, G. W., Sober, H. A. (1962) Biochim. Biophys. Acta *55* 217
Staehelin, M., Sober, H. A., Peterson, E. A. (1959) Arch. Biochem. Biophys. *85* 289
Staehelin, M.(1961) Biochim. Biophys. Acta *49* a/11, b/19, c/27
Staehelin, M. (1964) J. Mol. Biol. *8* 470
Szabolcs, M., Zsindely, A. (1963) Magyar Kémiai Folyóirat, *69* 515
Tankó, B. (1959) editor: Kovách, A. A nukleinsavak vizsgálata., in "A kísérletes orvostudomány vizsgáló módszerei" *4*, a/574, b/587, c/609
Tomlinson, R. W., Tener, G. M. (1962) J. Am. Chem. Soc. *84* 2644
Tomlinson, R. W., Tener, G. M. (1963) Biochemistry *2* 697
Wyatt, G. R. (1951) Biochem. J. *48* 584
Zsindely, A., Szabolcs, M., Tankó, B. (1959) Magyar Kémiai Folyóirat *65* 181
Zsindely, A. (1962) Acta Biol. Debrecina *8* 135

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 39—44 (1966)

Effect of Urea and Ca Ions on Pancreatic Amylase

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(Received November 23, 1965)

A simplified procedure for the isolation of crystalline hog pancreas amylase is described. The method makes use of the protective effect of an excess of calcium ions. In the presence of Ca ions amylase is reversibly inactivated by high urea concentrations.

The established method for the isolation of crystalline amylase (α-1,4-glucan 4-glucanohydrolase, E.C. 3.2.1.1) from hog pancreas (Fischer and Bernfeld, 1947, 1948) involves several steps including repeated precipitations with acetone and ammonium sulfate. One of the problems encountered during the recrystallization of the purified enzyme consists in its low solubility in neutral solutions and its lability at alkaline pH values, at which it is readily soluble. We have found that amylase crystals are rapidly dissolved in concentrated urea solutions. However, urea leads to inactivation unless Ca ions are present in excess. A similar protection of Taka-amylase activity by Ca ions in urea solution was observed (Okunuki, 1961). We have found that Ca ions exert a similar stabilizing action when the enzyme is exposed to organic solvents used during the purification procedure. A more drastic acetone treatment of the pancreas extract was therefore introduced, adding Ca ions to protect the enzyme. This procedure resulted in crystalline amylase after extraction and a single precipitation with acetone.

Methods

Amylase assay. Enzyme activity was routinely determined by measuring the decrease in the iodine colour of starch (Smith and Roe, 1949), the results were checked with the maltose-method (Bernfeld, 1955). The Noredux Standard starch used in the latter experiments was a gift of the Siegfried A. G., Zofingen. Other commercially available soluble starch samples were found to give about 10—20% lower figures. Recrystallized amylase had a maximal activity of 1300 maltose units or 4500 Smith-Roe units per mg protein.

Protein was determined by measuring the optical density at 280 mμ, using the value of $E^{1\%}_{1cm} = 24.1$ in a solution of 4 mM disodium glycerophosphate buffer,

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pH 7 (Hsiu et al. 1964). This value was controlled by determining the dry weight and N content of the preparations. The Lowry method (Lowry, 1951) of protein determination was occasionally applied, using a purified amylase solution as standard.

Results and Discussion

Isolation of amylase. 1 kg of hog pancreas freed from gross contamination of fat and connective tissue was homogenized in appropriate portions in a blendor with about 0.8 volume of a solution containing 3% butanol and 0.01 M $CaCl_2$. The pooled homogenate was rapidly heated to 37°C and kept at this temperature for 30 minutes. It was then mixed with acetone (final concentration of acetone 40% v/v) and cooled to 10°C by the addition of dry ice to the mixture. The resulting pulp was poured into a sack of thick cloth yielding a slightly opalescent yellow filtrate. This solution was cooled below 10°C and cold acetone was added to bring the acetone concentration to 67%. The resulting sticky precipitate was either immediately centrifuged or left to settle in the ice-box. In the latter case most of the supernatant could be decanted. The precipitate was suspended in a minimum amount of water and dialyzed first against running tap water overnight, then against several changes of a solution of 0.001 M $CaCl_2$. The dialyzed solution was cleared by centrifugation and left to crystallize. A few drops of a suspension of amylase crystals were added to promote crystallization. This first crystallization took about 4—6 days in the ice-box. The crystals were then centrifuged (the supernatant contains usually less than 5 mg/ml amylase), and the compact sediment was washed once with cold distilled water.

Recrystallization. The crystals were dissolved by adding a solution of 10 M urea containing 0.01 M $CaCl_2$. Care was taken to avoid a high urea concentration, the amount of 10 M urea should therefore not exceed that which would yield finally a 4 M solution. The clear solution was diluted to bring the protein concentration below 10% and the solution was dialyzed overnight against 0.001 M $CaCl_2$. At this stage crystallization is completed in 1 or 2 days. The crystals were centrifuged, washed and repeatedly recrystallized in a similar way, dissolving them with urea, etc.

Table 1

Isolation of amylase from 3.7 kg hog pancreas

Steps	Volume ml	Total protein g	Purity per cent
Dialyzed solution after acetone fractionation	750	93	31
First crystallization	500	45	49
Second ,,		27	82
Third ,,		23	96
Fourth ,,			100

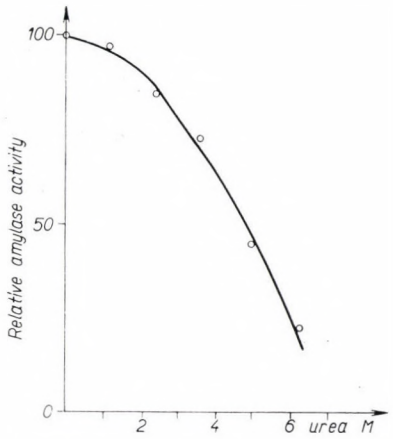

Fig. 1. Amylase activity measured in the presence of urea. Starch and amylase solutions were prepared to yield the appropriate urea concentration in 0.005 M $CaCl_2$ + 0.05 M Tris buffer pH 8.4. After 5 min. incubation the reaction was stopped by the addition of hydrochloric acid and the iodine colour value was determined

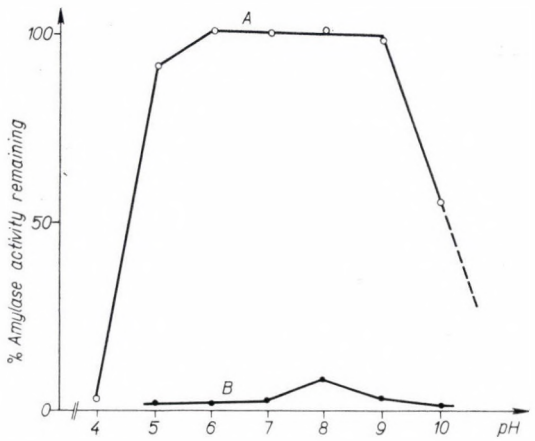

Fig. 2. pH stability curve of amylase. Crystalline amylase 3 mg/ml was incubated for 60 min. at 37 °C in the presence of 0.02 M buffer of the pH indicated. *A:* in the presence of 6 M urea and 0.01 M $CaCl_2$. *B:* in the presence of 6 M urea. After incubation the samples were diluted 100fold with 0.02 M Tris buffer pH 7.2 and amylase activity was determined under optimal conditions

The amylase activity of the first crystals varies between 1000—2000 Smith-Roe units and maximum activity is usually obtained after 3—4 recrystallizations (4500—4700 Smith-Roe units, or 1300 maltose units per mg protein).

The effect of urea on the amylase enzyme activity in the presence of an excess of Ca ions. When the activity of amylase was measured at 37°C in the presence,

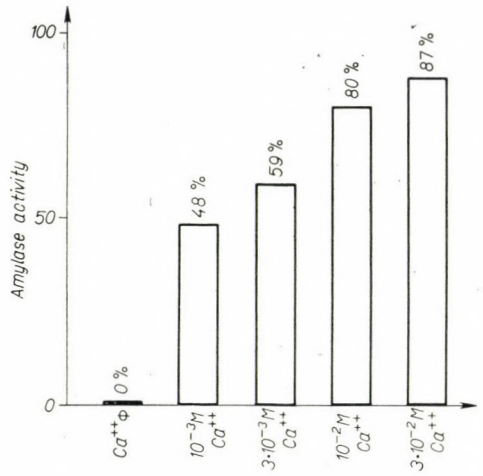

Fig. 3. Effect of Ca ions on the inactivation by urea. Crystalline amylase 3 mg/ml in Tris buffer pH 7.2 containing 6 M urea was incubated for 100 min. at 37°C in the presence of different Ca concentrations. After the incubation period samples were diluted 100fold with 0.02 M Tris buffer pH 7.2 and amylase activity was determined (100% = amylase activity under the above conditions in the absence of urea)

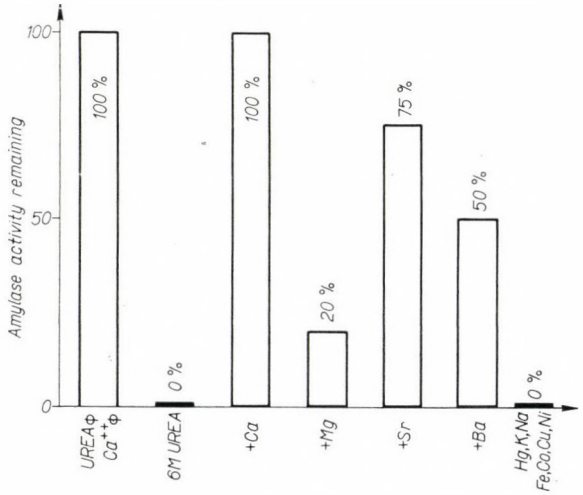

Fig. 4. Effect of different cations on the inactivation by urea. Crystalline amylase 3 mg/ml was incubated for 60 min. at 37°C in 0.02 M Tris buffer of pH 7.2 in the presence of 6 M urea and different cations: 0.01 M Ca, 0.01 M Mg, 0.01 M Sr, 0.01 M Ba, 0.001 M Cu, Ni, Co, Fe. After the incubation period samples were diluted 100fold with 0.02 M Tris buffer pH 7.2 and the amylase activity determined

of 0.02 M $CaCl_2$ and varying concentrations of urea, it was observed that urea progressively inhibited amylase activity. On the other hand, as shown by the results presented in Fig. 1 the inhibition of enzyme activity is negligible at urea concentrations below 1 M.

The experiments to be presented below show that amylase can be kept in the presence of 0.01 M $CaCl_2$ and 6 M urea at 37°C for about one hour without great loss of enzyme activity. In the latter case, however, a more concentrated solution is being exposed to urea and the measurement of activity is performed *after* diluting the mixture to obtain urea concentrations below 0.1 M. One may conclude therefore that amylase is reversibly inactivated at high urea concentrations.

The effect of bivalent cations on denaturation by urea. Fig. 2 shows the pH stability curve of amylase in buffered solutions in the presence of 6 M urea and 6 M urea + 0.01 M $CaCl_2$, respectively. The effect of Ca on the stability of the enzyme in the presence of 6 M urea is shown in Fig. 3.

Ca ions may be replaced to some extent by other alkaline earth metal ions. The stabilizing effect decreases in the order Ca > Sr > Ba > Mg (Fig. 4).

It has been established (Hsiu et al. 1964) that Ca ion is an integral constituent of pancreatic amylase. The protecting effect of high Ca concentrations against the denaturing effect of urea may be connected with the known effect of Ca to "tighten" the structure of the enzyme.

References

Bernfeld, P. (1955) in Colowick, S. P., Kaplan, N. O. (eds.) Methods of Enzymology, Academic Press, New York. Vol. 1. p. 149
Fischer, E., Bernfeld, P. (1947, 1948) Helv. Chim. Acta *30* 64; *31* 1831
Hsiu, J., Fischer, E., Stein, E. (1964) Biochemistry *3* 61
Lowry, O. H. (1951) J. Biol. Chem. *193* 265
Okunuki, K. (1961) Advanc. Enzymol. *23* 29
Smith, B. W., Roe, J. H. (1949) J. Biol. Chem. *179* 53

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 45–54 (1966)

Purification and Properties of Glutamine Synthetase from Chicken Liver

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(Received November 23, 1965)

The present work reports on the purification of glutamine synthetase L-glutamate: ammonia ligase (ADP) (E. C. 6.3.1.2.).

The ratio of glutamine synthetase and gamma-glutamyl transferase activity was constant during all phases of purification.

The purified enzyme was separated into 3 fractions by chromatography on DEAE cellulose. The ratio of glutamine synthetase and gamma-glutamyl trasferase activities was different in each fraction. Attempts to separate fractions with either glutamine synthetase or gamma-glutamyl transferase activity alone, have failed.

Glutamine synthetase was activated by Mg, Mn and Co ions as well as by cysteine. The activity of gamma-glutamyl transferase was enhanced by Mn and phosphatase ions and by adenosine-5'-diphosphate.

Our results suggest that the glutamine synthetase and gamma-glutamyl transferase activities are two different functions of one enzyme.

Glutamine synthetase is known to be present in animal, plant and bacterial organisms (Meister, 1962). The enzyme first isolated from pigeon liver (Speck, 1949) catalyzes the following reaction:

$$\text{Glutamic acid} + NH_3 + \text{ATP} \rightleftharpoons \text{glutamine} + \text{ADP} + \text{P} \qquad (1)$$

In some further studies it has been found that parallel with the purification of glutamine synthetase, also the activity of gamma-glutamyl transferase increased (Elliot, 1953). The latter enzyme catalyzes the reaction

$$\text{Glutamine} + NH_2OH \rightleftharpoons \text{gamma-glutamyl hydroxamate} + NH_3 \qquad (2)$$

The examination of glutamine synthetase preparations of different origin has regularly revealed the presence of gamma-glutamyl transferase activity, too (Meister, 1962).

The aim of the present work was to develop a method for the preparation of highly purified glutamine synthetase and to decide whether glutamine synthetase and gamma-glutamyl transferase were two different enzymes or two different activities of one single enzyme.

Materials and Methods

Analytical grade adenosine-5'-triphosphate (ATP) and adenosine-5'-diphosphate (ADP) were prepared from a commercially available preparation of ATP, using ion-exchange chromatography (Cohn, 1957). Adenosine-5'-phosphate (AMP), adenosine-3'(2')-phosphate, L-glutamic acid, L-glutamine, L-cysteine-HCl were all products of the firm "Reanal". Hydroxylamine hydrochloride and tris-hydroxymethyl-aminoethane were "Fluka" products. DEAE cellulose was a preparation of "Serva". The ribonucleic acid (RNA) was prepared by the sodium-laurylsulphate method (Schmidt, 1957).

Glutamine synthetase activity was measured in the following reaction mixture: 50 μM Tris-HCl buffer (pH 7.3), 12.5 μM ATP, 125 μM glutamic acid, 250 μM NH_2OH (freshly neutralized), 100 μM $MgSO_4$, 25 μM cysteine and an appropriate amount of enzyme in a final volume of 2.5 ml. The pH of ATP, glutamic acid, NH_2OH and cysteine solutions was adjusted to 7.3 before use.

The activity of gamma-glutamyl transferase was determined in the following system: 250 μM phosphate buffer (pH 6.3), 250 μM L-glutamine, 12.5 μM NH_2OH, 5 μM $MnSO_4$, 0.031 μM ADP and an appropriate amount of enzyme in a final volume of 2.5 ml.

The quantity of the enzyme in the reaction mixtures was always such as to prevent the production of more than 2 μM hydroxamic acid. Under such conditions the amount of hydroxamic acid produced was proportional to the amount of enzyme present in the system. The mixture was incubated at 38°C and the reaction was stopped by the addition of 0.75 ml of a mixture of 0.18 M $FeCl_3$ in 2.3 M hydrochloric acid and of 0.73 M trichloroacetic acid. After removal of the protein precipitate the amount of hydroxamic acid produced was determined at 520 mμ in an Unicam SP 600 spectrophotometer (Lipmann & Tuttle, 1945). The calibration curve was taken with a succinic acid-hydroxamic acid standard.

Protein determinations were made photometrically using a calibration curve taken of crystalline horse serum albumine as a reference standard (Lowry et al. 1951).

The amount of protein in the individual fractions of the chromatographically purified enzyme was measured in a Beckman DU spectrophotometer at 280 mμ.

The enzyme activity was measured by the amount of hydroxamic acid produced and expressed as specific activity. The amount of enzyme producing 1 μM hydroxamic acid in 20 minutes in the above described systems was considered as one unit. The specific activity was defined as enzyme activity units/mg protein.

Experimental Results

Purification of glutamine synthetase

The enzyme was prepared from chicken liver.

Step 1. Preparation of acetone-dried liver powder. 500 g of fresh chicken liver were homogenized in 2500 ml of acetone at 0°C. The homogenate was rapidly filtered with suction and the wet residue resuspended in one volume of chilled acetone, then filtered again. The final residue was finely ground and dried over $CaCl_2$ *in vacuo* at 0°C.

Step 2. Extraction of acetone-dried powder: 100 g of acetone dry powder was homogenized in 1800 ml of 0.1 M $NaHCO_3$ solution and allowed to stand overnight at 2°C. In case the pH would drop below pH 7.8, it should be readjusted by further addition of 0.1 M $NaHCO_3$. The extraction was followed by centrifugation at 3000 g and the sediment discarded. All further procedures were performed at low temperature (0 to + 2°C).

Step 3. Fractionation by changing the pH. The pH of the supernatant obtained in step 2 was adjusted to 6.1 by the addition of 1 M acetic acid under continuous stirring. After the formation of CO_2 had stopped, the precipitate was centrifuged at 3000 g and the sediment discarded. The pH of the supernatant was adjusted under continuous stirring to 5.3 by the addition of acetic acid. (The pH was measured with a glass electrode). The precipitate was collected by centrifugation and the supernatant was discarded. The sediment was extracted under continuous stirring by adding step by step a total of 350 ml of a solution of 0.09 M NaCl in 0.02 M $NaHCO_3$. The insoluble residue was removed by centrifugation.

Step 4. Fractionation with protamine. The pH of the supernatant obtained in step 3 was adjusted to 7.2 by the addition of 0.5 M Tris-HCl buffer. Subsequently aliquots of a 2 per cent solution of protamine sulphate were added until no further precipitate was formed. It is advisible to determine in a small sample the amount of protamine sulphate required for the total volume of the enzyme containing solution. The precipitate formed after the addition of protamine was centrifuged and discarded.

Step 5. Precipitation of the enzyme by nucleic acid. The pH of the supernatant obtained in step 4 was adjusted to 5.5 by adding 1 M acetic acid. This was followed by the addition of 15 to 20 ml of a 1 per cent solution of RNA. The pH was then shifted to 5.3 by adding further acetic acid under continuous control of the pH with a glass electrode. The addition of RNA was then continued until a small sample, after centrifuging, gave no further precipitate on addition of a drop of nucleic acid solution. At this step an additional 1/10 of the added volume of RNA solution was given to the mixture in order to provide for RNA-excess. The precipitate was centrifuged at 3000 g. The supernatant was discarded, the precipitate was extracted with a total of 30 ml of a solution of 0.09 M NaCl in 0.02 M $NaHCO_3$, in small portions under continuous agitation. If the pH exceeds the value of pH 7.2, it should be readjusted by the addition of acetic acid. The insoluble portion

Table I *Summary of the purifi-*

Stage No	Vol. ml	Protein concentration mg/ml	Glutamine synthetase	
			units / ml	total units
1. Crude extract.	2560	10.65	9.05	23 250
2. Precipitation at pH 6.1	2560	4.28	9.1	23 000
3. Precipitation at pH 5.2	350	5.8	45	15 700
4. Protamine treatment	300	4.5	43.6	13 100
5. RNA precipitation	72	6.0	140	10 780
6. $(NH_4)_2SO_4$ precipitation	41	2.2	150	6 150
7. 2nd protamine treatment	41	2.0	150	6 150

of the precipitate was centrifuged and reextracted by an additional 10 ml of the above solution. The two supernatants were pooled.

Step 6. Fractionation with ammonium sulphate. To the supernatant (step 5) 0.5 M phosphate buffer, pH 7.2, (4 ml buffer per 100 ml supernatant) was added. Saturated ammonium sulphate solution was given to the above mixture to obtain 0.37 saturation (22 g/100 ml). After 30 minutes the precipitate was centrifuged off and discarded. To the supernatant further ammonium sulphate was added until a 0.57 saturation (37 g/100 ml) was reached. If the original supernatant contains less than 3 mg/ml protein, it is advisable to adjust the ammonium sulphate to 0.65 saturation. The material was then centrifuged at 2500 g. The supernatant was discarded, the sediment dissolved in 40 ml glass distilled water and dialyzed against distilled water.

Step 7. Protamine fractionation II. After dialysis the pH of the enzyme solution was about pH 6.4 and was adjusted to pH 7.2 by the addition of 0.02 M $NaHCO_3$. A few drops of a 2 per cent protamine sulphate solution were then added until precipitate was formed. The inactive precipitate was removed and discarded.

Step 8. Fractionation on DEAE cellulose column. A DEAE cellulose column, 3.2 cm in diameter and 10 cm in length, was prepared and washed with 0.01 M Tris buffer (pH 8.0). The protein was adsorbed at a slow flow rate. Elution was carried out by a NaCl gradient in 0.01 M Tris buffer (pH 8.0) and fractions of 3 ml were collected. The flow rate of the eluent was adjusted to 4 ml per min. When the NaCl content of the effluent exceeded 0.1 N in concentration, the flow rate was readjusted to 1 ml/min.

Elution of the enzyme started at 0.130 N chloride concentration. Three enzymatically active fractions were obtained in the peaks at 0.142, 0.156 and 0.186N chloride concentrations. The glutamine synthetase and gamma-glutamyl transferase activity of the individual fractions was different. The ratio of glutamine synthetase/glutamyl transferase was 7, 5, and 3 in fractions 1, 2, and 3, respectively.

cation procedure

		Glutamyl transferase				Ratio, transferase / synthetase
Specific activity	Yield	units / ml	total units	Specific act.	Yield	
0.84	100	57.4	146 800	5.45	100	6.4
2.1	98	61.0	152 000	14.5	104	6.75
7.8	71	328	115 000	57.3	75	7.3
9.7	57	330	99 000	73.0	66	7.55
23.4	45	1072	77 184	178	53	7.6
68.0	26	1112	45 592	505	30	7.42
75.0	26	1108	45 428	554	30	7.45

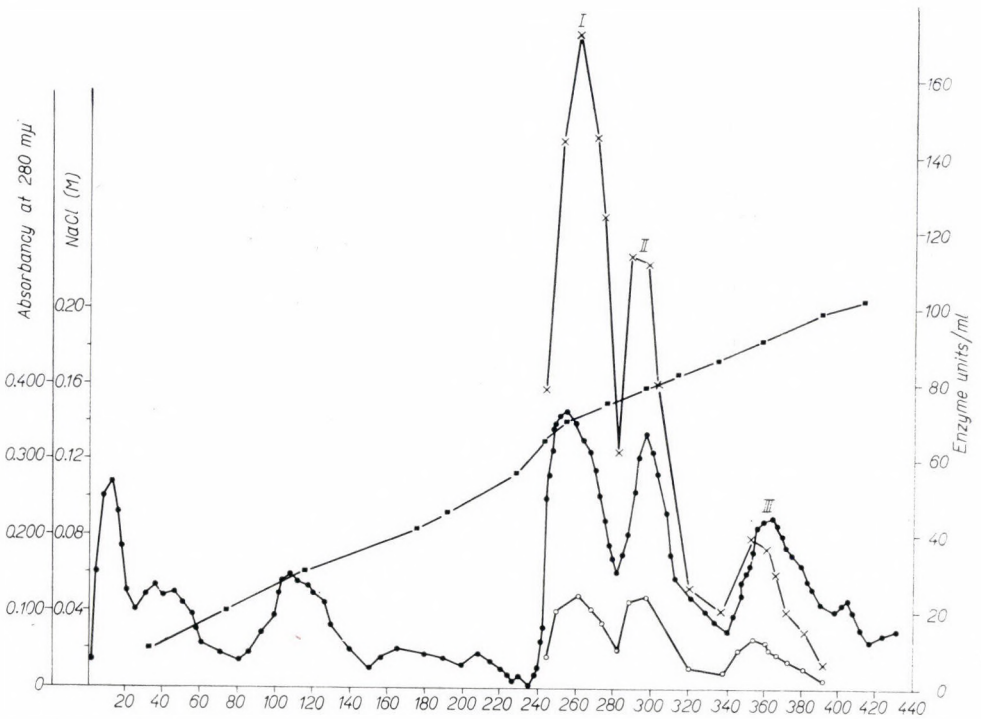

Fig. 1. Gradient elution of purified glutamine synthetase on DEAE-cellulose column.
OD at 280 mμ (protein) ●——●;
activity of glutamine synthetase in μM/ml: ○——○;
activity of glutamyl transferase in μM/ml: ×——×;
concentration of sodium chloride in M: ■——■

Remarks concerning the isolation of the enzyme. The ratio glutamyl transferase/glutamine synthetase activity was about 10 in both the acetate-treated (step 3) and RNA-treated (step 5) fractions at pH 7.2. The above ratio diminished slowly with time becoming 8 to 8.1 and 7.45 to 7.75 after 2 and 6 hours, respectively. This latter value equals approximately that observed prior to the adjustment of the pH to 5.3. Maintaining the precipitate at pH 5.3 for e.g. 6 hours prior to adjustment to pH 7.2 the ratio glutamyl transferase/glutamine synthetase turned out to be 15. The explanation of the above phenomena was found in the fact that

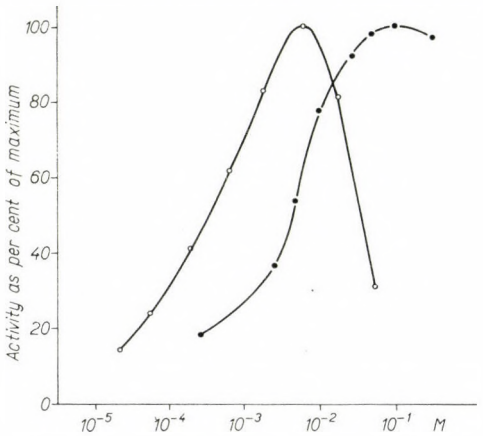

Fig. 2. The effect of L-glutamate (● ●) and of ATP (○ ○) on the activity of glutamine synthetase

at pH 5.3 the activity of glutamine synthetase diminished reversibly, while that of glutamyl transferase remained unchanged. The activity and activity ratio values given in Table I for steps 3 and 5 represent the values obtained with reactivated enzymes.

The activity of glutamine synthetase decreased markedly at pH values lower than 5.3. Thus at pH 5.1 a slow, at pH 5.0 a rapid and at pH 4.9 an immediate and irreversible inactivation of the enzyme occurred.

Table II

Specific activity of enzyme fractions obtained by DEAE-cellulose chromatography

Fractions No	NaCl conc. M	Specific activity		
		Glutamine synthetase	Glutamyl transferase	Ratio, transferase / synthetase
I	0.142	151	1110	7.35
II	0.156	209	1060	5.1
III	0.186	85	300	3.45

Another problem was encountered during the fractionation with RNA. Equal amounts of enzyme could be precipitated by both the commercial, low molecular weight RNA and by the high molecular weight RNA prepared by the sodium laurylsulphate method. The former preparation (or some contaminations in it) was, however, found to inactivate part of the glutamyl transferase activity. A further disadvantage of this preparation lay in the poor separation of fractions upon chromatography on DEAE cellulose column. These observations suggested the important role of the quality of RNA in purification steps 5 and 8.

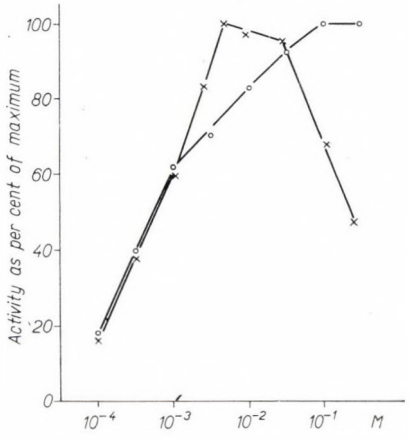

Fig. 3. The effect of hydroxylamine on the activity of glutamine synthetase (○——○) and glutamyl transferase (×——×)

As shown in Table I the activity ratio of glutamyl transferase/glutamine synthetase equals about 7, and this value exhibits but small changes during purification.

Factors influencing enzyme activity. Detailed examinations were performed only on the first enzyme fraction eluted from DEAE cellulose column at 0.142 N chloride ion concentration. The specific activity of this fraction was 151 U/mg protein and 1110 U/mg protein for glutamine synthetase and glutamyl transferase, respectively. The specific activity ratio of the two enzymes was 7. This value agrees well with that observed prior to chromatography. The optimal conditions for the measurement of enzyme activity were examined using this fraction and the reaction mixtures thus determined were used further in all stages of purification. It is possible that the other two fractions may have different optimal conditions for maximum activity.

The dependence of glutamine synthetase activity on the concentration of L-glutamic acid, ATP and hydroxylamine is shown in Figures 2, 3. The enzyme is activated by Mg, Mn and Co ions. As there is cystein present in the reaction mixture it is highly probable that the concentration of Co ions required for the activation of the enzyme is much lower than that actually measured. Cysteine had

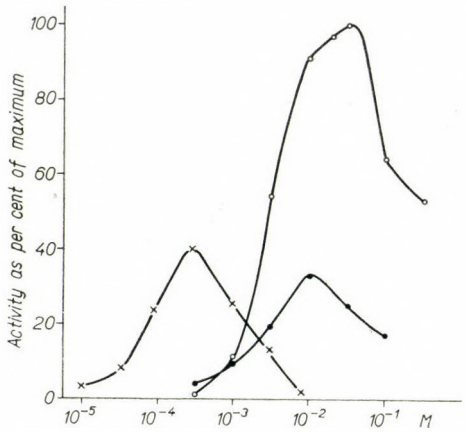

Fig. 4. Effect of Mg^{++} (○——○), Mn^{++} (×——×), Co^{++} (●——●) ions on glutamine synthetase activity

optimum activating effect in a concentration of 0.01 M. The pH optimum of glutamine synthetase activity was pH 7.4.

Gamma-glutamyl transferase activity was observable only in the presence of L-glutamine, D-glutamine was not utilized. In the presence of 0.1 M L-glutamine used in our experiments the optimum concentration for hydroxylamine was 0.003 M. The activity of glutamyl transferase was enhanced by adenine nucleotides. Nevertheless, the enzyme is active also in the absence of nucleotides. The latter activity is called "basal-activity". The most efficient activator was ADP. ATP and

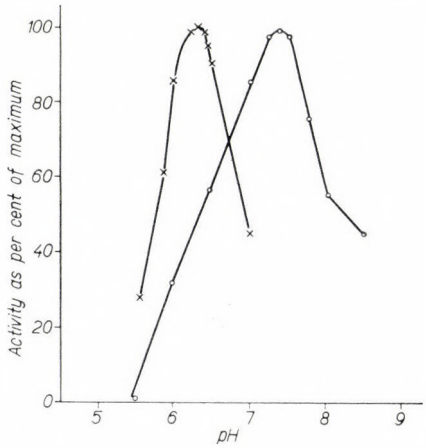

Fig. 5. The effect of pH on the activity of glutamine synthetase (○——○) and glutamyl transferase (×——×) activity

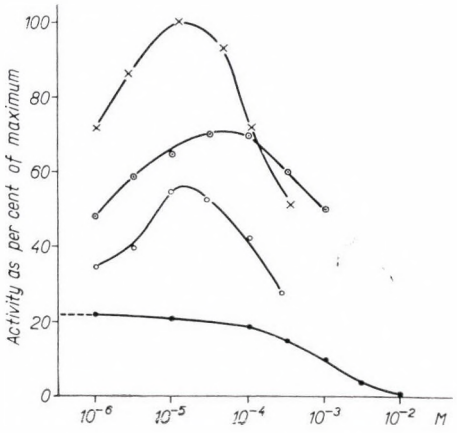

Fig. 6. Effect of ATP (⊙——⊙), ADP (×——×), AMP (○——○) and adenosine-3'-phosphate (●——●) on glutamyl transferase activity

AMP were less active. Adenosine-3'-phosphate had no activating effect, moreover in higher concentrations it even inhibited the "basal-activity". Transferase activity was activated by Mn ions. Mg and Co ions failed to produce an activating effect. The presence of phosphate ions was required for transferase activity. The optimal phosphate concentration was 0.01 M in the presence of 1.25×10^{-5} M ADP. The pH optimum of transferase activity was pH 6.3.

Discussion

The experiments described above suggest that glutamine synthetase and glutamyl transferase are not two separate enzymes. The two activities are very probably two different functions of one and the same protein molecule. By chromatography on DEAE cellulose column the enzyme could be separated into three fractions, each being eluted at well defined concentrations of chloride ions. All three fractions exhibit both enzymatic activities, the ratio of the two activities being different in each fraction, though. The question arises whether the enzyme is present in three forms also *in vivo* or the different forms are only artefacts produced during preparation. No unequivocal answer to this question can be given yet.

Further studies are being conducted in our laboratory in order to elucidate the molecular basis of the differences between the three fractions of glutamine synthetase and for their further characterization.

References

Cohn, W. E. (1957) in Colowick, S. P., Kaplan, N. O. (eds.) Methods in Enzymology, Academic Press, New York. Vol. 3. p. 867
Elliott, W. H. (1953) J. biol. Chem. *201* 661
Lipmann, F., Tuttle, C. L. (1945) J. biol. Chem. *159* 21
Lowry, O. H., Rosebrough, N. J., Farr, L. A., Randall, R. J. (1951) J. biol. Chem. *193* 265
Meister, A. (1962) in Boyer, P. D., Lardy, H., Myrback, K. (eds.) The Enzymes, Academic Press, New York. Vol. 6. p. 443
Schmidt, G. (1957) in Colowick, S. P., Kaplan, N. O. (eds.) Methods in Enzymology, Academic Press, New York. Vol. 3. p. 687
Speck, J. F. (1949) J. biol. Chem. *179* 1405

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 55–60 (1966)

Volume Changes during Muscle Activity

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(Received September 20, 1965)

Volume changes during tensionless twitches of m. sartorius and m. gastrocnemius (single stimulations) were investigated by Abbott and Baskin's method. Following the stimulation an almost completely reversible volume decrease took place, the duration of which was within 15 msec.

The problem of volume changes during muscle activity has been a much debated question (Ernst, 1963), though our findings that volume decrease occurs during activity have been generally confirmed in the literature. E. Fischer (1941) and recently Abbott and Baskin (1962), however, published some data about an initial volume increase during muscle activity. These findings seem to contradict ours. Fischer did not publish his method, nor has he continued these experiments. The experiments of Abbott and Baskin could not be fully evaluated by us, because neither calibration curves, nor original photographs were included in their work, (see Ernst, 1962). For this reason the problem was reinvestigated by us using their method.

Methods

The following important methodical viewpoints were kept in mind: 1. The muscle in action must not be in rigid connection with the stopper of the volume-welster, because measurable volume increase may occur if the muscle mechanically pulls the stopper inwards only just for 0.01 μ (Ernst and Mórocz, 1940). 2. Tensionless, i.e. free contraction, which is accompanied by volume decrease, should be investigated separately from contractions exerting tension, because the crystallization of the stretched muscle is accompanied by a greater and longer lasting volume decrease, which is superimposed on the initial volume decrease (Ernst et al. 1951; Ernst and Tigyi, 1951). 3. For recording the volume change instead of photographing the movement of the fluid meniscus contained in the capillary of the volumeter, a more appropriate method, i.e. the use of a piezoelectric transducer (Ernst et al. 1951; Ernst et al., 1954) should be used. Keeping these facts in mind, did we repeat these experiments by Abbott and Baskin's method, without those details which could doubtless cause error (e.g. rigid connection of the muscle with the stopper, stretching of the muscle, etc.).

Frog's sartorius and sometimes gastrocnemius were used in our experiments. The gastrocnemius was suspended in the volumeter immediately after dissection, the sartorius after keeping it in normal Ringer solution for 20 to 30 minutes, both in tensionless state. The volume change, i.e. the movement of the meniscus due to activity was recorded as the change of capacity.

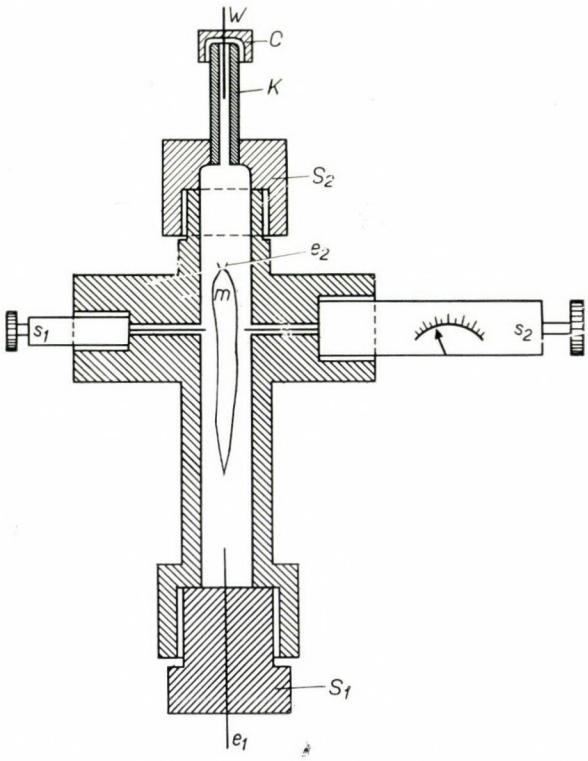


Fig. 1. Half schematic diagram of the volumeter

The volumeter is made of plexiglass and has a volume of about 5 ml. At the bottom (Fig. 1) it is fitted with a screw-plug (S_1) which holds one of the stimulating electrodes (e_1), while the other (e_2) is the hook for suspending the muscle (m). Both electrodes are of platinum. There are levelling screws (s_1, s_2) on both sides of the volumeter for bringing the meniscus to the desired level, roughly and precisely, respectively. At the top, the volumeter is closed with a screw-plug (S_2) made of plexiglass and containing a glass capillary (K) (∅0.8 mm).

The volumeter was filled up with normal Ringer solution after the muscle had been placed in, the plexiglass cover containing the capillary was screwed on, and cedar oil was spread on the top of the overflowing Ringer solution. The Ringer solution represented one plate of the condenser, the other plate was formed

by a platinum wire, which was conducted into the capillary. The capillary was covered up with a plastic cap (c) bored through in several places to equalize the pressure. This cap contained the wire, thus keeping it in a central position. The level of the meniscus was adjusted to a given distance from the end of the wire by s_1 and s_2. In such a way the capacity change is a measure of the movement of the meniscus.

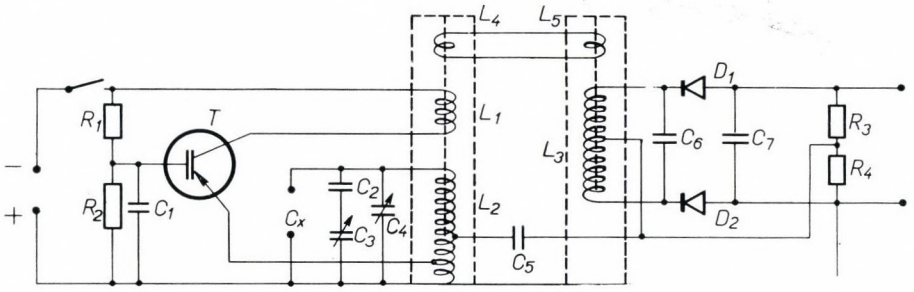


Fig. 2. The circuit of the capacity volumeter

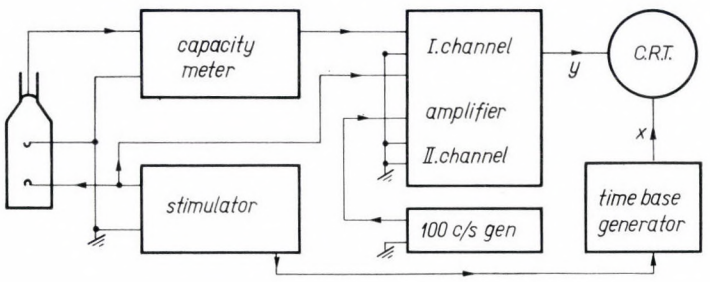


Fig. 3. Block diagram of the capacity volumeter

This condenser was connected parallel (Fig. 2) to the circuit of an oscillator of 7.10^5 csec^{-1} to bring about frequency modulation. The secondary circuit of the phase discriminator was connected to the oscillator circuit as to a primary circuit, in the usual way. When the self-frequencies of the circuits were equal, zero voltage appeared at the output after rectifying. This served as the base line on the screen of the oscilloscope (and in the figures). When the frequency of the primary circuit changed in consequence of the capacity change, d.c. voltage appeared at the output according to the increase or decrease of the frequency change. This d.c. voltage was led to the pair of deflecting plates of the cathode ray tube, after appropriate amplifying.

The amplifier of the oscilloscope was a direct current differential amplifier (Fig. 3). The voltage from the phase discriminator was connected to one of the grids of the differential amplifier and the earth, the other voltage, which was pro-

portional to the amplitude of the stimulation signal was connected to the other grid and the earth. In this way the two signals were superimposed on each other. A square wave generator of 100 c/s was used for time mark, and its oscillations were connected to the other channel of the oscilloscope. The muscle was stimulated with single stimuli through the Ringer solution, and the stimulation was carried out by square pulses, which lasted 0.5 msec each. The voltage of the stimulus was varied from 6 V to 100 V. The change due to the effect of stimulation, appeared on the screen of the oscilloscope, and was recorded by a camera. The beginning of the exposition produced the stimulation with a delay of 0.5 msec.

The movement of the meniscus was also recorded at the same time, with different methods: 1. Recording, after a 150fold magnification, on light-sensitive paper by photokimograph. 2. Photographing the movement of the meniscus on dia-film. The pictures were taken before, during, and immediately after the stimulation, with an exposition time of 2, 5, 10 and 20 msec. Here also the beginning of the exposition started the stimulation. 3. Recording themo vement of the meniscus by a 16 mm camera. The speed of the film in such experiments was 64 frames/sec.

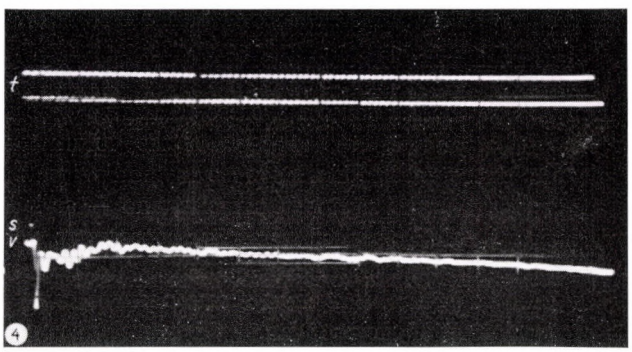
Experimental Results

1. 518 stimulations were performed with the described method. Volume decrease occurred after stimulation in 516 cases, volume increase could be observed only twice at the two first stimulations in one experiment. An oscilloscopical photograph is shown in Fig. 4. The upper line (t) — the 10 msec square pulses — is the time mark. The volume change (v) is indicated by the other ray of the oscilloscope (the lower line in the figure), and the stimulation signal (s) can be observed also at the point above the lower line. The deviation from the base line represents volume changes: volume increase upwards and volume decrease downwards, respectively. The volume decrease following the stimulation is well visible in the figure. (The damped oscillations following the volume decrease are presumably due to the liquid movement brought about by the muscle contraction.)

2. This volume decrease is reversible, and it seems that the recovery of the volume is exactly as rapid as the volume decrease. Thus a longer lasting volume change is out of question under the condition of these experiments. This can be judged by the course of the volume change: the slopes of the ascending, and descending parts of the curve are nearly equal.

3. These photographs enabled us to calculate the duration of the volume decrease: the time generator gave signals in every 10 msec, and the volume decrease took place during a single interval. Considering the speed of the film (64 frames/sec) in the experiments with moving films, the period of each shot was about 15 msec. Fig. 5 shows 3 film frames from such an experiment; the distance between the platinum wire and the meniscus is the greatest in the middle frame, the two outer frames show smaller distances. Thus the drop of the meniscus level, i.e.

the volume decrease was recorded on a single film frame. From this we can conclude that the period of the volume decrease does not exceed 15 msec.



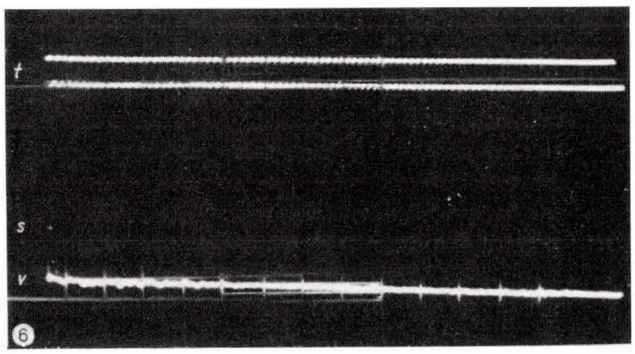


Fig. 4. Volume decrease of m. sartorius for a single stimulus
Fig. 5. Volume decrease of m. sartorius for a single stimulus as recorded on moving film
Fig. 6. Investigation of artefact during the stimulation of m. sartorius (see text)

In order to answer the question of how much of the volume decrease is an artefact, photographs were taken of muscles stimulated to death, at the end of each experiment. One of these photographs is shown in Fig. 6. There is no trace of any kind of volume change due to electric stimulation.

Discussion

The experiments carried out by using the above method, unequivocally show that volume decrease takes place in the muscle after stimulation and that it is almost quite reversible. This is in good agreement with our earlier results. We did not find, however, the initial volume increase described by Abbott and Baskin. According to them, this volume increase appeared 3 msec after the stimulation and was followed by a definitely longer lasting volume decrease. In these experiments the muscle was in a rigid connection with the stopper of the volumeter, and this could have been the cause of the volume increase (see methodical part). Furthermore, their experiments were done with stretched muscles, and — as it is known — in this case the volume change is influenced by other processes as well (e.g. crystallization).

In another article Baskin (1962) suggests, that the volume changes can be attributed to the molecular reorganization of the proteins of the muscle (see e.g. Ernst, 1963; Marsh, 1952).

Addendum: An abstract, recently published (Paolini and Baskin, 1965), shortly mentions a new method which "... containing a sensitive pressure transducer" seems to conform with the principles developed in our institute.

Acknowledgement

Authors express their thanks to Professor E. Ernst for encouragement and help in this work.

References

Abbot, B. C., Baskin, R. J. (1962) J. Physiol. London *161* 379
Baskin, R. J. (1962) Nature *195* 290
Ernst, E. (1962) Rev. Canad. Biol. *21* 249
Ernst. E. (1963) Biophysics of the Striated Muscle. Akadémiai Kiadó, Budapest
Ernst, E., Balog, J., Tigyi, J., Sebes, A. (1951) Acta Physiol. Acad. Sci. Hung. *2* 253
Ernst, E., Mórocz, E. (1940) Enzymologia *9* 139
Ernst, E., Tigyi, J. (1951) Acta Physiol. Acad. Sci. Hung. *2* 261
Ernst, E., Tigyi, J., László, M. (1954) Acta Physiol. Acad. Sci Hung. *6* 171
Ernst, E., Tigyi, J., Örkényi, J. (1951) Acta Physiol. Acad. Sci. Hung. *2* 281
Fischer, E. (1941) Biol. Symp. Muscle. Ed. J. Cattel *3* 211
Marsh, B. B. (1952) Biochim. Biophys. Acta *9* 247
Paolini, P. J. Jr., Baskin, R. J. (1965) Abstracts Biophys. Soc. p. 126

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 61–68 (1966)

Effect of Potassium on Automaticity of Frog Heart

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(Received November 30, 1965)

The role played by potassium in so many biological functions is a problem to be investigated in the field of quantum mechanics and electron biology. The more special question of "potassium and excitation" was investigated on frog hearts and pieces of the sinus venosus. Both were functioning according to their spontaneous automaticity in Ringer solution, but they *a)* stopped reversibly when they were treated with a Ringer solution lacking K (and had lost a certain part of their K-content) and *b)* afterwards restarted beating when treated with normal Ringer solution or even with one lacking Ca. Accordingly, the essential role of potassium in the automaticity of frog heart seems to have been demonstrated.

Since Ringer described (1882—83) the indispensability of some anorganic cations in the normal beating of the heart, and Howell and Duke called attention (1908) to the role played by potassium in the activity of the heart, many papers have dealt with the importance of potassium for several biological functions (Ernst, 1963). Thus the author and his co-workers (Ernst and Scheffer, 1928; Ernst and Csúcs, 1929) described that muscular activity was accompanied by a decrease in the K- and an increase in the Na-content of the muscles. Later, in consequence of the so-called Hodgkin's theory many data were published about these questions; recently, however, the role of K, as compared with those of Na and Ca (see e.g. Gergely, 1963) seems to have lost its importance. Therefore the author, having repeatedly emphasized the role of potassium in excitation, describes in the following some of the experiments performed over the past years on the role of K in the automaticity of the frog heart (Ernst, 1965).

Methods

The frog heart was treated with Ringer-solution first lacking potassium and then containing it. The hearts were prepared and tied to Straub cannules which contained a Ringer solution lacking K (6.8 g of NaCl, 0.2 g of CaCl, 0.2 g of $NaHCO_3$ in 1000 ml of water). For a certain time this solution seemed quite appropriate for the heart, but after being renewed 15—20 times every half hour with a new portion of the identical solution the hearts stopped beating. When the hearts failed to stop after a certain number of renewals of the K-less solution, they were

put in a cold room (2° C) and then, on the next day, their treatment was continued, until they stopped beating.

As described by Zwaardemaker (1921), the frog heart which stopped beating in a K-less solution, remains irritable by a very weak stimulus, even by a very little mechanical shock. Therefore, the hearts, having stopped beating, were used for further investigation only if they responded with a single contraction to a single stimulus; but if after one single stimulus they kept on beating, the treatment with K-less solution had to be continued.

The hearts in this state were continuously studied in three different ways as follows.

1. *Stimulation — Irritability**

Electrical stimulation of the heart (h) was performed with the equipment shown in Figure 1; the heart could be stimulated either with direct current (d.c) or with the other stimulator (s) depending on whether K_1 was switched to the left

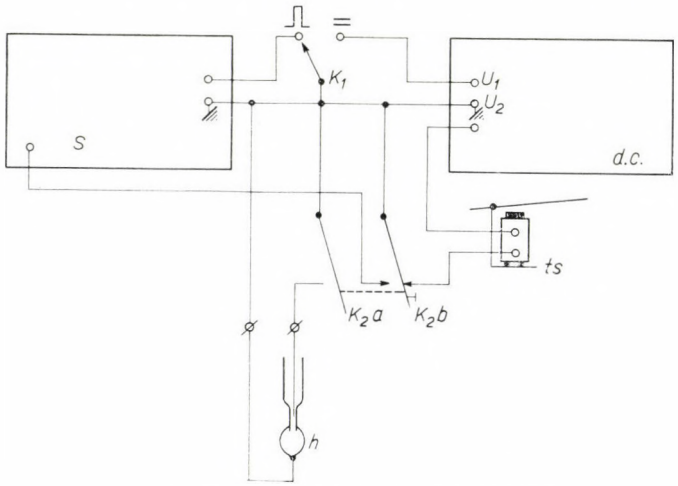

Fig. 1. Equipment for electrical stimulation

or the right. The stimulator could deliver electric shocks of different frequencies or voltages or intensities. Simultaneously with the stimulation, started with the switch K_2, the time of stimulation could be recorded by breaking the current of a special time-signal (ts). When for stimulation K_2 was switched to the left, first the circuit of the stimulating current was closed (by K_{2a}) and only hereafter (K_{2b}) was the stimulating impulse started.

*Co-workers: J. Örkényi, I. Papp.

2. *Restarting of automaticity**

Each experiment was carried out simultaneously on 6—20 hearts; some such series of hearts, the Straub cannules of which contained K-less solution and therefore had stopped beating, were dried at about 110°C and then incinerated at about 400°C in an electric stove. The ashes were dissolved and their K-Na-Ca contents determined by flame photometry.

Some other series of hearts,** having stopped beating due to the K-less solution, were used for further experimental investigations. First of all the question was to be settled, whether the hearts could be induced to restart beating by replacing the K-less solution by a normal Ringer solution. The earlier experimental results of Zwaardemaker, and the recent ones of Hoitink (Hoitink, Westhoff, 1956), and especially those of our own institute made it clear that the restarting of the automaticity found in some single hearts could lead to quite an unfounded conclusion. Namely that restarting would be attributable to the replacing of the K-less solution by normal Ringer solution. This is only one possibility, but the arrested heart can — as described above — restart beating also in response to any very weak stimulus, e.g. a mechanical effect caused by emptying and refilling the Straub cannule. Indeed, arrested hearts could repeatedly be observed to start beating when the K-less solution was replaced by a new portion of the same solution.

In view of this fact, the question to be investigated has been answered by adopting a statistical method. Some series of hearts arrested by a K-less solution were treated in the following way: Each heart with an even number e.g. the 2, 4, 6, 8 and 10 was given normal Ringer solution and each one with an odd number, i.e. the 1, 3, 5, 7 and 9, a K-less solution. Then they were observed say at intervals of 3 hours and it was recorded whether they were beating or not, e.g. as follows:

heart No	1	2	3	4	5	6	7	8	9	10
+ or —	+	+	—	+	—	—	—	+	—	+

Similar experiments were performed by replacing the K-less solution with a Ringer solution lacking $CaCl_2$.

Every series of hearts was observed 6—10 times and the results were evaluated by the chi-square (χ^2) test.

* Co-workers: E. Fór, A. Kollár.

** Some hearts which stopped beating were examined for irritability, as described above.

3. *Stopping — Restarting experiments on pieces of the sinus venosus**

The experiments in which the hearts stopped beating after being deprived of part of their potassium content and were made restart beating by replacing the K-less solution by one containing potassium, were performed — mutatis mutandis — on pieces of the sinus venosus as well. In general the sinus was cut into 4—5 pieces in a normal Ringer solution; the sinus-pieces were put in a normal Ringer solution and the number of their contractions per minute was recorded. Then the sinus pieces were put in a K-less solution (described above) in which, after a certain time, they produced fewer and fewer contractions and in 0.5—1.5 hours stopped contracting. Hereupon they were put in another vessel containing identical K-less solution, lest the restarting of contractions should be ascribed to the mechanical effect caused by moving the pieces.** If the sinus pieces did not restart to contract in the second vessel containing K-less solution, they were put in a vessel containing normal Ringer solution, where after a certain time, they restarted to contract. Similar experiments were performed in which the sinus pieces restarted to contract in the last solution containing no $CaCl_2$. The numeric evaluation of the results was the same as described above.

4. *General remarks*

The hearts tied to the Straub cannule containing normal Ringer solution remained in a good condition for 8—10 days if they were put in a cold room and brought to the laboratory only for the time of the treatments. The Ringer solution must not contain sugar or other "nutrient", nor be oxygenized. The sinus pieces (without oxygenization) continued contracting at room-temperature in normal Ringer solution for a day.

Results

1. First of all the question should be answered how the content of K, Na, Ca was changed by the long lasting treatment of the hearts with K-less solution.

Table 1

K, Na and Ca contents of the hearts mg/g dry substance (average)

	K	Na	Ca
nR	13.5	20.0	0.98
K-less	5.0	20.0	0.85

* Co-workers: E. Fór and M. Hajnal-Papp.

** Some sinus pieces which stopped beating after being for a certain period in normal Ringer solution, sometimes were restarted to contract by a mechanical effect.

Table 1 shows that the Na content increased to a very large extent, the K content decreased by its 1/2—2/3, while the Ca content did not change considerably.

2. In order to answer the question whether or not the regainment of automaticity of the hearts which had stopped beating due to the K-less solution is to be ascribed to replacing the K-less solution by normal Ringer solution, 7 series of hearts were investigated with the method described in the 2nd section of "Methods". The results are shown in Table 2.

Table 2

Effect of K-*less solution on the heart activity*
Number of hearts
beating (+), not beating (−)

No. of series	n Ringer		K-less solution	
	+	−	+	−
1.	36	13	13	36
2.	34	8	13	29
3.	33	16	11	38
4.	28	14	10	32
5.	24	18	9	33
6.	69	31	28	62
7.	28	0	13	15
Sum	252	100	97	245

According to the ratio between the number of the beating (N_+) and of all (N) hearts

$$\frac{N_+}{N} = \frac{349}{694}$$

the N_+ of the nR hearts is expected to be

$$N_+(nR) = 352\,\frac{349}{694} \sim 177$$

against the actually observed number of 252. According to the other numbers computed in this manner it follows that

$$\chi^2 = \frac{(252 - 177)^2}{177} + \frac{(100 - 175)^2}{175} + \frac{(97 - 172)^2}{172} + \frac{(245 - 170)^2}{170} \sim 130$$

as against the value (in the χ^2-table for $n = 1$ and $P = 0.001$) of 10.83. On the basis of this result, the fact that many hearts which stopped beating due to the

K-less solution, restarted beating, is attributable to the normal Ringer solution by which the K-less solution had been replaced. Since this latter solution differed from the first one only in containing K, the inference that K is an essential factor in the automaticity of the heart seems justified.

One heart of each series was used for recording its mechanical activity as demonstrated in Figure 2. The heart stopped beating due to the K-less solution, as can be seen on the upper line. Electric shock, shown by the lower line, caused one single contraction as seen on the upper line. After replacing the K-less solution by normal Ringer solution (nR), the heart restarted beating.

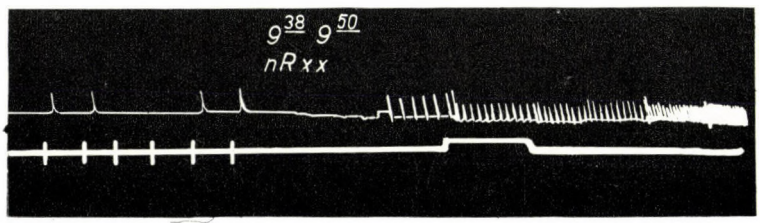

Fig. 2. Heart stopped beating due to a K-less Ringer solution, then stimulated to single contractions and then restored to automaticity by nR

3. Against this statement the objection can be raised that the heart which stopped beating due to the K-less solution may have lost also some Ca besides a large amount of K (see Table 1). Thus, the restored automaticity could perhaps be ascribed to the Ca content of the normal Ringer solution. In opposition to this objection first of all the fact may be mentioned that also the K-less solution contained as much Ca as the normal Ringer solution. In addition, separate experiments were performed to ascertain the decisive role of K in the cessation of automaticity: when K is absent in the solution, and in the restoring of automaticity: when the solution contains potassium. In these experiments the K-less solution

Table 3

Effet of $CaCl_2$-free solution on the heart activity
Number of hearts
beating (+), not beating (−)

No. of series	nR − $CaCl_2$		K-less solution	
	+	−	+	−
1.	24	21	10	50
2.	35	73	24	84
3.	131	61	27	162
4.	20	0	5	15
5.	26	4	5	25
Sum	236	159	71	336

was replaced by a Ringer solution without $CaCl_2$; Table 3 contains the numeric data of these experiments. The evaluation of these data by the chi-square test ($\chi^2 \sim 150$ against the value of 10.83) indicates that the restoring of automaticity is due to the fact that the K-less solution was replaced by one containing potassium and lacking Ca.

Fig. 3 shows an experiment in which mechanical activity was recorded; details are similar to those described in Fig. 2.

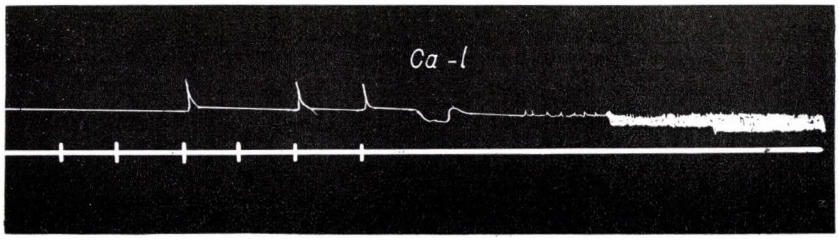

Fig. 3. The same as Figure 2, but the automaticity was restored by a Ca-less Ringer solution

4. Since the sinus venosus of the frog heart is known to start the cardiac impulse, it seemed necessary to investigate, whether the effect of potassium on the spontaneous automaticity of the sinus venosus was similar to what has been described above for the whole heart. *a)* From the sinus venosus of 19 hearts 75 pieces were excised and put in normal Ringer solution, where they contracted rhythmically. Then they were put in the K-less solution I, where they stopped contracting, afterwards they were put in the K-less solution II* where 71 remained actionless and 4 exerted contraction. Finally they were put in normal Ringer solution, where 69 contracted again and 6 did not.

b) In another series of experiments pieces of the sinus venosus of 18 hearts were treated in the same manner, except for the final phase, when they were put in a "Ringer solution" lacking $CaCl_2$. The numeric results were as follows: all 75 pieces contracted at the beginning in the normal Ringer solution, stopped contracting in the K-less solutions I and II, 61 pieces contracted again in the Ca-less solution, and 14 did not.

Mathematical evaluation indicates that potassium plays the leading role in these experiments, inasmuch as the pieces do or do not contract, depending on whether potassium is or is not contained in the bathing solution.

Discussion

The role played by potassium in biological functions is known to be a very complex problem, the different aspects of which can be treated in the future from a common point of view of quantum mechanics or electron biology. As an ex-

* see "Methods"

ample the fact may be mentioned that KN_bO_3 shows ferroelectricity changing to antiferroelectricity when Na takes the place of K, and electrostrictive effects occur. Both these phenomena, the exchange of Na for K and the volume diminution, together with the important role of K in excitation make up an essential part of the author's scientific work, nevertheless this question will not be discussed this time.

Two questions, however, should be mentioned in connection with the results described in this paper

1. How is it to be explained that the hearts stopped beating when they still contained 1/2—1/3 of their original K-content (e.g. 0.1—0.2 mg) but they restarted beating due to the new solution containing only 0.1 mgK/ml?

2. Why was it that the hearts stopped beating i.e. no excitation arose in them, though they contained much Na and still enough K to be exchanged?

References

Ernst, E. (1963) "Biophysics of the Striated Muscle". Academic Press, Budapest
Ernst, E., Scheffer, L. (1928) Arch. ges. Physiol. *220* 655
Ernst, E., Csúcs, L. (1929) Arch. ges. Physiol. *223* 663
Ernst, E. (1965) in press
Gergely, J. (1963) "Biochemistry of Muscle Contraction". Little, Brown and Comp., Boston
Howell, J., Duke, K. (1908) Amer. J. Physiol. *21* 51
Ringer, S. (1882—3) J. Physiol. *4* 22; *5* 247
Zwaardemaker, H. (1925) Erg. Physiol. *19* 326

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 69—72 (1966)

Potassium and Acetylcholine as Antagonists

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(Received November 30, 1965)

According to the preceding paper under certain conditions potassium plays a stimulating role in the automatic activity of the frog heart. Acetylcholine, on the other hand, is known to inhibit the cardiac automaticity. In the light of this antagonism it seemed worthwhile to investigate the effective concentration of acetylcholine in relation to the amount of potassium. The concentration of acetylcholine was determined by which the automatic activity of the frog heart could be inhibited in a normal or a potassium-less Ringer solution. In the latter the hearts were much more sensitive to acetylcholine than in normal Ringer solution.

In an old textbook of Pharmacology (Meyer—Gottlieb, 1910—1921) one could read as early as half a century ago (p. 301) that "Kalisalze die normale Reizerzeugung im Sinus anregen" (K-salts irritate the pacemaker in the sinus), and (p. 271) that acetylcholine exerted an inhibitory effect similar to that caused by stimulation of the nervus vagus. In the previous paper it has been described that potassium plays an essential role in the automaticity of the frog heart. Thus it seemed worthwile to investigate these antagonistic effects of potassium and acetylcholine on the activity of whole hearts and on pieces of the sinus venosus.

Methods

1. Two series of 5—10 frog hearts were prepared and tied to Straub cannules. These were filled with normal Ringer solution (6.6 g NaCl, 0.2 g each of KCl, $CaCl_2$ and $NaHCO_3$ in 1000 ml of water) in one of the series, and with K-less "Ringer solution" in the other. The hearts of both series continued to beat for many hours independently of whether their solutions remained unchanged or were renewed 10 times every half hour with the identical solution as originally used. After this preparatory treatment the solution of both series was replaced by the identical solution containing a certain amount of acetylcholine.

This was arranged in such a way that two series of normal and K-less Ringer solutions were prepared, and in each series the individual vessels contained different amounts of acetylcholine, as follows:

No. of vessels	1	2	3	4	5	6	7	8
g/ml acetylch. in nR or K-less solutions	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}

The normal or K-less Ringer solutions in the Straub cannules were replaced by the solution No. 1 containing acetylcholine at the concentration of 10^{-9}. Both series of hearts were observed for half an hour and it was noted which of them stopped beating. Hereafter solution No. 1 was replaced in both series by solution

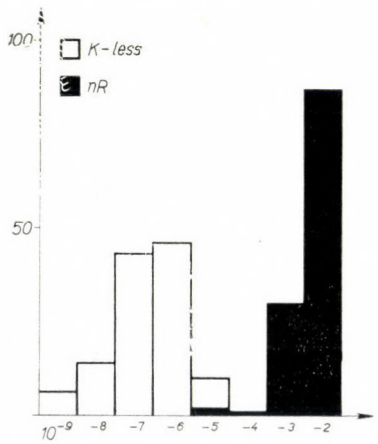

Fig. 1
Abscissa = concentration of acetylcholine;
ordinate = number of hearts that stopped beating

No. 2 and the number of the hearts which had stopped beating was noted again. This procedure was continued either until all the hearts had stopped beating, or did not stop beating even after having been treated with solution No. 8 containing acetylcholine at a concentration of 10^{-2}.

2. Two series of pieces of sinus venosus were also investigated to find out whether there was a difference between the concentrations of acetylcholine at which the pieces stopped contracting depending on whether they were put in normal or K-less Ringer solution. The sinus venosus was cut into 4—5 pieces, all beating in normal Ringer solution, then half of them was put in a vessel containing normal, the other half in another vessel containing K-less Ringer solution, and both contained acetylcholine at the concentration of 10^{-8}. In every five minute the pieces were observed (and the number of contractions/min. counted) whether or not they had stopped contracting.

Results

Fig. 1 shows the results obtained with 120—120 hearts; the numbers under the abscissa stand for the concentrations of acetylcholine, the quadrangles for the number of hearts which stopped beating. It can be seen that the hearts treated with a K-less Ringer solution generally stopped beating at a much lower concentration of acetylcholine than the hearts treated with normal Ringer solution.

On the basis of the data of Fig. 1 it seemed self-evident that the differences in the sensibility of the pieces of sinus venosus will be demonstrable by putting them in normal or K-less solutions containing acetylcholine at a concentration of 10^{-7} or 10^{-6}. But it turned out that at these concentrations of acetylcholine every piece stopped beating within the first five minutes. Hence the acetylcholine was used at the lower concentration of 10^{-8}. The numeric results are as follows: the percentage of the pieces that had stopped contracting in the first 5 minutes after having been put:

in normal Ringer solution (acetylcholine 10^{-8})	10 per cent
in K-less Ringer solution	75 per cent
In the first 40 minutes	
in nR solution	30 per cent
in K-less solution	100 per cent

More than 50 per cent of the pieces in normal Ringer solution (acetylcholine: 10^{-8}) did not stop contracting even after 3 hours. The overwhelming majority of the pieces that had stopped contracting in solutions containing acetylcholine restarted contracting after having been put in normal or K-less Ringer solutions which did not contain acetylcholine.

These facts seem to indicate that the hearts or pieces of the sinus venosus treated with a K-less solution were more sensible to acetylcholine than those treated with normal Ringer solution.

Discussion

Potassium has been shown in the preceding paper (Ernst, 1966) to play an essential role in the spontaneous automaticity of the hearts or of the pieces of the sinus venosus of the frog. This statement was based on the experimental fact that these systems stopped or restarted functioning rhythmically depending on whether they were treated with a K-less or a normal Ringer solution. (In both cases the Na- and Ca-contents were the same.) Furthermore, the hearts still contained 0.1—0.2 mg potassium i. e. one half or one third of their original K-content, when they stopped beating due to the K-less solution and restarted beating due to the replacing of the former by a Ringer solution containing 0.1 mg K/ml. Hence the inference that there is some functional difference between the original K-content of the heart and that given from outside with the Ringer solution, seems to be well-founded.

As a continuation of these considerations, the antagonism between the effects of potassium and acetylcholine, shown in this paper, lends itself to further comments. Acetylcholine is known to inhibit the functioning of the heart; this effect is similar to that of the lack of potassium in the milieu, as described above. Furthermore, this lack of K promotes the effect of acetylcholine inasmuch as it becomes effective at a much lower concentration. Now, since the effect of acetylcholine is generally dealt with from a neurological point of view (Eccles, 1964), the phenomena caused by the lack or presence of potassium in the milieu, were brought in connection with the function of the nervous system.

References

Eccles, J. (1964) The Physiology of Synapses. Springer-Verlag, Berlin

Ernst, J. (1966) Acta Biochim. Biophys. Hung. (in press)

Meyer, H. H., Gottlieb, R. (1921) Die experimentelle Pharmakologie. V. Aufl. Urban et Schwarzenberg, Berlin—Wien

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 73–80 (1966)

Crystallization of the Myosin (Actomyosin) in Stretched Fibrils

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(Received September 20, 1965)

The crystallization of muscle proteins was investigated by different optical methods. It was demonstrated that the solubility of the proteins strongly decreases by stretch. This supports our view concerning the crystallization of the proteins induced by stretch.

The crystallization of fibrous polymers induced by stretch and generally accompanied by a decrease of solubility is a well known phenomenon. Investigations on the crystallization of the polymer system in the muscle have been performed in our Institute for a long time (Ernst, 1963). In the present work the decrease of solubility produced by the crystallization has been investigated on single isolated myofibrils.

Materials and Methods

We used the indirect flight muscle of the bee, from which we prepared teared preparations on slides by means of needles, as described by Ernst and co-workers (Ernst et al., 1956). These preparations were checked by different methods before and after treatment with Weber-Edsall's solution. The treatment was performed under the microscope by perfusion. We chose areas in which there were unstretched, slightly and extremely stretched fibrils side by side.

One part of the work consisted in taking photomicrographs by the Baker's interference microscope before and after the extraction. The photographs were evaluated by a Zeiss Schnellphotometer. The density of the negative was determined in distance of 0.1 mm and from these values photometric curves were constructed. The second part of the micrographs was evaluated by a Kipp automatic photometer.

In other experiments we determined the dry-mass content of the stretched and unstretched fibrils before and after treatment, by means of a Sénarmont-compensator eye-piece attached to the interference microscope.

After the treatment with a salt solution of high ionic strength the material was observed in the electron microscope, too. After the treatment with the protein extracting solution the residue was fixed with dilute formol and subsequently a formvar layer was prepared on the slides. By removing the latter from the slide and mounting it on grids we obtained the replica or the pseudo-replica of the

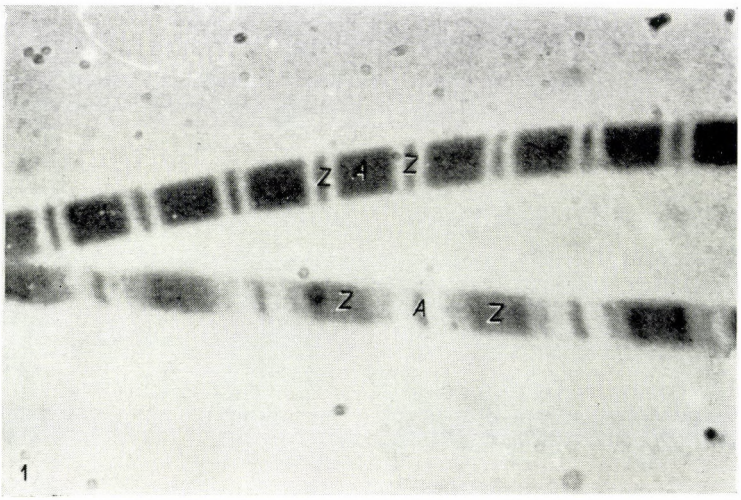

Fig. 1. An extremely (lower) and a slightly (upper) stretched fibril in native state. Interference microscope, 10 × 100. On the lower fibril: Z = anisotrope; A = Z line

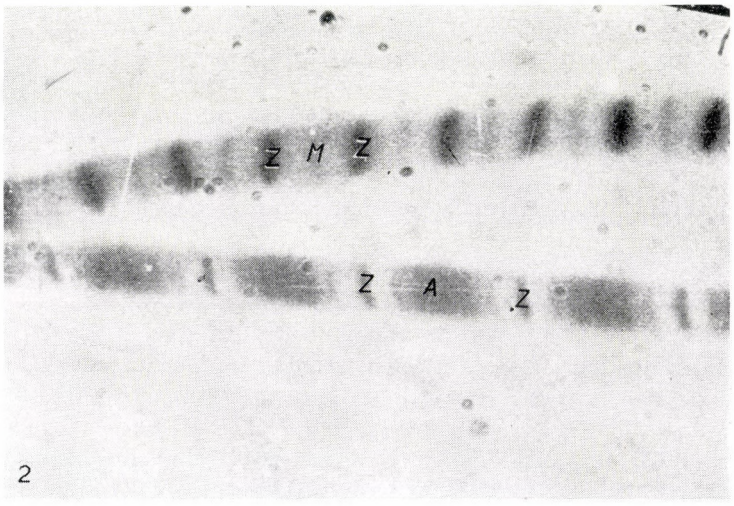

Fig. 2. The fibrils shown in Fig. 1, after their treatment with the Weber-Edsall's solution. Interference microscope 10 × 100

specimen, suitable for electron microscopic observation (Garamvölgyi et al., 1964).

The same problem was also studied by the polarizing-microscope. The experimental procedure was similar to that of the interference microscopy. The areas of the specimen containing stretched and unstretched fibrils, were photographed in a polarizing, as well as in a phase contrast microscope.

Results

Our results are shown on micrographs, on densitometric curves taken from the latter, as well as by interference microscopic measurements.

The first photomicrographs show different behavior of highly stretched and unstretched, (or slightly stretched) fibrils affected by the Weber-Edsall's solution.

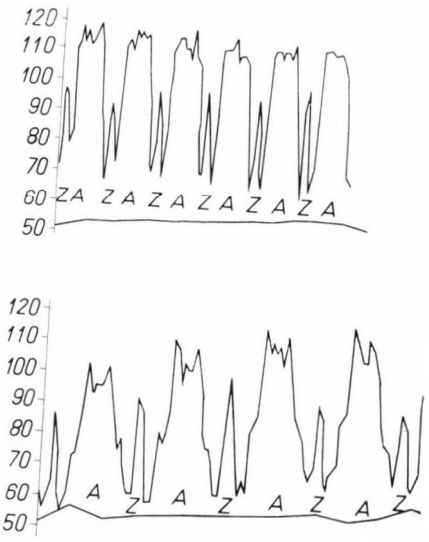

Fig. 3. Densitometric record of the fibrils shown in Fig. 1 in an extremely (bottom), and slightly (top) stretched state, respectively

On Fig. 1 two fibrils are visible, a highly stretched (S = 7.5 μ) and a slightly elongated (S = 4.3 μ) one, both in their native state. The content and the distribution of the substances, which may be estimated by the optical density of the fibril, show the usual pattern. After the treatment the relations are quite different. In Fig. 2 a strong decrease of the density of the A-band may be observed in the slightly stretched fibril. The density became almost equal with that of the ground reference field, while the densities of the Z- and M-lines persisted, that of the Z-line even slightly increased.

The negatives of Figs 1 and 2 have been evaluated also by densitometry, the result of which may be seen in Figs 3 and 4.

Fig. 3 shows the state of the fibrils before the extraction. The curve on the top of the picture represents the slightly stretched fibril, that on the bottom the extremely stretched one. The curves taken from the state after the extraction are shown in Fig. 4. The curve of the slightly stretched fibril (top) shows substantial changes as compared to the untreated state. The density of the A-band strongly

decreased, that of the Z-line increased, indicating that in the course of the extraction substances migrated to the Z-line, which have not yet been entirely dissolved (Tigyi-Sebes, 1966). On the curve of the stretched fibril (bottom) only a moderate decrease of the density of the A-band may be observed.

Our experiments performed by the compensation method also support our observations mentioned above. The average result of 37 measurements is presented in Table 1.

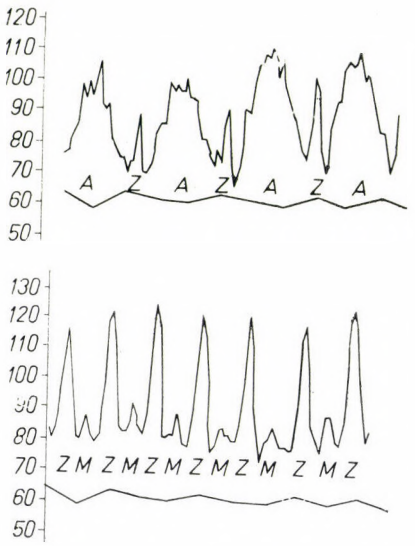

Fig. 4. Densitometric record of the fibrils shown in Fig. 2. Similar to Fig. 3, but showing the state after the extraction

The amount of the substances which survived in the fibrils stretched to a different extent and subjected to extraction, can be demonstrated also on electronmicrographs, taken from pseudoreplicas. The striation pattern of the extremely

Table 1

Optical density of the A-band of fibrils, before and after treatment
The values represent the differences of the two readings of the analyzer position (reference area and specimen) expressed in degrees

	Slightly stretched	Highly stretched
Before treatment	17 ± 2	16 ± 2
After treatment	0	10 ± 2

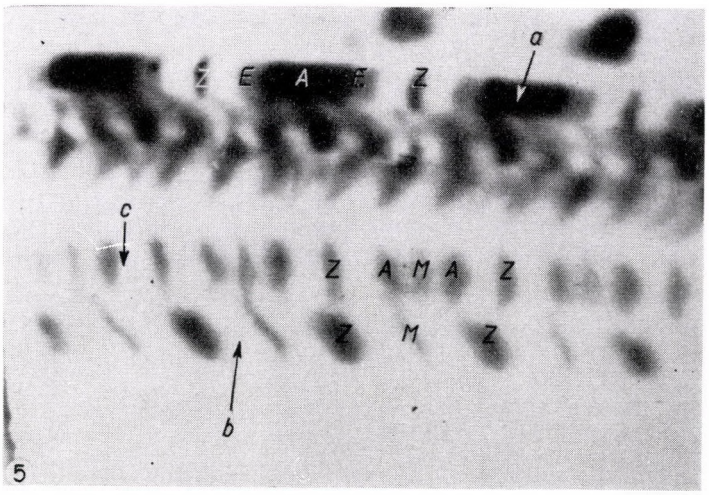

Fig. 5. Pseudoreplica of parallelly oriented fibrils in extremely (a), slightly (b), intermediately (c) stretched states and extracted with the Weber-Edsall's solution. (Electronmicrograph, magnification: 5000 ×)

stretched fibril (a) persisted, while from the slightly stretched one (b) only the thick Z-lines and the finer M-lines survived. Fibril c represents an intermediate state, in which some traces of the A-band can still be observed (Fig. 5).

Our polarizing microscopic observations similarly support our findings (Figs 6—9). Figs 6 and 8 represent the same area by means of the phase con-

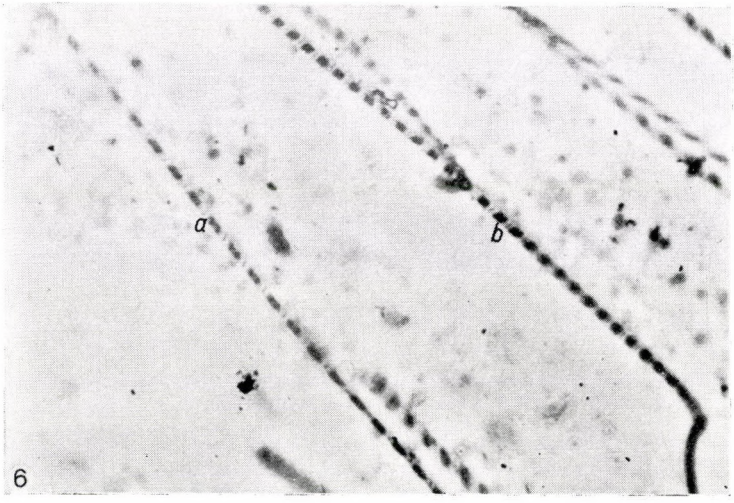

Fig. 6. Native fibrils, highly (a), or slightly (b) stretched. Phase contrast micrograph (40 × 10)

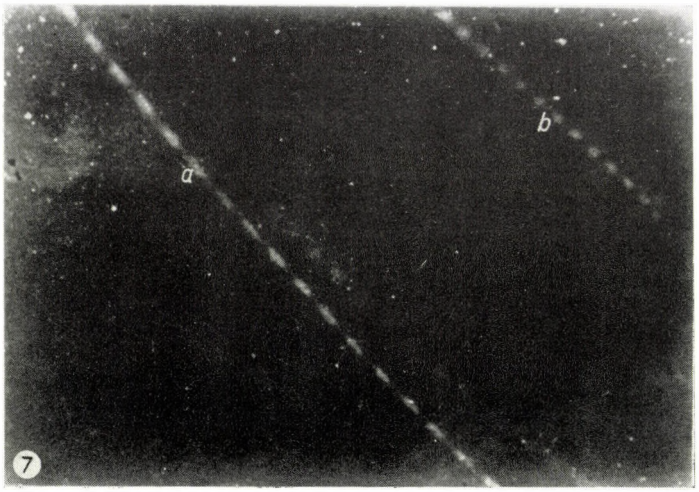

Fig. 7. The same fibrils as in Fig. 6, after treatment. (40 × 10)

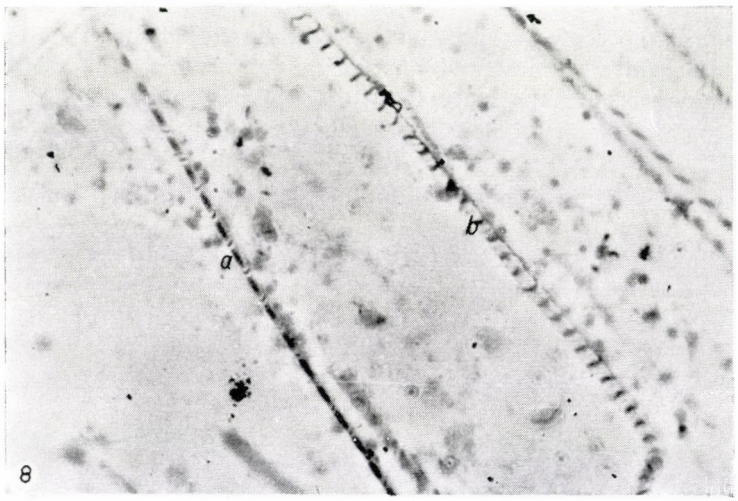

Fig. 8. The fibrils of Fig. 6 in the polarizing microscope (40 × 15)

trast microscope, as shown in Figs 7 and 9 in polarized light. Figs 6 and 7 were taken before, Figs 8 and 9 after extraction. The slightly stretched fibril (b) lost the bulk of its substances, contrary to the highly stretched one. In the same time the slightly stretched fibril obviously lost its double refraction, while the extremely stretched fibril lost only a part of it.

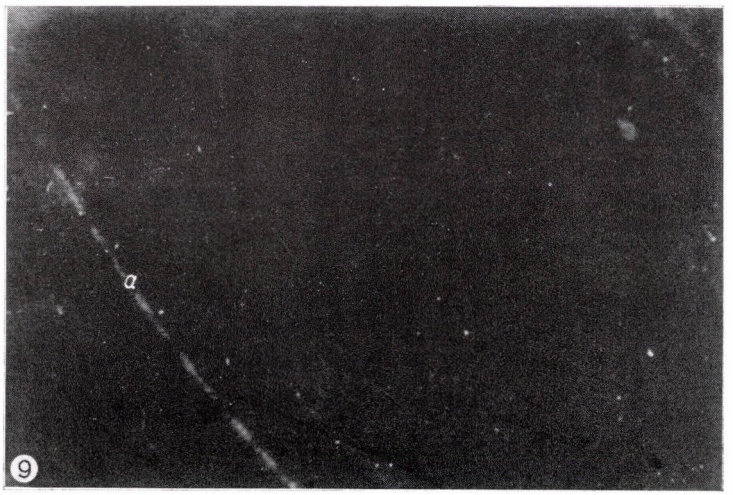

Fig. 9. The same fibrils as in Fig. 8, after extraction. Polarizing microscope (40 × 15)

Discussion

The decrease in the solubility of the proteins of single, isolated myofibrils, induced by stretch, seems to support our earlier considerations concerning the crystallization of the actomyosin in stretched muscle. However, we did not find linear relation between the solubility and the length of the sarcomere.

On the basis of the "sliding"-hypothesis of Huxley and Hanson (1960) we could expect that the solubility of the fibril would increase proportionally to the increase of the sarcomere length, because the number of the reaction sites of the actin-myosin interaction decreases and the zones of interaction (the overlap zones) become shorter. Our work shows that the solubility of the fibril doe, not depend on the extent of the supposed overlap, because the solubility decreases with increasing sarcomere length and there is a minimum solubility in the fibrilss in which, according to the "sliding"-hypothesis, no more overlap could exist.

Acknowledgements

Thanks are due to Professor E. Ernst for calling my attention on this topic, as well as for his support during the work, and to N. Garamvölgyi for his advices in methodical problems.

References

Ernst, E. (1963) Biophysics of the Striated Muscle. Akadémiai Kiadó, Budapest

Ernst, E., Bélecki, L., Nagy, J. (1956) Acta Physiol. Acad. Sci. Hung. *9* 7

Garamvölgyi, N., Kerner, J., Cser-Schultz, M. (1964) Acta Physiol. Acad. Sci. Hung. *24* 381

Huxley, H. E., Hanson, J. (1960) in The Structure and Function of Muscle. G. H. Bourne (ed), vol. I. p. 183. Acad. Press, New York

Tigyi-Sebes, A. (1966) In press

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 81–88 (1966)

The Ultrastructure of the Insect Flight Muscle Fibril Ghost

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(Received November 15, 1965)

Flight muscle fibrils of the bee were extracted with Weber-Edsall's solution and the surviving "fibril ghosts" were examined in the electron microscope, both in transverse and longitudinal sections. The effect of the myosin (or actomyosin) extraction on the structure of the different structural constituents of the myofibril (i. e. Z-lines, primary, secondary and S-filaments) is discussed. The structural changes caused by the extraction are brought in connection with the localization of the contractile proteins, a question of great functional importance.

The myofibrils deprived of their myosin content by means of a salt solution of high ionic strength are known as fibril ghosts, a designation analogous to the erythrocyte ghost, a framework surviving hemolysis. The fibril ghosts are lacking of the dense A-band substance, a fact, from which the localization of the myosin in the A-band was concluded (Hasselbach, 1953; Hanson and Huxley, 1953; Huxley and Hanson, 1954; Guba, 1954). The majority of these works were performed on fibrils of muscle homogenate, but very few electronmicrographs taken from ultrathin sections are known (Hanson and Huxley, 1953; Hanson and Huxley, 1956; de Villafranca et al. 1959; Guba et al. 1964; Garamvölgyi, 1965). This may be due to difficulties in the extraction of intact (unhomogenized) fibers on the one hand and to incommodities in the manipulation of extracted, fragile material during the process of fixation and embedding on the other hand.

Earlier and recent papers from our Institute are dealing with the structural changes induced by the extraction by Weber-Edsall's solution and observed in the light microscope during the whole process. According to these works the extraction begins with the appearance of a light zone in the middle of the A-band (Ernst et al. 1956). Subsequently the split substance of the A-band migrates to both Z-lines (Tigyi-Sebes, 1964; Tigyi-Sebes, 1966). In the course of prolonged extraction the dense zones on both sides of the Z-lines successively shrink and disappear (Tigyi-Sebes, 1966). Although we are dealing with the possible mechanism of the migration of the A-band substance in this paper, we do not bring this migration in connection with the contractile mechanism.

In previous works of our team (Ernst et al., 1958; Garamvölgyi et al., 1962) we reported on our experiments which lead to the isolation of Z-lines, using extrac-

tion by lactic acid. We obtained similar isolated Z-lines (Garamvölgyi et al. 1962) and later whole "Z-networks" (Garamvölgyi, 1962) by prolonged extraction by Weber-Edsall's solution. In a recent paper (Garamvölgyi, 1965) the problem of the S-filaments (Huxley and Hanson, 1954) has been discussed on the basis of observations made on ultrathin sections. Most recently a paper of one of us (Kerner, 1966) dealt with the effect of Weber-Edsall's solution on slightly and highly stretched fibrils.

Materials and Methods

Bundles of some fibers from the flight muscle of the bee were carefully prepared, preserving a small part of the chitin exoskeleton on both ends. The bundles became stretched and were fixed on the surface of a cork by sticking pins through the chitin plates. The corks were reversed and laid into a glass vessel, containing Weber-Edsall's solution. The extraction lasted from one to eighteen hours. After this a drop of 1 per cent osmiumtetroxyde was added to each preparation. After a preliminary fixation of 10 to 20 minutes the fibers were cut down from the corks and were put into 1 per cent osmiumtetroxyde for additional fixation of one hour. The specimens were embedded into araldite, sectioned by an LKB "Ultrotome" ultramicrotome and stained in an alcoholic solution of PTA for 5 minutes. For examination a Zeiss-Jena Elmi D 2, or a Tesla BS 242 electron microscope was used.

Observations

1. *Cross-sections*

The transverse sections of extracted fibrils show a great number of thin filaments, without any regular array. Particularly interesting are those fibrils in which there are still island-like areas with the double array of both kinds of filaments not yet affected by the extraction. Some primary filaments have begun to shrink already, due to their dissolution. In the same time their density strongly decreased. Such damaged filaments can be identified only on the basis of their site inside the hexagonal array, because their size is not very different from that of the secondary filaments. It is therefore difficult to recognize the individual thin filaments and it is similarly difficult to state that the primary filaments disappear without any surviving trace. It is, however, clearly visible that the alteration of the primary filaments is accompanied by the collapse of the regular array of the fibril (Fig. 1).

Since the existence of the two kinds of filaments has been revealed (Huxley 1953), it is generally accepted that it is the set of the primary filaments which contain all the myosin of the sarcomere. The earlier concept of the amorphous A-substance has been nearly forgotten. Our electron-micrographs demonstrate the immediate influence of the myosin extracting salt solution on the primary filaments, so

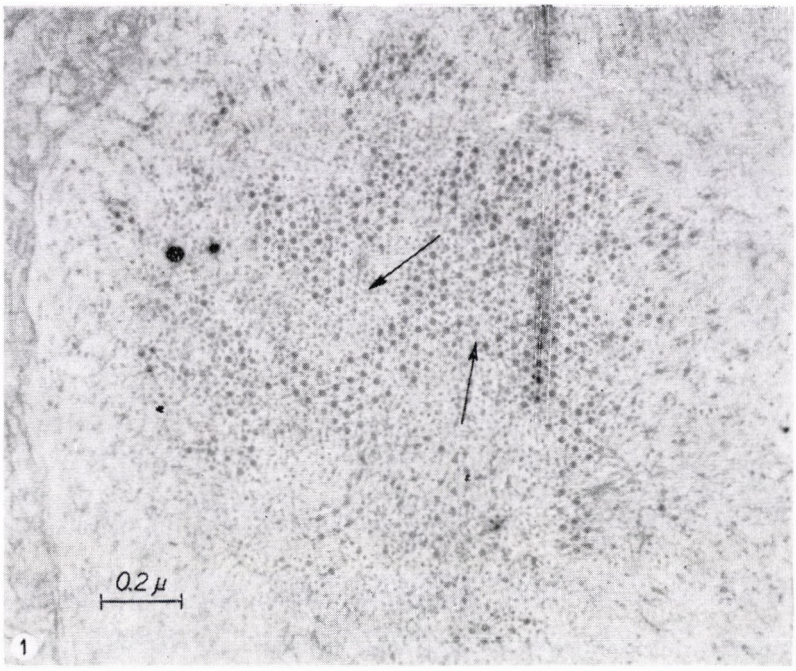

Fig. 1. Transverse section of a myofibril subjected to extraction by Weber-Edsall's solution. Note the areas still containing the double hexagonal order of both kinds of filaments. Arrows mark areas with primary filaments showing already signs of dissolution

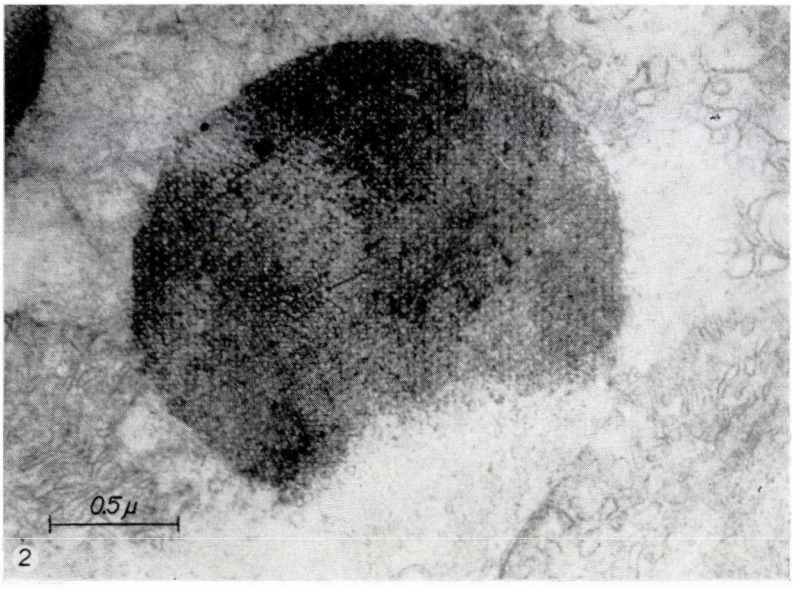

Fig. 2. Transverse section through the Z-line of a myosin-extracted myofibril

we have no reason to deny that the primary filaments consist mainly of myosin.

Other transverse sections show the structure of the Z-lines of fibrils subjected to myosin extraction (Fig. 2). No changes as compare to the Z-lines of unextracted fibrils are visible. The preserved hexagonal array, as well as the holes formed by the pleated secondary filaments (Auber and Couteaux, 1963) may be recognized. No sign of any loss of substances could be observed by this method.

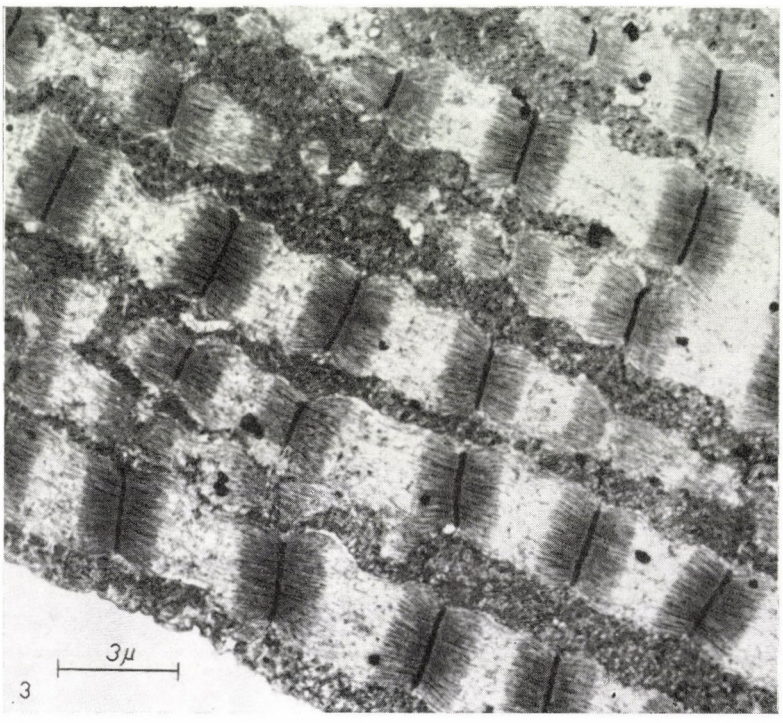

Fig. 3. Low magnification electronmicrograph of a longitudinal section of fibril ghosts. Note the uniform length of the dense zones on both sides of the Z-lines

2. *Longitudinal sections*

The striation pattern of ghost fibrils is quite the same as that observed by the light microscope. There is a light gap in the middle of the sarcomere (Ernst et al. 1956) and there are dense zones on both sides of the Z-line, which doubtlessly correspond to the dense zones resulting from the migration of the A-substance to the Z-lines (Tigyi-Sebes, 1966). The dense zones are symmetrically located to the Z-lines. They consist of very thick filaments, the length of which seems fairly uniform over large areas of the specimen (Fig. 3). In general the dense zones have a double besom-like appearance (Fig. 4) and their position and regularity suggests

that they represent the I-segments consisting of the set of the secondary filaments, to which the removed A-substance has been attached in one, or another way. If this is true, the medium empty zone would correspond to the original H-zone. The space between two dense zones is occupied by thin, but well recognizable filaments, which still connect both dense zones. These thin filaments correspond topographically to the S-filaments (Huxley and Hanson, 1954) supposed in order to explain the fact that the fibril ghosts do not fall into isolated I-segments as

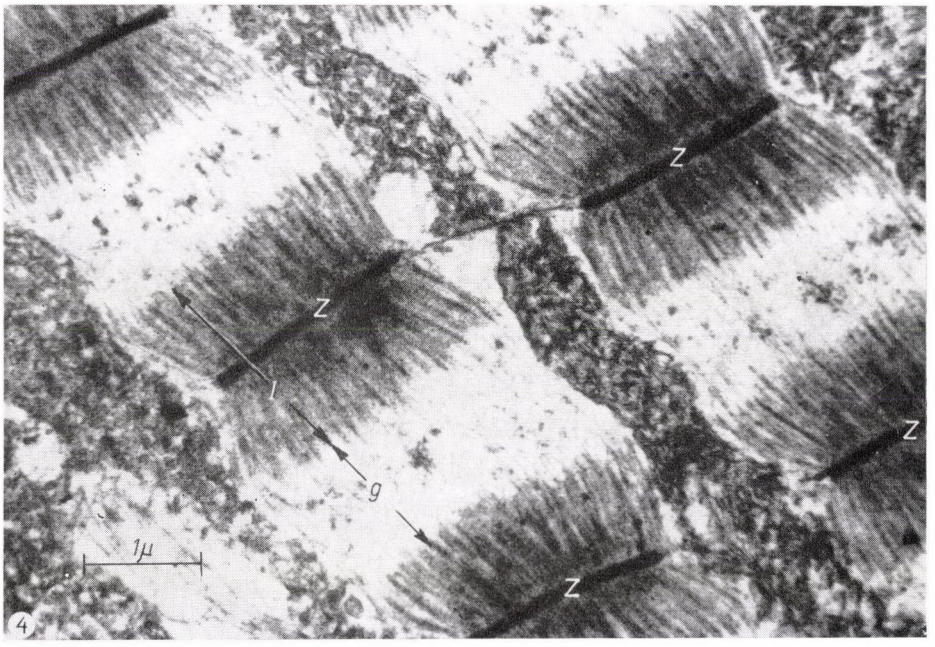

Fig. 4. Similar to Fig. 3, but higher magnification. Note the extremely thick filaments inside the dense zones. g: gap, corresponding to the H-zone

a result of the removal of the A-band filaments. In fibrils having been highly stretched and having sarcomere lengths higher than the "no overlap point", the light zones are very long, but still occupied by S-filaments (Fig. 5), a fact, the significance of which has been discussed in a previous paper (Garamvölgyi, 1965).

Our electronmicrographs perhaps do not represent the final state of the extraction. It was shown in our Institute that the migrating A-substance successively disappear in the further course of the treatment (Tigyi-Sebes, 1966). We did not follow the course of the extraction systematically, we found, however, areas with broadened light zones and shrinking dense zones, the latter with unequal filament lengths (Fig. 6). The light zones of increased length still contain S-filaments, consequently they are not restricted to the H-zone, but they probably

form a continuous framework of the sarcomere. Concerning this basic framework Guba came to a quite similar result on rabbit muscle (Guba et al., 1964). The fact that the extraction was performed by us on fibrils under tension, decreases the possibility of the artificial formation of the S-filaments during the extraction process (Garamvölgyi, 1965).

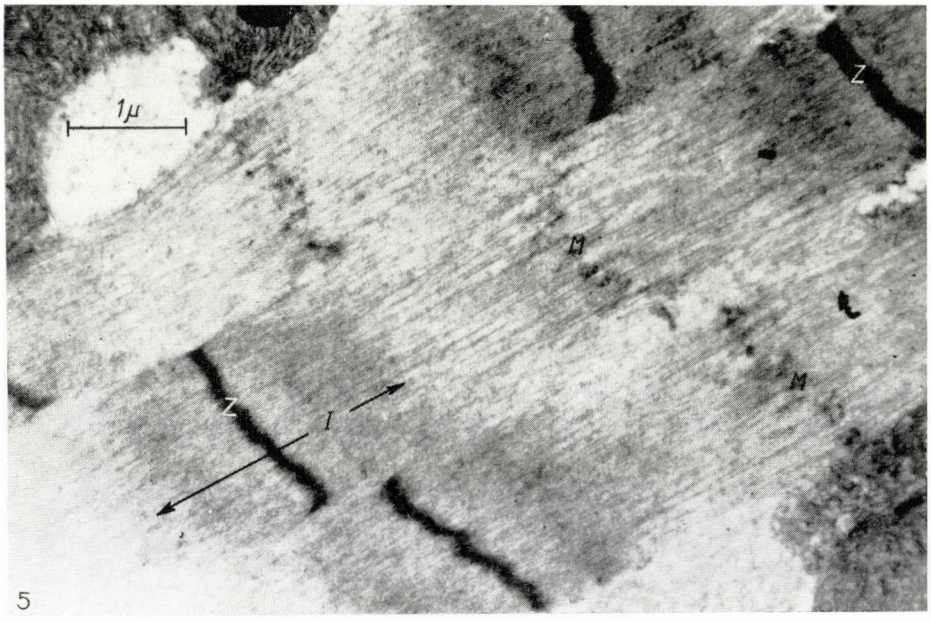

Fig. 5. Longitudinal section of fibrils extracted in a stretched state

Discussion

The problem of the S-filaments has been already discussed in a paper by one of us (Garamvölgyi, 1965). Here we mention only that we still use the term "S-filaments", although this auxiliary hypothesis has been abandoned by H. E. Huxley due to the phenomenon of the double overlap of the secondary filaments (Huxley, 1965). On the other hand, we do not express the opinion that the S-filaments should necessarily belong to the secondary filaments. The S-substance may also be a constituent of the primary filaments.

Concerning the mechanism of the migration of the A-substance there is a possibility, by which this phenomenon may perhaps be explained. If the I-segments consist of actin, the substance of the A-band liberated by the dissolution of the primary filaments may — at least partly — interact with the actin content of the I-segments, thus forming an actomyosin complex. The other part of the

Fig. 6. Longitudinal section of myofibrils showing broadened gaps and shrunken dense zones. Note the surviving S-filaments inside the gap

A-substance may leave the fibril representing the pure myosin (Myosin A) fraction. In the course of a prolonged extraction the actomyosin complex can similarly be extracted. It is well known that as a result of a prolonged extraction by Weber-Edsall's solution we do obtain the Myosin B (i. e. actomyosin) fraction (Banga and Szent-Györgyi, 1942). The extremely thick filaments inside the besom-like dense zones indicate that the A-substance can be really bound to the filaments of the I-segment.

References

Auber, J. and Couteaux, R. (1963) J. de Microscopie *2* 309
Banga, I. and Szent-Györgyi, A. (1942) Studies Inst. Med. Chem. Szeged *1* 5
Ernst, E., Garamvölgyi, N. and Guba, F. (1958) Acta Physiol. Acad. Sci.Hung. *14* Suppl. 40
Ernst, E., Béleczki, L. and Nagy, J. (1956) Acta Physiol. Acad. Sci. Hung. *9* 7
Garamvölgyi, N. (1962) Acta Physiol. Acad. Sci. Hung. *22* 235
Garamvölgyi, N. (1965) J. Ultrastructure Research *13* 409
Garamvölgyi, N., Metzger-Török, G. and Tigyi-Sebes, A. (1962) Acta Physiol. Acad. Sci. Hung. *22* 223

Guba, F. (1954) Electronmicroscopic Investigation of Muscle. (in Hungarian) Theses of Cand. Biol. Sci. Budapest.

Guba, F., Harsányi, V. and Vajda, E. (1964) Proc. Europ. Reg. Conf. on Electr. Microscopy, Prague, Vol. B. p. 77. Publ. Czechoslovak Acad. Sci. Prague, 1964.

Hanson, J. and Huxley, H. E. (1953) Nature *172* 530.

Hanson, J. and Huxley, H. E. (1956) Symp. Soc. Exp. Biol. *9* 228.

Hasselbach, W. (1953) Z. Naturforschung *8. b* 449.

Hodge, A. J. (1955) J. Biophys. Biochem. Cytol. *1* 361.

Huxley, H. E. (1953) Biochim. Biophys. Acta *12* 387.

Huxley, H. E. (1965) in: Muscle — Proc. Symp. Univ. Alberta, 1964. p. 3—28. Pergamon Press, New York.

Huxley, H. E. and Hanson, J. (1954) Nature *173* 973.

Kerner, J. (1966) Acta Biochim. Biophys. Hung. 1.

Tigyi-Sebes, A. (1964) Abstr. 3rd Meeting Hung. Biophys. Soc. p. 5. Budapest. (in Hungarian).

Tigyi-Sebes, A. (1966) in press.

Villafranca, G. W. de, Scheinblum, T. S. and Philpott, D. E. (1959) Biochim. Biophys. Acta *34* 147.

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 89—100 (1966)

Elongation of the Primary Myofilaments in Highly Stretched Insect Flight Muscle Fibrils

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(Received September 30, 1965)

The changes in the cross-striation of myofibrils indicate the elongation of the primary myofilaments. The appearance of E- (elongation) zones beyond a sarcomere length, close to the "no overlap point" is accompanied by a successive shortening of the central dense A-zones. In transverse ultrathin sections the E-zones exhibited decreased filament diameters. Some E-zone filaments preserved their "tubular" structure, indicating that they are elongated primary filaments. The additional E-zones which appear in the middle of the A-band similarly consist of primary filaments with a reduced diameter.

In our previous papers we reported on some observations on highly stretched insect flight muscle fibrils (Garamvölgyi et al. 1964; Garamvölgyi, 1965 a). We assumed that the length of the A-band is constant in a certain range of the sarcomere length, but beyond the upper limit of this range E-zones appear at the A-I-junctions, which result from the passive elongation of the primary filaments. This view seemed to be supported by the fact, that the optical path difference, measured by the interference microscope, was found to be higher than that of the I-band.

Earlier observations from our Institute on the flight muscle of the house-fly (Ernst et al. 1956) have shown that in highly stretched myofibrils the A-bands suffered distortions. In this connection the authors raised two possibilities, either the substance of the A-band may be drawn out into the I-band, or one part of the filaments might have been broken by the stretch exerted on them.

The sarcomere length beyond which the E-zones become visible is fairly close to the "no overlap point" following from the theory of the sliding filaments, i. e. to the estimated sarcomere length, at which the successively broadening H-zones would occupy the entire A-band. This sarcomere length is about 5 μ in the case of the flight muscle of the bee. Inspecting isolated myofibrils of the bee by the light microscope we noted that sarcomere lengths between 5 and 7 μ are very rare (Garamvölgyi et al. 1964). This means that the fibrils either do not stretch until the "no overlap point," or their length increases very easily until extreme sarcomere lengths. In glycerol-extracted muscles sarcomere lengths slightly beyond the no overlap point occur more frequently and we are able to reconstruct the subsequent changes of the striation pattern.

Materials and Methods

For electron microscopy stretched muscle fibers of the indirect thoracic muscle of the bee were prepared as described in a previous paper (Garamvölgyi, 1965 a). The fixation was performed by 1 per cent osmium tetroxide. The specimens were embedded into araldite, sectioned by an LKB "Ultrotome" ultramicrotome and stained with phosphotungstic acid. The electonmicrographs were taken by a Tesla BS 242 electron microscope.

For phase contrast microscopy the fibres were teared by needles on slides. In the case of glycerol extracted muscle the fibres were previously extracted in a 15 per cent, or 50 per cent solution of glycerol. After 2, or 3 hours of extraction they were washed by Pringle's solution and prepared on slides in the same way as the untreated muscle. The length of the sarcomeres and those of the different bands were determined on photographs, on the basis of a micrometer slide of 10 μ intervals, photographed with the same magnification (90 $\times$ 16).

Observations

1. *Changes of the cross striation beyond the "No overlap point"*

In order to demonstrate the length changes of the different bands of the cross-striation in relation to the sarcomere length, a series of phase contrast micrographs has been presented (Fig. 1), showing the band pattern characteristic to the corresponding sarcomere lengths.

Fibril *a* has a sarcomere length of 4 μ and A-bands of 2.7 μ. It represents the striation pattern below the "no overlap point".

Fibril *b* shows the state just beyond the critical 5 μ limit, where the E-zones appear and simultaneously the dense zones of the A-band become shorter (1.9 μ). The sarcomere length is 5.4 μ and the A-band length (i. e. that of both E-zones plus the medium dense zone) is 3.2 μ.

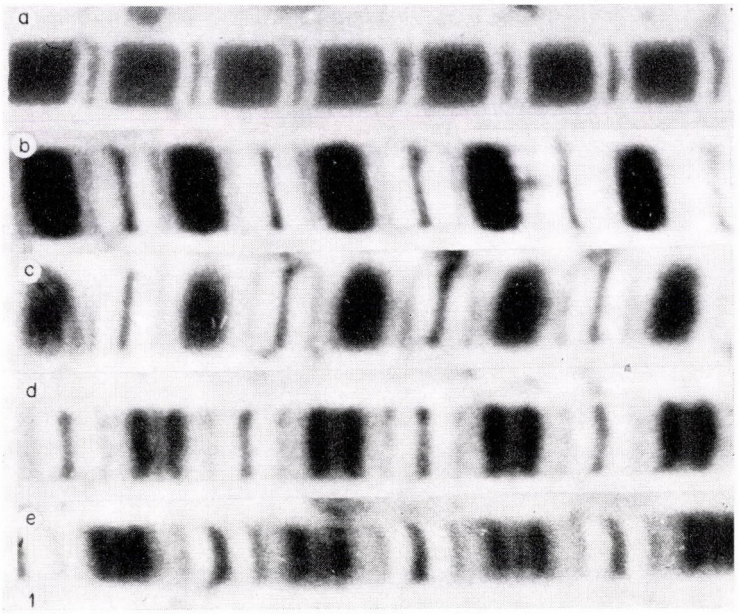
Fibril *c* has a sarcomere length of 6.2 μ and A-band length of 4 μ. The medium A-zone suffered a further decrease, it has a length of 1.6 μ.

Fibril *d* shows the appearance of additional E-zones in the middle of the A-band and because of this the medium zone begins to stretch, too. The sarcomere length is 6.7 μ, that of the A-band is 4.6 μ.

Fig. 1 a-e. Phase contrast micrographs of myofibrils showing the striation pattern characteristic to the different sarcomere lengths. For details see the text

Fig. 2. Phase contrast micrograph of a myofibril having dense A-zones of extremely reduced size

Fig. 3. Low magnification electronmicrograph showing cross sections of highly stretched myofibrils. Note the different bands, lines and zones, of the cross striation inside the same fibril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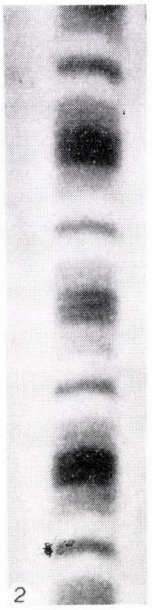
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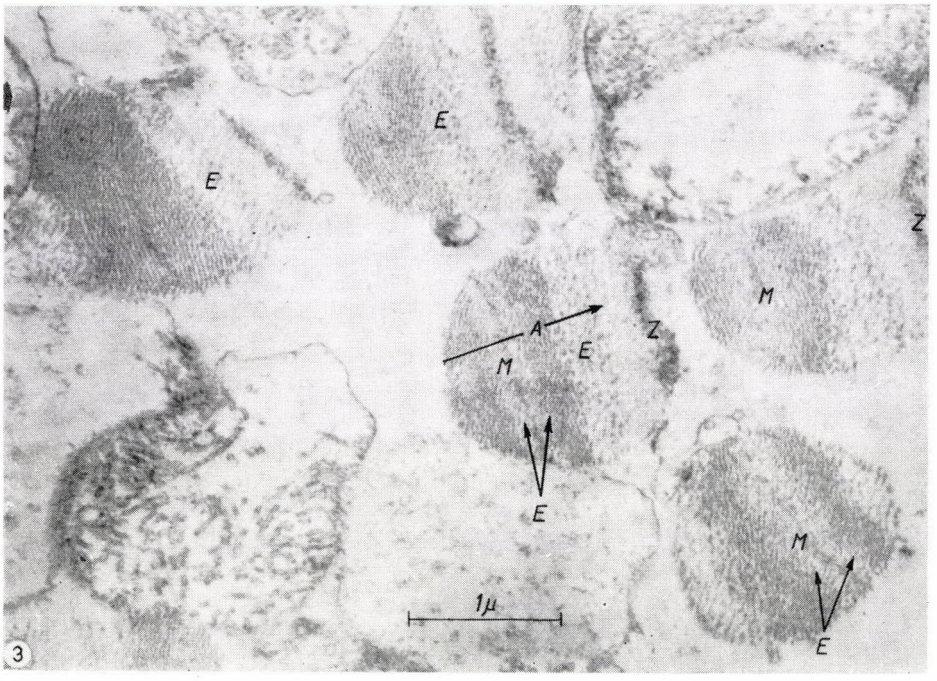
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Fibril *e* represents the striation pattern of the longest occurring sarcomeres, with a length of 8.1 μ, from which the A-band occupies 5.4 μ.

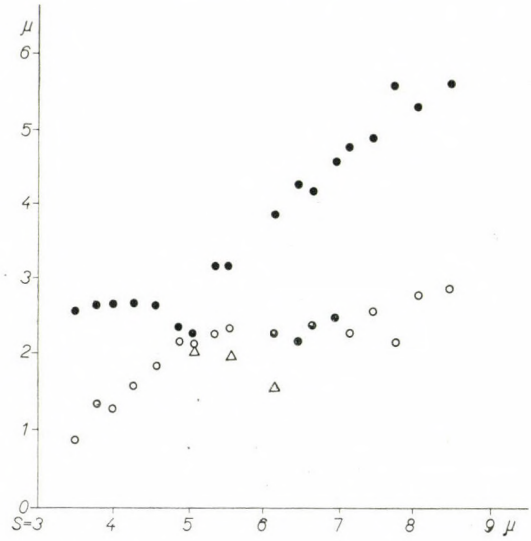
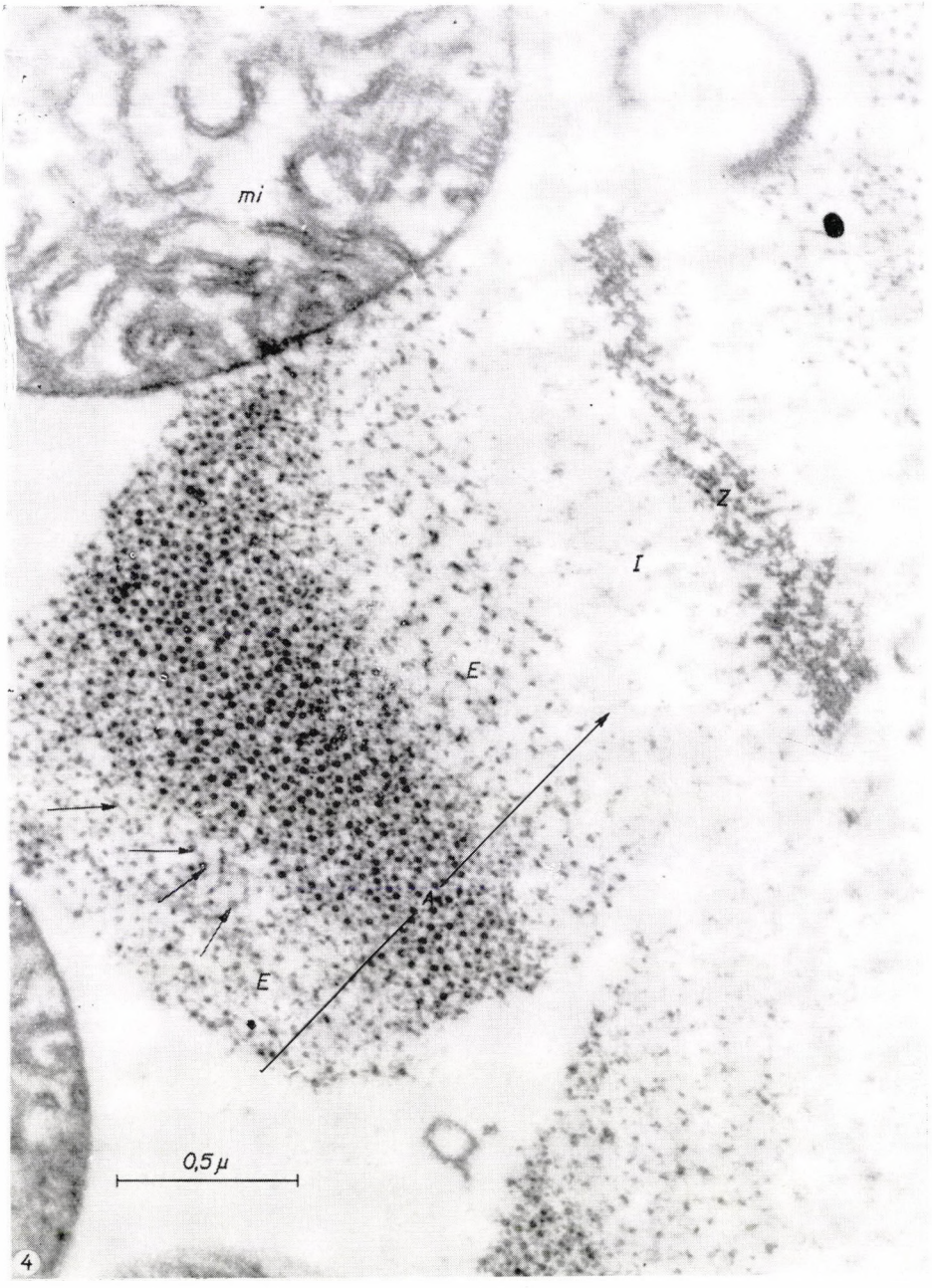


Diagram 1. The length of the different bands in relation to the sarcomere length. The dots mark the length of the A-band, the empty circles that of the I-band and the triangles that of the medium dense A-zone

The changes characterized above are represented in Diagram 1. The upper curve marks the length of the A-band, the lower one that of the I-band (i. e. both halves belonging to a sarcomere). Each point represents the average value of several fibrils. It is clearly visible that until the sarcomere length is of 5 μ the A-bands have a fairly constant length, while the length of the I-band increases nearly linearly. Beyond this critical limit of sarcomere length the I-bands suffer only a slight elongation. From the same sarcomere length on the total A-band increases in form of E-zones at the A-I junctions and in the same time the medium zone of the A-band decreases in length down to a minimum value of 1.6 μ. The triangular marks of the diagram represent the length of the medium dense zone of the A-band. At the sarcomere length of about 6.5 μ additional E-zones appear on both sides of the M-line and the further elongation of the A-band is brought about in this way. In some cases we could observe that the central dense A-zone became extremely reduced and only two fine lines survived (Fig. 2), while almost the entire A-band seems uniformly elongated.

Fig. 4. Transverse section of a myofibril of a highly stretched fiber. Note the "tubular" primary filaments inside the dense A-zone and the filaments with decreased diameter in both E-zones. Arrows mark some E-zone filaments in the E-zone in the middle of the sarcomere, with their still preserved "tubular" structure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2. *Cross-sections of highly elongated myofibrils*

Observing transverse sections of myofibrils fixed in a stretched state we obtained many times pictures showing not a single band (Fig. 3). Probably two factors contributed to this. On the one hand the sections could have been cut sligthly obliquely, on the other hand the striation itself could be oblique by the stretch exerted on the fibrils. In this latter case we obtained different band structures inside the cross-section of the same myofibril, even in the case of exact transverse cutting direction. The Z-lines may serve as the sites of orientation. Related to their position the different zones of the A-band (dense A-zones, as well as both kinds of E-zones, at the A-I junctions and in the middle of the A-band, respectively) may be identified.

In the dense A-zones (i. e. in zones with preserved high density) there are primary filaments with their characteristic "tubular" structure (Fig. 4). Their hexagonal array is generally disordered. Between the primary filaments there are very often also secondary ones, a fact, the significance of which will be discussed later. In the E-zones at the A-I junctions, as well as in the middle of the A-band, the filament diameters seem considerably lower than in the dense A-zones (Figs 4—6). Notwithstanding, some E-zone filaments still show their "tubular" structure (Fig. 4 and 5, marked by arrows) indicating that they belong to the set of the primary filaments.

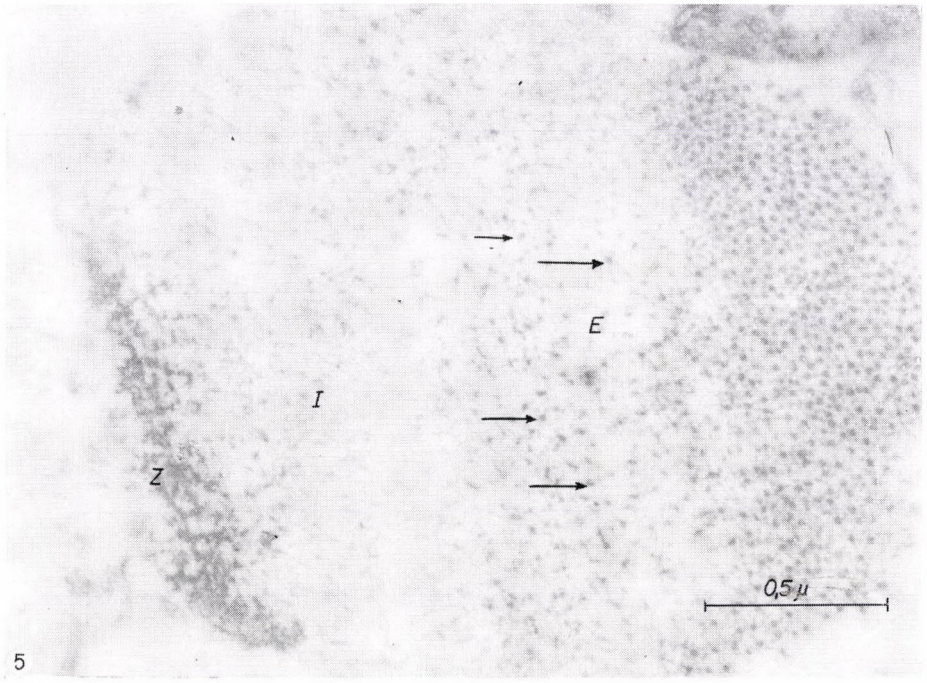
The Z-lines of highly stretched myofibrils show a very loose structure. The regular hexagonal order of the Z-line observed in unstretched fibrils (Auber and Couteaux, 1963) cannot be recognized. The density of the Z-line area seems decreased and the individual Z-filaments can be distinguished. Their general appearance in transverse sections corresponds to their structure which is visible in longitudinal orientation (Garamvölgyi, 1965 a), This indicates that some material has been drawn out from the Z-lines (Fig. 7).

3. *Fibrils broken by extreme stretch*

In our specimens of muscle fixed in a strongly elongated state we could find broken fibrils, particularly at the periphery of the fibres. They can be recognized easily by their unusual orientation, i. e. single fibrils longitudinally cut in a specimen of transverse sections, or reversed. In transverse sections of broken myofibrils we found a disarray of the primary filaments and the absence of the secondary ones (Fig. 8). The sarcomere lengths are about those of the unstretched fibrils (Figs 9 and 10). There are no I-bands, more correctly both sides of the Z-line are occupied by diffuse dark bands, consisting perhaps of broken filaments.

Fig. 5. Similar to Fig. 4. There are "tubular" E-zone filaments in the E-zone at the A-I junction (arrows)

Fig. 6. Similar to Figs 4 and 5. Note the decreased filament diameters in the E-zones



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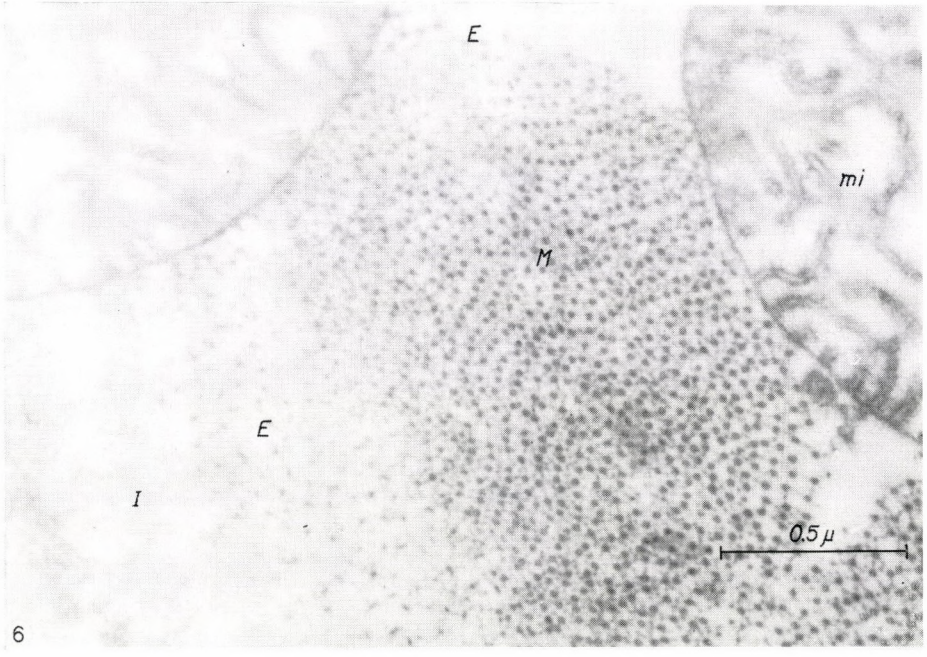

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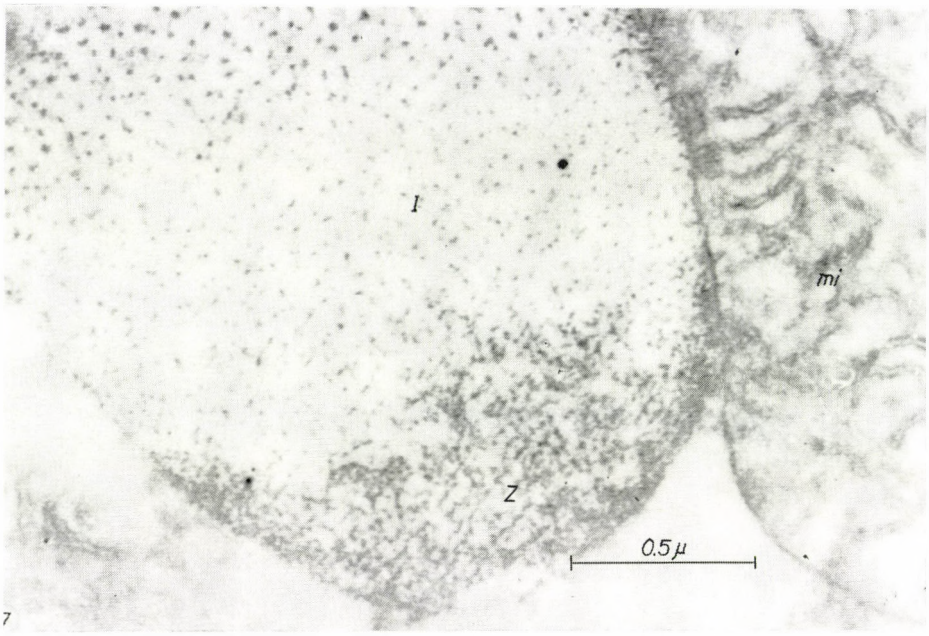

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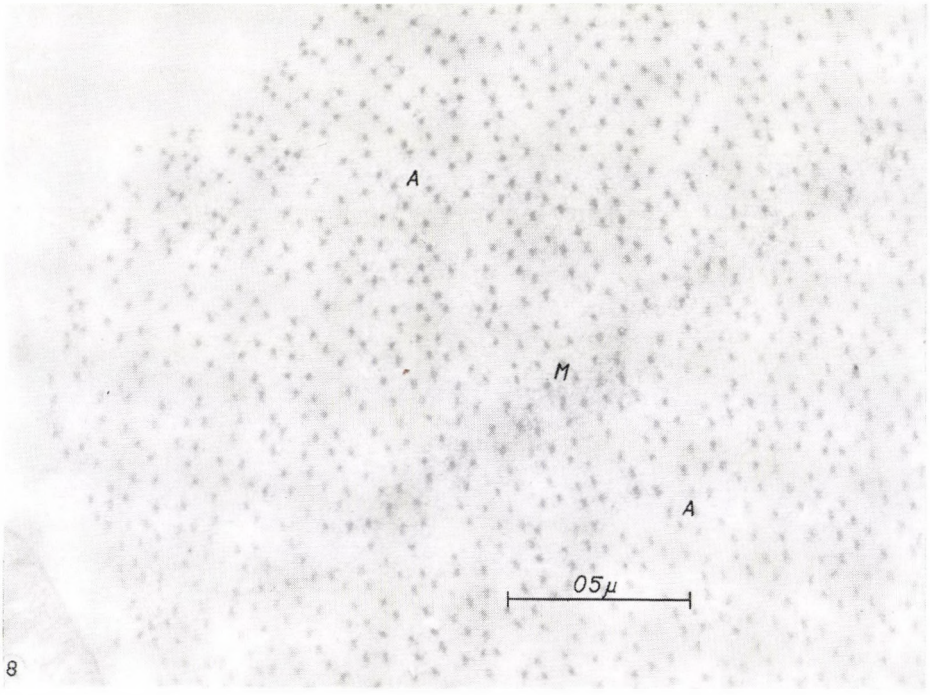

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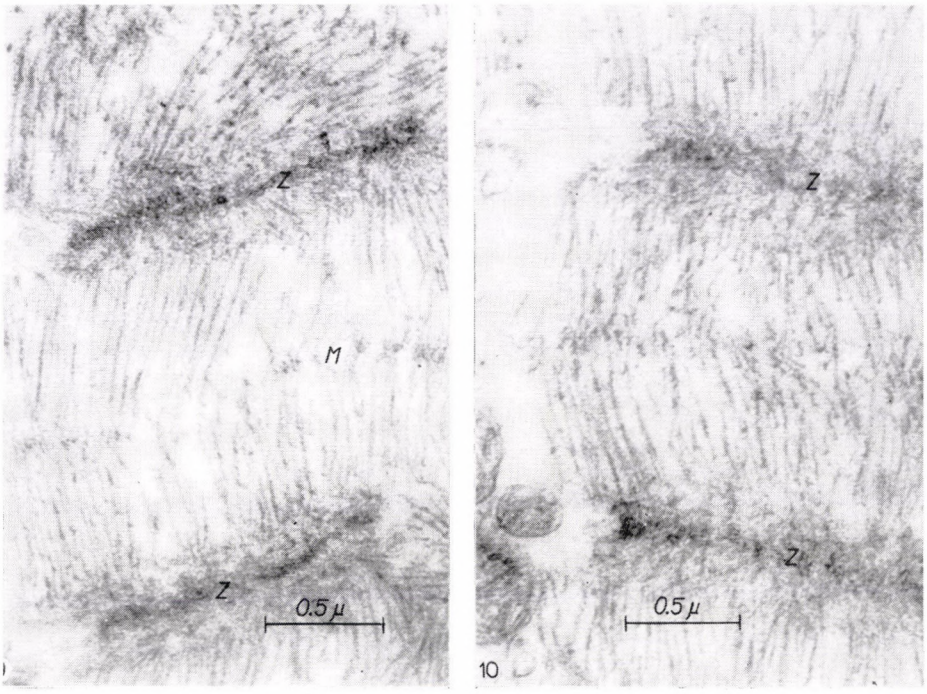

Figs 9 and 10. Longitudinal sections of myofibrils broken by stretch. Note the diffuse dense zones on both sides of the Z-line. The sarcomere lengths are about 3μ

Broken fibrils very often have a beaded shape corresponding to the "Perlenschnurstruktur", frequently mentioned in earlier papers (Ernst et al. 1956). This phenomenon is probably due to a swelling, which occurs in the course of the passive shortening. The passive shortening of myofibrils broken by strong elongation demonstrates that even very high degrees of passive stretch do not cause irreversible changes in the sarcomere length.

Fig. 7. Cross-section of a stretched myofibril containing also an area in the Z-line
Fig. 8. Cross-section of a myofibril broken by strong stretch. Note the absence of the secondary filaments

Discussion

It is a very important question, by which structures the elongation of the A-band can be brought about. We have shown that the very thick and dense filamental portions inside the Z-lines are parts of the primary myofilaments (Garamvölgyi, 1963). Thus the primary filaments in the A-band of the insect flight muscle are not simply connected with the Z-line (Auber and Couteaux, 1962; 1963) in general, but with the distinct primary filaments inside the Z-line. This relation may be demonstrated in slightly stretched fibrils, too, which have short, but already well expressed and typical I-bands (Garamvölgyi, 1965 a). By stronger elongation the primary Z-filaments suffer further alterations, their diameter decreases and the shape of the Z-line indicates that their substance has been drawn out from the Z-line to both adjacent halves of the I-band. This assumption seems confirmed by the disturbed structure of the Z-line, as seen in cross sections (Fig. 7). This means that by strong elongation of the myofibril the primary filaments of the A-band, as well as those of the Z-line are influenced in the same manner. Thus we have no reason to suppose that the thin elements connecting the primary filaments of the A-band to the corresponding elements of the Z-line were merely loose connections, which could be interrupted by the slightest force. They seem to be able to bring about the elongation of the primary filaments, even, when there are primary and secondary filaments side by side in the A-band (Fig. 4). Moreover, the elongation of the primary filaments is not restricted to their A-I junction, but they also stretch in their middle (Figs 4 and 6), a fact which — in my opinion — unambiguously excludes any confusion of the primary and secondary filaments inside the E-zones.

Our previous observations (Garamvölgyi, 1963; 1965 a and b) served as basis for a functional concept involving the own shortening of certain portions of the myofilaments themselves. The changes of the cross-striation below 5μ sarcomere length found by us are quite the same as those, which served as the structural basis of the theory of the sliding filaments (Huxley and Hanson, 1954; Hanson and Huxley, 1965). We regularly observed also the two different kinds of filaments (Huxley, 1953). We are not in conflict with the constant length of the A-band and the I-segment (Page and Huxley, 1964) inside a medium range of sarcomere length. Even the most recent observations of the team of H. E. Huxley (Huxley et al. 1965 and simultaneously Elliott et al. 1965) on the constancy of axial spacings during contraction, are not incompatible with our concept. According to us the structure of the myofibrils may serve as basis for a structural mechanism different from the sliding of filaments. Our view is based on the continuity of the primary filaments and the present findings are in agreement with our assumption.

Remarks

The reports of G. Hoyle and Carlsen, Fuchs and Knappeis submitted to the Information Exchange Group No. 4. (Molecular Basis of Muscle Contraction) have been discussed in my paper presented to the same Group (IEG Nr. 4. Sci. Memo Nr. 40)

I am very grateful to Professor H. E. Huxley (Cambridge) for interesting and useful discussions.

References

Auber, J. and Couteaux, R. (1962) C. R. Acad. S. *254* 3425
Auber, J. and Couteaux, R. (1963) J. Microscopie *2* 309
Elliott, G. F., Lowy, J. and Millman, B. M. (1965) Nature (London) *206* 1357
Ernst, E., Béleczki, L. and Nagy, J. (1956) Acta Physiol. Acad. Sci. Hung. *9* 7
Garamvölgyi, N. (1963) J. Microscopie *2* 107
Garamvölgyi, N. (1965 a) J. Ultrastructure Research *13* 409
Garamvölgyi, N. (1965 b) J. Ultrastructure Research *13* 425
Garamvölgyi, N., Kerner, J. and Cser-Schultz, M. (1964) Acta Physiol. Acad. Sci. Hung, *24* 381
Hanson, J. and Huxley, H. E. (1956) Symp. Soc. Exp. Biol. *9* 228
Huxley, H. E. (1953) Biochim. Biophys. Acta *12* 387
Huxley, H. E. and Hanson, J. (1954) Nature (London) *173* 973
Huxley, H. E., Brown, W. and Holmes, K. C. (1965) Nature (London) *206* 1358
Page, S. and Huxley, H. E. (1964) J. Cell Biol. *19* 369

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 101—106 (1966)

Scintillations in Living Nerve Induced by β-Radiation of Tritium

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(Received November 15, 1965)

The ischiadic nerves of frogs (Rana esculenta) were incubated in Ringer's containing 0.1 mC/ml tritiated water, and the scintillation induced by the β-radiation in the nerve tissue itself was measured by photomultiplier. The tritium-treated nerves were compared with the untreated ones. A significant increase in scintillations was detectable in the nerves containing tritium. The β-counting efficiency of the nerves was calculated as 0.05 per cent. The measuring of scintillations induced by ionizing radiation in living tissues seems to create a new methodological possibility to investigate the changes and amount of natural scintillating substances.

In spite of the outstanding results of radioautography, the problem of the microlocalization in living cells cannot be considered to be solved yet. All of the used methods of radioautography have the apriori disadvantage that the cell has to be killed before localization. During the last years several excellent methods have been developed for fixation (Hill, 1964; Winegrad, 1965; Caro, 1962) but looking for a method which would permit to localize the isotopes, and their changes in the living cell during the living processes, seems to be a very fruitful perspective.

As far as an adequate detector is concerned, the Geiger-tube cannot be taken into account because of its dimensions (Selverstone and Robinson, 1960). The use of the crystal counter, discovered in 1947 by Seitz (Seitz, 1947) would be a hopeful method for microlocalization, but the technic of producing the necessary pinpoint crystal chamber is the task of the future. The scintillation method offers the simplest way of the detection. Instead of using artificial scintillators, the study of the scintillations themselves in the living tissue seems to be a good approximation of the problem. Hevesy and Paneth in their old textbook (Hevesy and Paneth, 1921) are also mentioning the fluorescence of the lens, corpus vitreum and the retina of the human eye, which is caused by γ-radiation. The intensive studies on the organic scintillators (Schramm, 1963) have made expectable the scintillations in biological tissues caused by ionizing radiations. Because of the physical properties of the radiation, the β-radiation of the tritium was chosen for the introductory experiments described in this paper.

Methods

Ischiadic nerve of frog (Rana esculenta) was used. The nerves were freshly dissected. One of the parallels was incubated in Ringer solution containing tritiated water, whereas the control was kept in an isotope free Ringer solution. The incubation time was 24 hours, the temperature 2—4 °C. The radioactivity of the tritiated Ringer solution was between 0.1—0.4 mC/ml.

After the 24 hours' incubation, the nerves were blotted with filter paper, and placed on the bottom of an aluminium preparation holder, according to Fig. la. The wet nerve was stuck to the black paper placed on the bottom of the aluminium

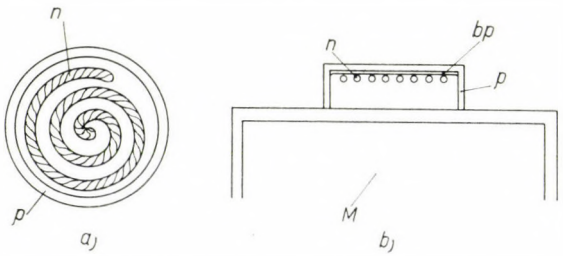

Fig. 1. Arrangement for measuring β induced scintillations in the nerve.
a) n: ischiadic nerve; p: Al. planchette.
b) M: multiplyer; n: ischiadic nerve; bp: black paper; p: Al. planchette

planchette without any sticking medium. As shown in Fig. 1b, the planchette with the nerve was placed on the window of the photomultiplier. This arrangement is without a liquid optical coupling to avoid the contact of tritium with the glass material of the tube. The distance of the nerve from the photomultiplier's surface was 5 mm, the range of the β-radiation of tritium in the air was 3 mm. This inserted air was enough to absorb perfectly the β-particles coming from the tritium-treated nerve. This arrangement reduced the efficiency of the measurements but it was advantageous to eliminate any possible accidental effect.

The photomultiplier was a Zeiss type M12-FS35 in Gamma type H5-IM lead chamber, computed with a scaler Orion EMG type 1872.

Besides the control nerve kept in normal Ringer solution, another control was also used. In such a case the nerves were substituted with a flax thread, which was treated with tritiated Ringer solution similarly to the nerve.

All specimens were counted 10 times for 2 minutes. At least 80 000 scintillations were counted each time. For the determination of the efficiency of counting the absolute activity of the tritium taken up by the nerves, an I.D.L. tritium scintillation counter, type 6012 was used. The tritium was extracted from the nerve by shaking it with 0.1 N HNO_3 for 48 hours.

Results

Table 1 shows the results of 6 parallel experiments. These nerves were kept previously in a Ringer solution of an activity of 0.1 mC/ml. The nerve samples were of an average fresh wet weight of 200 mg. In all the experiments the tritium treated nerves were compared with the untreated control ones, to avoid geometrical

Table 1

Scintillation induced by β-radiation of incorporated tritium in the nerve

No	Nerves treated with tritium		Control		$N_t - N_k$	$\frac{N_t - N_k}{N_k} 100$
	counts/2 Min, N_t	u_t	counts/ 2Min, N_k	u_k		
1	1532	20	1258	10	274	21.8
2	800	10	648	9	152	23.4
3	1044	11	897	15	147	16.4
4	1095	11	871	9	224	25.8
5	894	9	762	8	132	17.4
6	802	10	679	12	123	18.1

differences. It can be seen that in every case the number of scintillations is significantly higher in the tritiated nerves than in the control ($P \leq 0.001$). The average value of the differences was 20 per cent. The background in these experiments was 696 ± 10 counts/2 min. Table 2 gives the data of the nerve-free control. There is

Table 2

Control experiments with flax threads

Specimen	Counts/2Min	u
linen thread with incorporated tritium	128	$\pm$ 4
inactive thread	131	$\pm$ 3
empty planchette	120	$\pm$ 3

no significant difference between the tritium-treated and the control flax threads. Fig . 2 represents the relation between the tritium concentration and the number of scintillations.

These nerves have been treated in a series of Ringer solutions containing 0.1, 0.2, 0.3, and 0.4 mC/ml of tritium, respectively. The incubation time was 24 hours, the temperature 2—4°C.

In first approximation, there is a linear relation between the values of scintillations and the tritium concentrations. Attempts were made to calculate the ef-

ficiency of scintillations caused by the β-particles of tritium in the nerve. To this end the effective number of scintillations measured in the above way, and the absolute activity of the incorporated tritium in the nerve, were taken into consideration. These approximative calculations gave:

$$\eta = \frac{\text{number of scintillations measured in the nerve}}{\text{number of } \beta\text{-particles incorporated in the nerve}}$$

$$\eta = 0.01 - 0.05$$

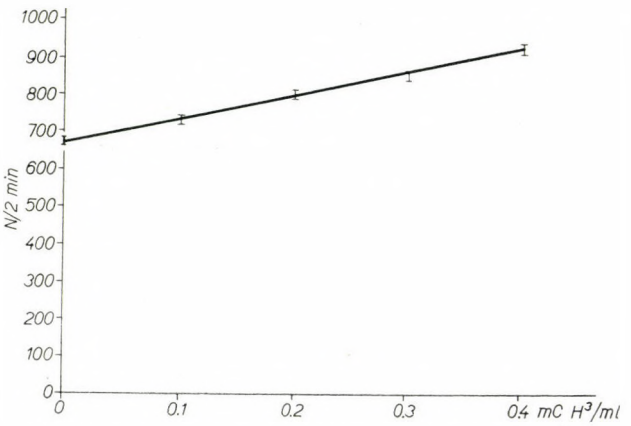

Fig. 2. Relation between tritium concentration and number of scintillations in the nerve

Discussion

Our results show that the ionizing radiation of tritium causes well detectable scintillations in the nerve tissue. These scintillations are supposed to be the signs of excitations in the electron shells of the molecules of the nerve tissue (Schramm, 1963). We have chosen the β-particles of tritium, because its energy equals 18 keV, and does not generate Cherenkov radiation (Cherenkov, 1937) in the water which is present in about 80 per cent in the nerve tissue. The velocity (v) of these electrons (neglecting the relativity correction) would be

$$v = 6 \times 10^7 \times \sqrt{18.000} = 8{\cdot}1 \times 10^9 \text{ cm/sec.}$$

This allows to calculate the necessary optical refractory index to create Cherenkov radiation. The calculation gives an optical refractory index $n = 4$, so we can completely exclude the role of Cherenkov radiation in the effect measured by us.

As far as the spectral range of these scintillations is concerned, all what can be said is that the sensitivity range of the photomultiplier contains it, or at least a part of it. The spectral sensitivity of the photomultiplier, type M12-FS35, ranges between 300—600 mμ (Keszthelyi, 1964). It is possible, that the low efficiency of the scintillations is caused by the difference between the spectral ranges of the scintillations and the multiplier sensitivity, respectively. The other reason of low

efficiency would be the above mentioned (p. 102) poor optical coupling between the nerves and multiplier window. Our later measurements have shown that we can get a significant increase of the effect given in Table 1. This increase amounts to a factor of about 2. The highest efficiency measured in these experiments was 0.1 per cent. Of course, a possible reason for the low efficiency can be the small amount of scintillating material in the nerve tissue.

The analysis of the data in Table 1 shows a difference between the control and the background values. This difference could be caused by chemiluminescence of the nerve tissue (Tarusow: Personal communication).

One of the most important problems concerning these experimental results is the decision which molecules of the nerve tissue are responsible for the scintillation. The very detailed studies on scintillation detectors (e.g. J.A.E.A. 1960) give some basis for speculations about this problem, but none of these is well enough founded to our purpose. According to our opinion, the only way which could lead to the solution of this problem would be the fractional extraction of different compounds for the nerve tissue. Similar studies are in progress in our laboratory, and are also made on cross-striated muscle.

We hope that the future development of this method: the analysis of scintillation in living tissues induced by ionizing radiation will give a new possibility to approach and explore some details concerning the biological functions in the cell.

References

Caro, L. G. (1962) J. Cell. Biol. *15* 189
Cherenkov, P. A. (1937) Phys. Rev. *86* 685
Hevesy, G. a. Paneth, F. (1921) Lehrbuch der Radioaktivität. A. Barth, Leipzig
Hill, D. K. (1964) J. Physiol. *175* 275
Keszthelyi, L. (1964) Scintillations-counters (Hungarian). Műszaki Könyvkiadó, Budapest
Schramm, E. (1963) "Organic Scintillations Detectors". Elsevier Publ. Comp.
Seitz, F. (1947) Phys. Rev. *73* 549
Selverstone, B. a. Robinson, C. V. (1960) in Glasser O. (ed.) Medical Physics. The Year Book p. 328
Winegrad, S. (1965) J. Gen. Physiol. *48* 455

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 107—112 (1966)

Enrichment of Polysomes in the Liver upon X-Irradiation in vivo*

(Preliminary Report)

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(Received December 30, 1965)

The functional relationship between ribosomes and messenger-RNA has been recognized recently and led to a deeper knowledge of the mechanism of protein synthesis.

As there are some conflicting results concerning the effect of X-irradiation on protein synthesis (Hevesy, 1949; Butler et al., 1956; Richmond et al., 1957; Smit and Stocken 1963), we decided to examine the changes of the ribosomal system upon X-irradiation *in vivo* (Hidvégi et al., 1964; Hidvégi et al., 1965).

Male guinea-pigs, body-weight 350—400 g, fasted overnight were irradiated with 600 R (200 kV, 15 mA, 0.5 Cu filter, focus-skin distance 50 cm, dose rate 52,1 R/min., whole body). The animals were killed at appropriate intervals, livers quickly excised and homogenized with teflon-glass homogenizer in 0.25 M sucrose containing medium-A in the presence of bentonite (medium-A: 5 mM $MgCl_2$, 50 mM KCl, 6 mM 2-mercaptoethanol, 0.02 M Tris buffer, pH 7.8). The homogenate was centrifuged at 15 000 × g for 15 min. and the supernatant at 105 000 × × g for 60 min.

Microsomes were suspended in 0.25 M sucrose containing medium-A, treated with sodium deoxy-cholate (1 per cent) and analysed by sucrose density gradient centrifugation.

As it appears from Fig. 1, the ribosomal fraction isolated from unirradiated animals contained a high amount of monomers (50—60 per cent). Already 2 hours after irradiation, the amount of polysomes increased. 12 to 15 hours after irradiation, the monomer ribosomes constituted an appreciably minor part (30—40 per cent) of the ribosomal fraction.

Evaluation of electron microscopic observations and analytical ultracentrifugal analysis confirmed these results.

The radiation-induced enrichment of polysomes cannot be taken for some artificial aggregation of ribosomes, since they proved to be degradable equally either by endogenous or exogenous ribonuclease digestion.

* This work was supported in part by the International Atomic Energy Agency.

To examine the protein synthesizing ability of ribosomes, total particles were isolated (Munro et al., 1964) and incubated with ^{14}C-amino acid in a cell-free system.

Results are shown in Table 1. The incorporation of amino acids by the ribosomes was found to be slightly higher 2 and 4 hours after irradiation as compared to the unirradiated control and kept on increasing between 9 to 15 hours.

These data give some evidence that the enrichment of polysomes in the cytoplasm is connected with the increased functional activity of the ribosomal system.

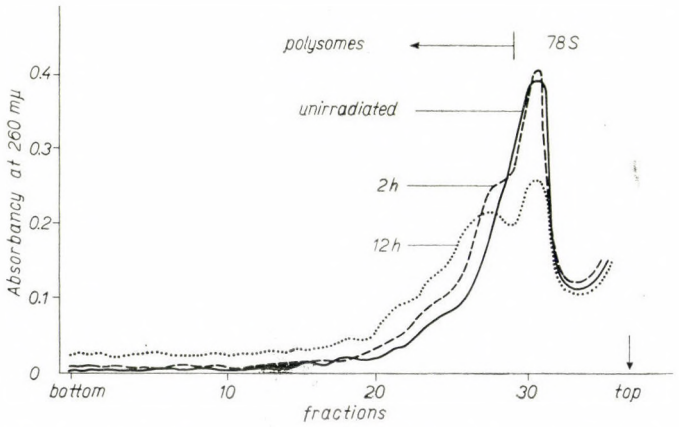

Fig. 1. Density, gradient analysis of liver ribosomes isolated at various times after 600 R X-irradiation. The deoxy-cholate treated microsomes were layered on sucrose gradient (linear, 15—30%). (Sucrose was dissolved in Medium-A containing but no 2-mercaptoethanol.) Centrifugation was performed in rotor SW 39 at 37 000 rpm for 40 minutes. Two drops fractions were collected. Absorbancy was read at 260 mμ. Correction was made for ferritin (Munro el al., 1964) and for sucrose solution. The position of monomer ribosomes was determined by limited digestion with pancreatic ribonuclease (1 μg/ml, 5 min. at 37°)

The experiments raised the problem whether or not the phenomenon exists *in vivo*, too. Therefore, ^{14}C-amino acids were injected to the animals for a short time (60 min) after irradiation with a higher dose (1 800 R, killed 11 hrs later). Indeed, more radioactivity was found in the ribosomal preparate isolated from the liver of X-irradiated animals. Most of radioactivity appeared in the range of polysomes.

The mechanism responsible for the shift to more polysomes in the liver after irradiation connected with increase in the amino acid incorporating ability is unsolved. The enrichment of polysomes involves the presence of more messenger-RNAs in the cytoplasm. As a working hypothesis we may suppose that irradiation enhanced either the synthesis of messenger-RNAs or their transfer from the nucleus to the cytoplasm, or even both. Either of them alone would explain the enrichment of polysomes and increased amino acid incorporation.

Table 1

Effect of X-irradiation on the incorporation of ^{14}C-arginine into ribosomes

Ribosomes obtained from livers of irradiated (600 R) and unirradiated animals were incubated at 37° in a total volume of 1 ml. The mixture contained: ribosomes (1.6 mg RNA), pH 5 fraction preparated from the livers of unirradiated animals (1.5—2.0 mg protein), 20 μmoles Tris-HCl puffer (pH 7.8), 10 μmoles $MgCl_2$, 50 μmoles KCl, 6 μmoles 2-mercaptoethanol, 0.5 μmoles ATP, 0.05 μmole GTP, 5 μmole phospho-enolpyruvic acid, 20 μg pyruvate kinase, 1 μC ^{14}C-L-arginine (uniformly labelled 24.2 mC/mM). The same pH 5 fraction was used for one and the same experimental series. Incubation was stopped by the addition of 5% trichloroacetic acid and the precipitate washed with cold and hot trichloroacetic acid and lipid solvents. The specific radioactivity of protein was expressed in counts/min/mg protein. The numbers in brackets express the percentual changes of radioactivity as compared to the controls

Experiment	Period between X-irradiation and killing.	Specific activity	
		Incubation 60 min.	Incubation 105 min.
1	Unirradiated	2 010 (100)	2 230 (100)
	2 h	2 330 (116)	2 480 (111)
	4 h	2 520 (125)	2 630 (118)
2	Unirradiated	1 250 (100)	1 380 (100)
	9 h	1 760 (141)	2 110 (153)
3	Unirradiated	1 680 (100)	1 770 (100)
	15 h	2 010 (120)	2 510 (142)

The nucleus and in particular the nucleolus are known to react to irradiation by increased activity (Peters, 1963; Braun, 1963). As they are also known to play a basic role in the synthesis of ribosomes and messenger-RNA, the radiation effect on the RNAs in the nucleus and nucleolus should be studied.

Acknowledgement

The authors wish to thank Professor G. Hevesy, Dr. P. Alexander and Dr. F. Antoni for their helpful suggestions during the course of these experiments or for their reading the manuscript.

References

Braun, H. (1963) in Scherer, E., Stender, H. S. (editors), Strahlenpathologie der Zelle, Georg Thieme, Stuttgart, p. 233

Butler, J. A. V., Cohn, P., Crathorn, A. R., (1956) in Hevesy, G.,Forssberg, A. G., Abbatt, J. D. (editors), Advances in Radiobiology, Oliver and Boyd, London, p. 33

Hevesy, G. (1949) Nature, *164* 269

Hidvégi, E. J., Kálmán, E., Lónai, P., Szabó, L. D., Lovas, B., Várterész, V. (1964) Abstract Paper at the Sixth International Congress of Biochemistry, New York

Hidvégi, E. J., Kálmán, E. , Lónai, P., Bölöni, E., Holland J., Várterész, V. (1965) Abstract Paper at the 2nd Meeting of Federation of European Biochemical Societies, Vienna

Munro, J., Jackson, R. J., Korner, A. (1964) Biochem. J., *92* 289

Peters, K. (1963) in Scherer, E., Stender, H. S. (editors) Strahlenpathologie der Zelle, Georg Thieme, Stuttgart, p. 32

Richmond, J. E., Ord, M. G., Stocken, L. A. (1957) in Biochem. J., *66* 123

Smit, J. A., Stocken, L. A. (1963) Biochem, J., *89* 10

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 111–112 (1966)

Bound Water in Muscle

(Preliminary Report)

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(Received October 23, 1965)

In the opinion of our institute, the muscle is not an "osmotic sack" but at least partly a swollen system (Ernst, 1963). Dydynska and Wilkie (Dydynska and Wilkie, 1963), however, recently have reinvestigated this problem, and have suggested that the muscle behaves as a solution.

In order to settle this problem we accepted the working hypothesis that the *own water content* of the muscle has to be decreased, because, the smaller the water content, the stronger it is "bound". Therefore the own water of the muscle was examined in our experiments. Similar investigations already were done (Ernst et al., 1950), and now we approached the problem by studying the density of the muscle water. If the muscle dries up step by step, the density of the muscle water must increase. This proves the increase in its "boundedness".

Frog's gastrocnemius was used in these experiments. The muscles were dried by putting them into a little open glass vessel and then placed into a vacuum exsiccator containing $CaCl_2$.

The single muscles were weighed in different phases of drying. The densty of the muscles was measured by the floating method (other methods are not discussed this time). The floating method is carried out by placing the muscles inio a chloroform-benzene mixture. (The temperature of the mixture was 23°Ct.) For this procedure a series of mixtures with increasing densities was prepared. The density of the mixture in which the muscle was floating was measured by areometer. The measurement of density was followed by drying; after drying weighing and measurement of density followed, then again drying, and so on. The results of one experiment are given schematically in Table 1.

Table 1

Change of the mass (M) and the density (d) of muscle during drying

Phase	M(g)	d(g/cm³)
1.	0.96	1.065
2.	0.78	1.085
3.	0.50	1.130
4.	0.25	1.275

The density of muscle water can be expressed at a certain degree of drying by the formula

$$d = \frac{M - m}{V - v},$$

where M and V are the mass and volume of the muscle in a certain case of water decrease, while m and v are the mass and volume of the dry substance of the muscle, respectively. Supposing that there is only water loss during the drying of muscle, the content of the dry substances remains constant. The weight of the dry mass is 0.190 g, its density is 1.3 g/cm^3. Thus according to the data of Table 1, the density of the muscle water in the 3rd phase is

$$d_3 = \frac{0.500 - 0.190}{\frac{0.500}{1.130} - \frac{0.190}{1.300}} = 1.047,$$

in contrast to the initial value, which is

$$d_1 = \frac{0.960 - 0.190}{\frac{0.960}{1.065} - \frac{0.190}{1.300}} = 1.019$$

In relation to this result we refer to Meyer and Marks investigations (Meyer and Mark, 1928), according to which 1 g caoutchouc binds 32 g benzene or 29 g chlor-benzene. The bound amount of fluid does not bring about osmotic pressure ("real" osmotic pressure), it rather plays a role in the swelling process (Ostwald, 1919) According to Katz's investigations (Katz, 1924), a swollen system is able to produce several thousands of atmospheric pressure at a relative water content of 0.1 to 0.03. On the other hand, in the case of great pressure, the water, despite its small compressibility ($\sim 5 \times 10^{-5}$ at^{-1}) may undergo a significant increase tn density.

The results presented in this paper may be subject to criticism. Therefore, this work should be considered as an initial step taken to solve a complex problem.

Acknowledgement

The author expresses his thanks to Professor E. Ernst who called his attention to this question.

References

Dydynska, M. a. Wilkie, D. R. (1963) J. Physiol. *169* 312
Ernst, E. (1963) Biophysics of the Striated Muscle, Academic Press, Budapest. p. 98
Ernst, E., Tigyi, J., Zahorcsek, A. (1950) Acta Physiol. Acad. Sci. Hung. *1* 5
Katz, J. R. (1924) Ergebnisse der exakten Naturwissenschaften III. Verlag Von Julius Springer, Berlin, p. 385
Meyer, K. H., Mark, H. (1928) Ber. dtsch. chem. Ges. *61* 1939
Ostwald, W. O. (1919) Kolloid Z. *24* 68

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 113–114 (1966)

Na—K Exchange in Active Muscle

(Preliminary Report)

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(Received February 10, 1966)

On the basis of the literature and our own experiments the following concept has been adopted by us (Ernst, 1963): The potassium content of the striated muscle is electively accumulated in the anisotropic bands of the fibrils and is partly replaced by sodium during activity. The very extensive literature on this problem published since our first papers dealing with this question (Ernst and Scheffer, 1928; Ernst and Csúcs, 1929) will not be discussed in this short preliminary note; it is sufficient to say that it has not provided a decisive proof of this concept so far. Having succeeded in preparing single fibrils from frog's striated muscle, we tried to perform autoradiographic experiments. Hind legs of frogs (Rana

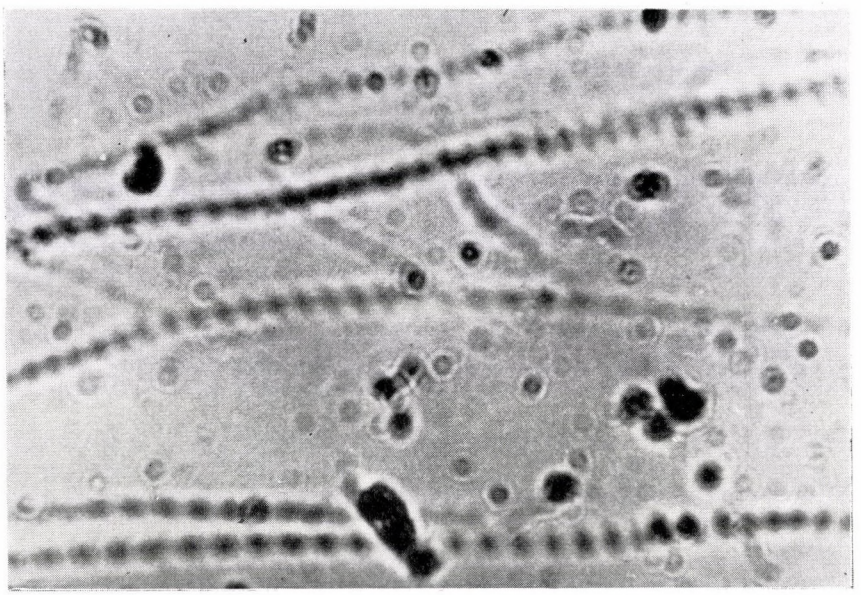

Fig. 1. Fibrils of sartorius muscle; some radioautographic traces belonging to the anisotropic bands (in order to make the traces more conspicuous the fibrils are less clear-cut)

esculenta) were perfused with Ringer's solution containing Na^{24} and stimulated directly till fatigue. From the sartorius taken from such legs and kept in formaldehyde for an hour single fibrils were prepared on a slide and covered with photographic emulsion. Fig. 1 shows a part of such a slide after the emulsion has been developed; several traces caused by Na^{24} and belonging to the anisotropic bands can be seen. These experiments should be extended in different directions (e. g. electronmicroscopy), on the one hand, and evaluated very cautiously, on the other.

References

Ernst, E. (1963) Biophysics of the Striated Muscle. Akadémiai Kiadó, Budapest
Ernst, E. and Scheffer, L. (1928) Arch. ges. Physiol. *220* 655
Ernst, E. and Csúcs, L. (1929) Arch. ges. Physiol. *223* 663

Printed in Hungary

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

A kézirat nyomdába érkezett: 1966. II. 2. — Terjedelem: 10 (A5) ív, 64 ábra

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31. V. 1966 Index: 26.030

VOLUME 1
NUMBER 2
1966

Acta biochimica et biophysica

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 115–126 (1966)

Studies on Proteins and Protein Complexes of Muscle by Means of Proteolysis II.

Preliminary Characterization of the Proteolytic Fragments of Myosin Obtained Under Untraditional Conditions

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(Received October 26, 1965)

The ultracentrifuge pattern of myosin digests, obtained at low ionic strength or at low ionic strength in the presence of 0.01 M $CaCl_2$ is rather similar to the patterns of traditional digests i.e. ones obtained by digestion of myosin in 0.5 M KCl. Further observations, however, showed that especially during digestion in the presence of Ca protein fragments other than the meromyosins were formed: When myosin is digested at low ionic strength in the presence of Ca, protein and ATP-ase* remain largly undissolved. Viscosity of the whole digest decreases to a significantly lower degree than without Ca. The protein going in solution has a high specific ATP-ase activity and an extremly low intrinsic viscosity. The protein undissolved has an intristic viscosity higher than that of myosin.

It is suggested that in the presence of Ca fragmentation ensues at an other region of the myosin molecule than in the traditional case: a "HMM subfragment-1"-like protein is formed directly from myosin and correspondingly a molecule remains, which encloses a greater part of the "tail" of the myosin molecule than ordinary LMM does.

Introduction

We have reported in a previous paper that the rate of tryptic proteolysis of myosin is greatly influenced by the ionic milieu, more precisely by the kind and concentration of cations present (Biró and Bálint, 1966). At low concentration of KCl myosin (being precipitated under these circumstances) is digested substantially faster than when dissolved at high ionic strength i.e. under conditions of the preparation of meromyosins. When $CaCl_2$ or $MgCl_2$ is added to the myosin suspension precipitated in 0.02 M KCl, the proteolysis is markedly depressed. The analysis of the time course of proteolysis gave results which suggested that the changes in kinetics might be explained by changes of the secondary and/or tertiary structure of myosin caused by cation binding. This suggestion leads to

* List of abbreviations:
DFP = diisopropylfluorophosphate, HMM = heavy meromyosin, LMM = light meromyosin, HMM, fr. 1. = heavy meromyosin fraction 1, ATP-ase = adenosinetriphosphatase.

the hypothesis that under our "untraditional" conditions fragments differing from the meromyosins hitherto studied are formed. In this paper we report on some experiments which seem to support this assumption.

Materials and Methods

For the preparation of myosin (Portzehl et al., 1950) and of actin (Bárány et al., 1954) conventional methods were used. Crude actin extract was purified by ultracentrifugation (Mommaerts, 1952).

Before digestion myosin was dialyzed overnight against 0.02 M KCl + 0.02 M borate-NaOH buffer of pH 8.2. (In the following: "borate-KCl".) The resulting fine precipitate was distributed by pipetting for the different experiments. If needed it could be kept in this state for several days without deterioration, as judged by its ATP-ase activity.

LMM and HMM were prepared by the precedure of Szent-Györgyi (1953) with the slight modification described in our foregoing paper (Biró and Bálint, 1966).

Trypsin and chymotrypsin were lyophilized preparations of the firm *Kőbányai Gyógyszergyár*. The standardization of trypsin was described in our foregoing paper (Biró and Bálint, 1966).

In general Worthington soya-bean trypsininhibitor was used to stop the action of trypsin. On the preparative scale and with chymotrypsin proteolysis was stopped by the addition of DFP (0.01 M in the case of trypsin, 0.001 M with chymotrypsin).

ATP-ase activity was measured in the following test system: 4 mM $CaCl_2$, 50 mM tris-HCl buffer of pH 7.0, 0.5 M KCl, 22°C. The time of incubation was 3 to 5 minutes, depending on activity, the protein content was 0.5–1.5 mg/ml.

Removal of Ca from the Ca containing systems was carried out by dialyzing the samples overnight against the desired saline and adding to the outer fluid a substantial amount of an iminodiacetate resin in K^+ phase. ("KKO 1" – an experimental product of the Institute for Plastics Research, Budapest.)

Protein content was measured by the biuret method of Gornall et al. (1949), phosphorus estimated by the method of Fiske and SubbaRow (1925).

Viscosity measurements were carried out in an Ostwald type viscosymeter in a volume of 4 ml having an outflow time with saline at 0°C of about 1 min.

Preparative ultracentrifugation was carried out in a "Spinco, Modell D" ultracentrifuge, analytical ultracentrifugations with a "Beckman–Spinco-E-HT" ultracentrifuge.

Results

During the kinetic studies described in our previous paper (Biró and Bálint, 1966) a striking difference was observed between the digestion of myosin in 0.02 M borate of pH 8.2 + 0.02 M KCl (in the following: "borate-KCl") and the above

mixture supplemented with 0.01 M $CaCl_2$. In borate-KCl myosin dissolves completely in the first minutes of trypsin treatment, whereas in the presence of Ca the protein remains precipitated even when digestion is continued for a considerable time.

The difference is not due simply to a depressed rate of proteolysis in presence of Ca. The difference persists if proteolysis in presence of Ca is continued up to an equivalent or substantially higher base consumption than in the former

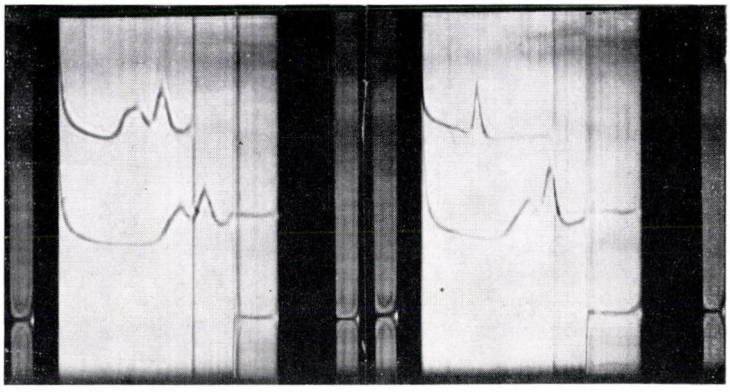

Fig. 1. Ultracentrifugation of different digests

Digestion at 22°C for 18 minutes at a myosin concentration of 10 mg/ml, trypsin concentration: 86 μg/ml in 0.02 M KCl and 0.01 M borate-NaOH buffer, pH 8.2, with the following additions: A, *lower curve:* none, A, *upper curve:* 0.5 M KCl, B, *lower curve:* 0.01 M $CaCl_2$, B, *upper curve:* undigested myosin. Digestion was stopped by addition of trypsin inhibitor to a final concentration of 160 μg/ml. The sample containing Ca was dissolved by addition of 0.5 M KCl and was stirred subsequently with 0.1 volume (wet) aminodiacetate resin in K^+ form for two hours at 0° and centrifuged. All four samples were dialyzed for 24 hours against 20 vols of 0.5 M KCl + 0.01 M borate-NaOH buffer, pH 8.2. Protein concentration of the samples subjected to ultracentrifugation digestion in presence of $CaCl_2$, 7.2 mg/ml; undigested myosin, 2.6 mg/ml; the two others 6.6 mg/ml. Centrifuged at 59,780 r.p.m. (256,000 g) at 20°C. Photographed at A: 50 min., B: 52 min. after reaching full speed; bar angle: 55°

case. It is to be noted that the rate of proteolysis in the presence of Ca is not much lower than that in 0.5 M KCl. It seems thus quite possible that fragmentation takes place also in the presence of Ca.

The patterns obtained in the analytical ultracentrifuge with the three kinds of digests (i.e. with myosin digested in borate-KCl, in borate-KCl + Ca and in 0.5 M KCl, respectively) do not show any rough differences. As it is seen in Fig. 1 in all three cases a rather homogeneous slower and a more or less complex faster peak is obtained. Table I shows the s_{20} values as well as the approximate relative amounts of the proteins as evaluated on the basis of area measurement. As judged by the sedimentation constants, the slow component could be in all three cases LMM. These data do not permit to draw a definite conclusion on the nature of

Table I

Data on the analytical ultracentrifugation of digests shown in Fig. 1
The components are numbered in the order of increasing sedimentation velocity

Sample		s_{20}	Relative area, per cent
Undigested myosin	1.	5.14	89
	2.	—	11
Digested in borate-KCl + 0.5 KCl	1.	2.51	48
	2.	5.14	52
	3.	5.71	
Digested in borate-KCl	1.	2.59	43
	2.	4.65	57
Digested in borate-KCl + 0.01 M $CaCl_2$	1.	2.57	53
	2.	4.32	47

Table II

Solubility and ATP-ase activity of myosin fragments obtained by tryptic digestion under different conditions

Digestion of myosin suspended in 10 ml borate-KCl at 22°C. Trypsin to protein ratio: 1 : 116. Digestion was stopped by addition of trypsin inhibitor in twice the amount of trypsin present, in a volume of 0.2 ml. Separation of undissolved proteins by centrifugation at 10,000 g for 30 min. "Ca" designates 0.01 M $CaCl_2$, borate-KCl: 0.02 M KCl, 0.02 M borate-NaOH, pH 8.2; — in the separations at pH 7.0 borax-borate buffer of pH 7.0 was used. Removal of Ca (if effected) as described in "Methods". Experiments 2—5, and 6—10, respectively, were carried out with two different stock suspensions of myosin. Experiment 1: a mixture of LMM plus HMM in a proportion of 1 : 3

Conditions		Initial conc-s		Conc-s in the supernatant			
of digestion	of separation	protein mg/ml	ATP-ase $\mu m\ P$ / ml.min.	protein mg/ml	per cent of total	ATP-ase $\mu m\ P$ / ml.min.	per cent of total
1. 0.5 M KCl, pH 8.8, 18 min.	borate-KCl + Ca	3.92	1.13	3.00	77	1.14	101
2. borate-KCl, 18 min.	borate-KCl	3.60	0.91	3.60	100	0.93	103
3. ,,	borate-KCl + Ca	3.60	0.91	2.80	78	0.83	91
4. borate-KCl, 12 min.	borate-KCl + Ca	3.60	0.91	2.65	73	0.81	89
5. borate-KCl + Ca, 18 min.	borate-KCl + Ca	3.60	0.91	1.08	30	0.29	32
6. borate-KCl + Ca, 16 min.	borate-KCl + Ca	5.00	0.95	1.59	32	0.38	40
7. ,,	pH 7.0, Ca removed	3.20	0.75	2.95	93	0.72	96
8. ,,	pH 7.0, Ca removed and readded	3.20	0.72	1.02	32	0.29	39
9. ,,	borate-KCl, Ca removed	3.20	0.72	2.95	92	0.73	102
10. ,,	borate-KCl, Ca removed and readded	3.20	0.72	1.14	36	0.25	35

the faster component, but the s_{20} values alone do not exclude the possibility of its being HMM.

The analytical ultracentrifuge patterns obtained suggest that the fragments formed in borate-KCl and in this milieu completed with Ca are essentially the well known meromyosins and that the poor dissolution of the proteins in the latter case is caused simply by the presence of Ca. It has been described that Ca^{++} and Mg^{++} lowers the solubility of HMM (Szent-Györgyi, 1953). In Nanninga's (1957) experiments in a concentration of 10 mg/ml HMM starts to dissolve from pH 8.0 onwards, if 0.01 M Ca is present. A detailed study of the solubility of proteins in the different digests, summarized in Table II excludes the possibility that low solubility of the proteins is caused simply by the precipitating action of Ca.

In the experiments of Table II the digests obtained under different conditions were separated into dissolved and undissolved proteins and the amounts of ATP-ase and protein in the former were measured. In some aliquots of the digests this separation was carried out without changing the milieu of digestion while in other samples from the same digests the separation was carried out after changing the ionic composition of the saline by dialysis (or if this was possible by simply adding the ingredients intended). The results shown in Table II point to the following facts:

A model mixture of traditionally prepared meromyosins (LMM : HMM = = 1 : 3) is practically completely soluble in the Ca containing saline used in the experiments (1st row in Table II). If Ca is added *after* digestion in borate-KCl, there is only a negligible enhancement of precipitation (2nd and 3rd row). The greatest part of the proteins as well as of ATP-ase activity remains in the solution. This is not due to the enhanced proteolysis in this milieu leading to an excessive degradation which could possibly prevent precipitation by Ca. If digestion in borate-KCl is conducted for a shorter period, i.e. up to a base consumption equivalent to that measured in digests which have been obtained in the presence of Ca, we get essentially the same distribution between precipitate and supernatant upon addition of Ca (4th row). However, when Ca is readded to digests obtained in the presence of this ion and previously freed of it the protein precipitates, whereas with digests obtained in the absence of Ca no precipitation occurs upon addition of this ion (experiments 8 and 10).

The data presented in Table II clearly show that the fragments formed under different conditions of digestion are not identical, but they give no information about the extent to which they differ. Altered solubility can possibly be due to rather small changes in the finer structure of the fragments, the overall build up being essentially the same as suggested by the ultracentrifuge patterns. Viscosimetric studies of the digestion process in the presence of Ca, however are indicative of a markedly different shape of the fragments formed under these conditions. In Fig. 2 we present the viscosity changes of myosins digested by trypsin under the three conditions in question. As it is seen, the presence of Ca depresses markedly the decrease of viscosity as compared to the viscosities in borate-KCl

and in 0.5 M KCl. This latter digestion shows essentially the picture observed in earlier studies by other authors (Mihályi and Szent-Györgyi, 1953; Gergely et al., 1955). The results of ultracentrifugation seem to exclude the possibility

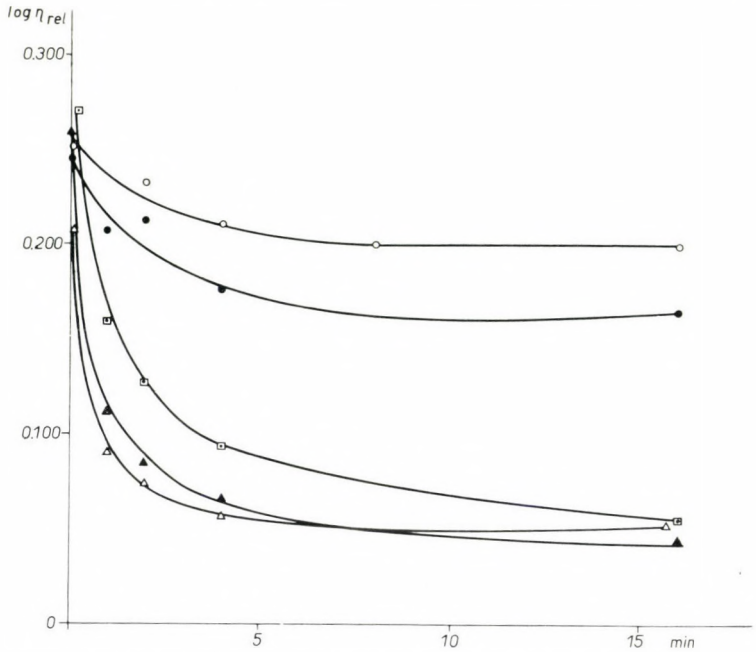

Fig. 2. Changes in the viscosity of myosin during digestion by trypsin and chymotrypsin in different salines

Digestion was carried out at 22°C in 0.02 M borate buffer, pH 8.2. Tryptic digestion was performed at a concentration of 10.4 mg/ml myosin and 0.104 mg/ml trypsin, chymotryptic digestion at myosin concentration of 11.4 mg/ml with 0.502 mg/ml chymotrypsin. At the time intervals indicated on the abscissa aliquots were pipetted into a certain volume of the same saline containing trypsin inhibitor (twice the amount of trypsin) or DFP (in the case of chymotrypsin). The samples were made up to 0.5 M with respect to KCl, diluted to a total protein concentration of 2.5 mg/ml, the decrease in protein concentration upon digestion being neglected, and the viscosity was measured. Ordinate: $\log \eta_{rel}$. Circles: digestion in presence of 0.01 M $CaCl_2$. Open circles: trypsin; full circles: chymotrypsin. Triangles: digestion in 0.5 M KCl; open triangles: trypsin; full triangles: chymotrypsin. Squares: digestion in 0.02 M KCl with trypsin

that the depressed viscosity change is due simply to a low extent of fragmentation. If we take the viscosity obtained in 0.5 M KCl at 16 min. as the value characteristic for complete fragmentation, the viscosity measured in the presence of Ca would suggest 60 per cent of unfragmented myosin (for the foundation of this type of calculation see: Mihályi, 1953). This amount certainly would not escape detection in the ultracentrifuge. Besides this reasoning the experiments performed with chymotrypsin (circles and triangles in Fig. 3) are pointing likewise against

a lack of fragmentation in presence of Ca. It was reported in our foregoing paper (Biró and Bálint, 1966) that Ca does not inhibit the proteolysis of myosin by chymotrypsin (as assessed by proton liberation). In contrast to this observation

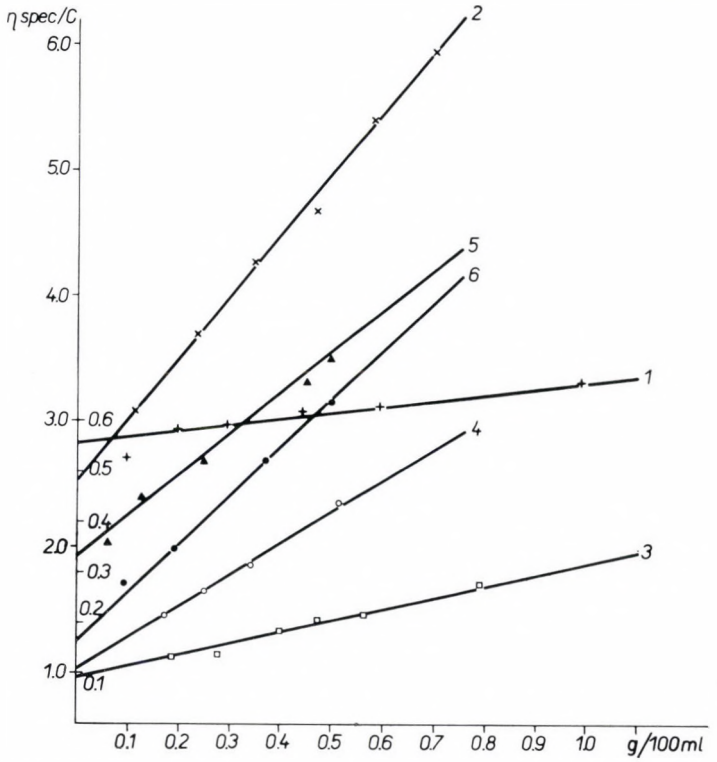

Fig. 3. Reduced viscosity plotted against concentration with different myosin fragments Abscissa: concentration in g/100 ml; ordinate: reduced viscosity. For curves 1 and 3 scale on the inside, for the others scale on the outside of the ordinate. Curve 3: soluble fraction, — curve 2: precipitate fraction, — curve 1: HMM, — curve 4: LMM, — curve 5: myosin, — curve 6: a mixture of LMM and myosin in a proportion of 2 : 1. Preparation of the soluble and precipitate fractions: a myosin suspension 14 mg/ml was digested in the saline containing 0.02 M KCl, 0.02 M borate-NaOH buffer (pH 8.8), 0.01 M $CaCl_2$, at 22°C for 20 minutes. Myosin to trypsin ratio 100 : 1. Digestion was stopped by adding DFP to a final concentration of 0.01 M. — The digest was separated by centrifugation. The precipitate was dissolved and reprecipitated as follows: dissolution in 0.5 M KCl, removal of Ca by adsorption on resin, dialysis against borate-KCl, pH 8.2, plus 0.01 M $CaCl_2$. For viscosimetry both preparations were freed from Ca and brought to 0.5 M with respect to KCl (in the case of the soluble fraction by dialysis)

Fig. 2 shows that the change of viscosity is depressed by Ca in the case of this proteolytic enzyme, too.

If we evaluate the intrinsic viscosities of the proteins going into solution and of those found in the precipitate there remains no doubt that the differences

observed in the experiments of Fig. 2 are due to far reaching differences in the molecular shape of the fragments formed in the presence of Ca. In Fig. 3 we have plotted the reduced viscosity as a function of concentration for the soluble fraction (curve 3) and for the precipitate fraction (c. 2). For the sake of comparison the results of analogous measurements for HMM (1) for LMM (4) for myosin (5) and for a mixture of myosin plus LMM (1 : 2 by weight; – 6) are also included in Fig. 3. The curves obtained can be extrapolated to a quite low value for the soluble fraction (somewhat below 0.1) and to the surprisingly high value of 2.5 for the precipitate fraction. The curves for myosin, LMM and HMM give values in accordance with literary data. Curve 6 is included to show that the precipitate cannot be a mixture of undigested myosin and of LMM. This curve as expected, can be extrapolated to 1.3, i.e. to the weighted average of the intrinsic viscosities of myosin and LMM ($0.33 \times 2 + 0.66 \times 1 = 1.32$).

The fractions used in the experiment of Fig. 3 were prepared from a digest obtained at pH 8.8 and the precipitate fraction was dissolved and reprecipitated again as specified in the legends to Fig. 3. Nearly all of the protein (92 per cent) was recovered. It should be noted that practically the same intrinsic viscosities were obtained for the two fractions concerned when digestion proceeded at pH 8.2 and when the precipitate fraction was not freed from Ca, but simply dissolved in 0.5 M KCl.

On the basis of the above findings there can be no much doubt that at low ionic strength in the presence of Ca trypsin treatment of myosin liberates fragments which differ substantially from ordinary meromyosins. According to some preliminary data the soluble protein (or a substantial part of it) is rather similar to the "subfragment 1" obtained by Mueller and Perry (1961, 1962) by digesting HMM. It has a high specific ATP-ase activity. When it is ultracentrifuged in the presence of polymerized actin, 50–80 per cent of the ATP-ase activity is sedimented. It shows in the ultracentrifuge a paucidisperse peak with a well defined average sedimentation velocity, giving an s_{20} of 4.6 at a protein concentration of 6 mg/ml, i.e. a value similar to that given by Mueller and Perry (1962) for subfragment 1. The value of intrinsic viscosity found by us for the soluble fraction is practically the same as that reported for "subfragment 1" by Young et al. (1964). The precipitate shows in the ultracentrifuge the presence of three major components. In the preparative ultracentrifuge, when centrifuged with actin roughly half of the protein and 80 per cent of ATP-ase is sedimented.

Discussion

The rather detailed picture we have on the architecture of the myosin molecule (see e.g. Woods et al., 1963) allows to make some hypotheses on the process of fragmentation in the presence of Ca. We assume that the protein in the soluble fraction resembling subfragment–1 of HMM of Mueller and Perry (1961, 1962) is formed directly from myosin during its digestion in the presence of Ca and not

via HMM. Digestion of HMM is but negligibly influenced by Ca (Biró and Bálint, 1966). Therefore it is quite improbable that the primary digestion product would be HMM, which in the presence of Ca would be completely degraded to a subfragment – 1-like protein of low asymmetry. There is no doubt on the other hand that in the precipitate fraction of digests obtained in the presence of Ca there is at least one component substantially more asymmetric than myosin. This protein again cannot be formed by a further degradation of one of the meromyosins. The digestion of HMM even in the presence of Ca is accompanied by a great viscosity drop. We did not evaluate the intrinsic viscosity of HMM digested in the presence of Ca, but if we extrapolate using the data given in our foregoing paper by help of the Arrhenius formula $\left([\eta] = \frac{2.3 \times \log \eta_r}{c}\right)$ we obtain a value of 0.13. When on the other hand LMM is digested in the presence of Ca (under conditions where its proteolysis is strongly depressed) we obtain an insignificant lowering of viscosity even if the undissolved proteins are separated and dissolved. As on digestion in the presence of Ca neither LMM nor HMM give rise to the highly asymmetric molecule found in the same type of digests of myosin we assume that this fragment comprises substantial segments of the myosin molecule which in the case of traditional fragmentation are in *both* fragments. On the basis of this reasoning we suggest that during the course of digestion in the presence of Ca, at least in a substantial part of the myosin molecules, the random "neck", the splitting of which leads to the formation of meromyosins (Mihályi and Harrington, 1959; Woods et al., 1963) remains intact and fragmentation occurs in a region situated near to the globular part of the molecule. This mechanism would produce a molecule less asymmetric than HMM on one hand and a molecule similar to LMM but much longer on the other. This picture is in agreement with the results of our kinetic studies. These experiments show first, that in the presence of Ca the splitting of the "random" region is minimum or nil, second, that at low ionic strength (as compared to the digestion in 0.5 M KCl) in HMM there are more bonds accessible for splitting (Biró and Bálint, 1966).

This picture on the fragmentation in the presence of Ca raises the question whether the "beheading" of the myosin molecule alone could give rise to molecules which have higher intrinsic viscosities than myosin. In order to explain the high intrinsic viscosity observed it could be assumed, that concomitant with the loss of the subfragment part of the molecule some unfolding occurs, which gives rise to molecules longer than myosin. As far as we know the dependence of viscosity on the shape has not been studied yet theoretically with such an asymmetric molecule as myosin is suggested to be. There remains, however, the possibility to use the well established formulas for rodlike molecules.

We have computed the intrinsic viscosity for the "tail" remaining after "beheading" of the myosin molecule, assuming different lengths and diameters. In Table III we show the values obtained with a diameter of 18 Å, regarded as the most probable value (Young et al., 1964). The intrinsic viscosity of the asymmetric component in a purified state is not yet known, but it cannot be less than 2.5,

Table III

Calculated intrinsic viscosity values for molecules of different length and a diameter of 18 Å

The figures in column 3 were calculated on the basis of the Kirkwood-Auer equation for string of beads model, those in column 4 on the basis of the Scheraga—Mandelkern equation. The molecular weights were calculated assuming a uniform cylinder model and aspecific gravity of 1.41 for the dry protein. (See: Young et al., 1964.) For the calculation of the viscosity values the formulas and tabulated functions given by Yang (1961) were used.

Length in Å	Molecular weight in 10^5 g	Intrinsic viscosity	
		Kirkwood—Auer equation	Scheraga—Mandelkern equation
1200	2.60	1.96	1.38
1400	3.02	2.58	1.84
1600	3.46	3.34	2.28
1800	3.90	4.12	2.82
2000	4.33	4.95	—

i. e.the intrinsic viscosity found for the whole inhomogeneous precipitate fraction. As it is seen, when calculating with the formula for a "string of beads" model (Kirkwood—Auer equation) at 1400 Å, with the method of equivalent hydrodinamic ellipsoid (Scheraga—Mandelkern equation) at about 1700 Å we reach the intrinsic viscosity values of 2.5. [The calculations were carried out using the formulas and tabulated functions as given by Yang (1961)]. The molecular weights corresponding to these two lengths (calculating with a density of 1.41, see Young et al., 1964) are 302000 and 368000 respectively, i.e. accepting 600000 as the molecular weight of myosin a sufficient portion of the molecule remains for the formation of the subfragment (or subfragments?) having probably a molecular weight of 100000 (Young et al., 1964). For the whole length of the myosin molecule electronmicrographes reveal average values ranging from 1546 Å to 1680 Å (Rice, 1964; Zobel and Carlson, 1963; Huxley, 1963) but according to the last named author it cannot be excluded that the molecules of average length seen in the preparations are in fact all degraded, due to some structural peculiarity which may lead to a systematic breakage during the course of preparation. Hence, according to this author the upper limit of possible length is 2000 Å.

Regarding the considerations presented above we suggest that in the case of tryptic fragmentation of myosin at low ionic strength in the presence of Ca, the molecule is broken into two parts just under the globular portion.

It is interesting to note that this peculiar type of fragmentation is found with chymotrypsin as well (if one can conclude this from the reduction by Ca of the viscosity change). It follows that there is no direct causal link between the depression by Ca of the number of peptide bonds split on one hand (which is observed only in the case of trypsin) and the peculiar mechanism of fragmentation caused by the presence of Ca on the other. If it were so, the fragmentation of

myosin ought to be regarded more specific with respect to the individual peptide bonds than it was hitherto assumed. In the case of chymotrypsin the difference of molecular change in the presence of Ca must be attributed to the lack of splitting of only a few bonds, which is negligible relative to the bulk of bonds split. There is another possibility, namely, that in the presence of Ca chymotrypsin would split other bonds, roughly in the same number as in absence of Ca. In connection with these speculations a recent paper of Chao and Leiner (1965) has to be mentioned in which the effect of Ca on the activation of trypsin, i.e. on a proteolytic process, was shown to be remarkebly specific: In the presence of this ion the splitting is restricted (at least in half of the molecules split) to a single peculiar lysin-isoleucine bond.

Acknowledgements

We are indebted to Ing. A. Czuppon and to P. Kovács, Research Institute for Chemical Structure of the Hungarian Academy of Sciences, for performing the analytical ultracentrifuge experiments.

Thanks are due to Dr. E. Mühlrad for stimulating discussions.

References

Bárány, M., Biró, N. A., Molnár, J. and Straub, F. B. (1954) Acta Physiol. Ac. Sci. Hung. *5* 369
Biró, N. A. and Bálint, M. (1966) Acta Biochem. Biophys. Ac. Sci. Hung. *1* 13
Chao, P. L. and Leiner, I. E. (1965) Biochim. Biophys. Acta *96* 508
Fiske, C. H. and SubbaRow, Y. (1925) J. Biol. Chem. *66* 375
Gergely, J., Gouvea, M. A. and Karibian, D. (1955) J. Biol. Chem. *212* 165
Gornall, A., Bardawill, C. J. and David, N. J. (1949) J. Biol. Chem. *177* 751
Huxley, H. E. (1963) J. Mol. Biol. *7* 281
Mihályi, E. (1953) J. Biol. Chem. *201* 197
Mihályi, E. and Harrington, W. F. (1959) Biochim. Biophys. Acta *36* 447
Mommaerts, W. F. H. M. (1952) J. Biol. Chem. *198* 445
Mueller, H. and Perry, S. V. (1961) Biochim. Biophys. Acta *50* 509
Mueller, H. and Perry, S. V. (1962) Biochem. J. *85* 431
Nanninga, L. B. (1957) Arch. Biochem. Biophys. *70* 346
Portzehl, H., Schramm, G. and Weber, H. H. (1950) Z. Naturforschung *5b* 61
Rice, R. V. (1964) in Biochemistry of Muscle Contradiction, Gergely, J. (editor), Little, Brown and Co., Boston, p. 46
Szent-Györgyi, A. G. (1953) Arch. Biochem. Biophys. *42* 305
Woods, E. F., Himmelfarb, S. and Harrington, W. F. (1963) J. Biol. Chem. *238* 2374
Yang, J. T. (1961) in Advances in Protein Chemistry, vol. 16, Anfinsen, C. B., Anson, M. L., Bailey, K. and Edsall, J. T. (editors), Academic Press New York, London, p. 323
Young, D. M., Himmelfarb, S. and Harrington, W. F. (1964) J. Biol. Chem. *239* 2822
Zobel, G. R. and Carlson, F. D. (1963) J. Mol. Biol. *7* 78

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 127–138 (1966)

Complex Formation of Light Meromyosin with Actin at Low Ionic Strength

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(Received October 27, 1965)

1. If LMM dissolved in 0.25 M KCl is precipitated by dilution at pH 7.0 in the presence of polymerized actin a substantial amount of actin precipitates along with LMM *. The presence of actin in the precipitate was shown by the use of ^{45}Ca-labelled actin, and by its activating effect on myosin (Mg)-ATP-ase, respectively.

2. LMM forms under these conditions a well defined complex in the stoichiometric ratio of 1.3 to 1.5 (actin: LMM). This complex is not decomposed to an appreciable extent by repeated washings at 0.05 ionic strength and pH 7.0.

3. The binding is specific: LMM denatured by heat or by incubation at pH 4.1, further LMM Fr. 1. do not bind measurable amounts of actin.

4. By increasing the ionic strength to 0.15 or the pH up to 8.0 the complex dissociates. The complex is dissociated by ATP, ADP, ITP and pyrophosphate in micromolar concentrations. EDTA (0.01 M) and phosphate (0.0137 M) have no effect.

5. No influence of LMM on the interaction between actin and HMM could be demonstrated under the conditions tested.

Introduction

The results of proteolytic fragmentation of myosin have greatly contributed to our present knowledge of functional muscle structure. The characteristic properties of the fragments formed have been established by the works of several authors. It has been generally accepted, that the ability of myosin to form complexes with actin is due entirely to the H-fragment and that there is no interaction between actin and LMM. During our experiments on tryptic digestion of myofibrils (to be reported in a following paper) we have made some observations which did not fit into the generally accepted views. Experiments along these lines have shown that LMM if precipitated at low ionic strength forms a well characterizable complex with actin. The experiments supporting this finding are presented in this paper.

* Abbreviations: LMM = light meromyosin, LMM Fr. 1. = light meromyosin, fraction 1, ATP-ase = adenosinetriphosphatase.

Materials and Methods

Meromyosins were prepared from myosin obtained by conventional methods (Portzehl et al., 1950) essentially as described by Szent-Györgyi (1953) with the difference, that the action of trypsin was stopped by the addition of 0.01 M DFP (final concentration) instead of by trypsin inhibitor. (A valuable suggestion by Dr. J. Gruda, Warsaw.)

In some experiments LMM dissolved in 0.25 M KCl was centrifuged at 100,000 g for two hours in order to remove some impurities. LMM treated in this way lost its turbidity and resembled myosin solutions. The absence of myosin in our LMM preparation was tested by its ATP-ase activity which was found to be low in agreement with literary data, and also by adding about 20 per cent (by weight) of actin to the protein dissolved in 0.25 M KCl. At this ionic strength actomyosin should be precipitated, but no precipitate was observed. On one occasion LMM dissolved in 0.5 M KCl was mixed with an equal amount (by weight) of actin and centrifuged at 105,000 g for three hours. When tested in our standard system for actin binding LMM treated in this way behaved as our usual LMM preparations.

LMM Fr. 1. was prepared according to Szent-Györgyi et al. (1960).

Actin was extracted at 0 °C (to avoid contaminations by tropomyosin, see Drabikowski and Gergely, 1962) from dried muscle powder prepared according to Bárány et al. (1954) and was purified further by the procedure of Mommaerts (1952).

Actin labelled with ^{45}Ca was prepared essentially as described by Bárány et al. (1962). Radioactivity of samples dried on small aluminium plates was counted in a Frisecke – Höpfner scaler with a gas flow GM-tube.

In all experiments actin polymerized with KCl was used.

Ca-ATP-ase and Mg-ATP-ase activities were measured in the following reaction mixtures: 0.01 M Tris, pH 7.0, ATP 0.002 M, $MgCl_2$ 0.002 M, or 0.05 M Tris-buffer, pH 7.0, ATP 0.004 M, $CaCl_2$ 0.004 M, KCl 0.4 M. The 2 ml samples containing varying amounts of protein, and incubated at 22°C for different time periods (both specified in the legends for the individual experiments) were deproteinized with 2 ml of a 10 per cent TCA solution. Inorganic phosphorus was estimated in 2 ml aliquots of the filtrate according to Fiske and SubbaRow (1925).

The protein content of the stock solutions was measured by the conventional biuret method (Gornall et al., 1949). With diluted protein solutions obtained in experiments on complex formation the color given by the biuret reaction was read at 290 mμ, where it is about 10 times more sensitive than at the wavelength used in conventional methods. Under these conditions different substances, especially those giving a complex with Cu can seriously interfere. Therefore in the case of this "sensitive biuret reaction" appropriate blanks were used. The readings in U. V. light were standardized to an LMM solution which had been purified by ultracentrifugation and the protein content of which had been measured by the traditional biuret method.

The experiments on complex formation were carried out at 0°C usually as follows: the appropriate protein mixture was prepared using protein stock solutions in 0.25 M KCl. The mixture was made up to 1.5 ml with 0.25 M KCl and was subsequently diluted five fold with 0.02 M borax-borate buffer, pH 7.0 (the pH was carefully controlled and readjusted before use, if needed). The resulting precipitate was separated by centrifugation at 20,000 g for 20 min. After the separation the supernatant and/or the precipitate were subjected to further tests, aimed at the qualitative and quantitative analysis of the proteins present.

Results

The protein concentration in the supernatant of an actin-LMM mixture precipitated by dilution is substantially less than it would be expected on the basis of actin present in the system. If there were no interaction between actin and LMM in a saline of low ionic strength all actin would stay in the supernatant. In our experiment we have found less protein in the supernatant owing to the precipitation of some actin along with the precipitating LMM. In Table I results of two experiments carried out with different sets of protein preparations seem to support this finding. As it is seen when increasing amounts of actin are added to a constant amount of LMM the increments of protein in the supernatant are substantially less than the increments of added actin. After a certain actin concentration has been reached the protein content of the supernatant begins to raise more rapidly i.e. the precipitating LMM becomes saturated with actin.

Table I

Co-precipitation of actin with LMM

	Amount of actin in the system (mg)	Amount of unprecipitated LMM + actin (mg)	Amount of protein in the supernatant (mg)	Amount of actin precipitated (mg)	Actin : LMM ratio	
					in the total system	in the precipitate
	a	*b*	*c*	$d = b - c$	*e*	*f*
	A) Amount of LMM in the system: 6.12 mg; unprecipitated on dilution: 1.16 mg; precipitated (by difference): 4.96 mg					
1	3.06	4.22	2.48	1.74	0.5	0.35
2	6.12	7.28	3.36	3.92	1.02	0.79
3	9.18	10.34	5.75	4.59	1.53	0.93
	B) Amount of LMM in the system: 4.17 mg; unprecipitated on dilution: 1.28 mg; precipitated (by difference): 2.89 mg					
1	4.30	5.58	3.12	2.46	1.07	0.85
2	6.50	7.78	3.24	4.54	1.56	1.60
3	8.65	8.93	5.15	3.78	2.08	1.65
4	13.10	14.38	9.15	5.23	3.16	1.81

Table II

Complex formation between LMM and labelled actin

Total amount of LMM in the system 2.1 mg; unprecipitated on dilution: 0.65 mg; precipitated (by difference) 1.45 mg

No of sample	1	2	3	4	Average
a) Amount of actin in the system (mg)	2.52	3.22	4.43	5.25	—
b) Counts/min with actin only, diluted to the final volume	352	442	630	780	—
c) Counts/min per mg actin	145	137	142	135	138
d) Counts/min in the supernatant	130	202	292	472	—
e) Counts/min in the precipitate ($b - d$)	222	240	338	308	—
f) Precipitated amount of actin estimated by radioactivity measurement in the supernatant (mg) $\frac{(b - d)}{\text{average of } c}$	1.60	1.74	2.54	2.22	—
g) Amounts of added actin + unprecipitated LMM (a + 0.65 mg)	3.17	3.87	5.08	5.90	—
h) Amount of protein in the supernatant (mg)	1.50	2.25	3.14	4.05	—
i) Amount of actin precipitated, estimated by protein measurement in the supernatant (mg) ($g - h$)	1.67	1.62	1.94	1.85	—
j) Actin: LMM ratio in the precipitate, estimated by radioactivity measurement	1.10	1.20	1.75	1.53	1.39
k) As *j)* estimated by protein test	1.15	1.12	1.34	1.27	1.27
l) Actin: LMM ratio in the total system	1.20	1.54	2.11	2.50	—

The evidence obtained in the experiments shown in Table I is weakened to some extent by the fact that it is based simply on protein estimation, a method which does not discriminate between the two proteins present in the system. Though it is very improbable indeed that the deficit in the protein content of the supernatant would result from a decrease in the solubility of LMM caused by the presence of actin it seemed to us necessary to test this hypothesis with more specific methods. The experiment shown in Table II is analogous to the first two experiments but actin labelled with Ca was used and in the supernatant in addition to protein content radioactivity was measured as well. In this way we have two independent methods to estimate the amount of actin precipitating along with LMM. (Table II, f and i.) The two results are in a very good agreement. Table II shows further that in spite of raising the amount of actin nearly two fold the amount

of actin precipitated remains practically constant. The average of actin: LMM ratio when estimated by protein determinations is found to be 1.27, on the basis of radioactivity measurements a value of 1.39 is found.

The presence of actin in the acto-LMM precipitate was shown also by the effect of the protein precipitated to enhance the Mg-ATP-ase activity of myosin. When this precipitate was dissolved in 0.5 M KCl and increasing amounts of this solution were added to myosin a gradual increase in the Mg activated ATP-ase activity of myosin was observed as expected when actin is added (Table III). It is known that LMM does not influence the ATP-ase activity of myosin and has but a negligible enzyme activity of its own. Therefore the activation observed was due to the actin present.

Table III

Activation of myosin-ATP-ase by acto-LMM

Acto-LMM (a mixture of 5.98 mg actin and of 4.45 mg LMM precipitated as in the foregoing experiments) was dissolved in 0.5 M KCl. The composition of this solution was (calculated as in the experiments presented in Table I and Table II) 2.94 mg actin and 4.66 mg LMM pro ml. Different amounts of this solution as indicated were added to 4.48 mg myosin in 1 ml. Mg—ATP-ase measurements were carried out taking 0.8 ml aliquots of the mixtures. Specific activity expressed as μM P/min/mg *myosin*

Added actin-LMM mixture (ml)	Myosin : actin	Specific activity
none	—	0.038
0.15	10.2	0.043
0.30	5.0	0.061
0.48	3.2	0.080
0.63	2.4	0.127

The results presented above suggest that when LMM is precipitated in the presence of actin a considerable quantity of actin goes into the precipitate despite the fact that actin alone is completely soluble under the above experimental conditions. This was verified in separate experiments using the same actin preparations. The experiments to be described are intended to prove that the phenomenon is due to the formation of a specific complex between actin and LMM.

The observation that the bond between actin and LMM is rather strong seems to support this assumption. Repeated washings of the acto-LMM precipitate (with 0.05 M KCl + 0.01 M borax-borate of pH 7.0) did not solubilize measurable amounts of protein.

The specificity of binding is substantiated by the quite definite stoichiometric ratio between LMM and the amount of actin bound. If we compare the results of quite different experiments made with different protein preparations on different occasions by plotting the LMM: actin ratio found in the precipitate as a function of the same ratio in the total system (Fig. 1) it is apparent that LMM

always binds a defined quantity of actin. Above a certain actin: LMM ratio there is saturation. In spite of the scattering of the data the maximum binding ratio lies between 1.3 – 1.6 (actin/LMM; weight by weight).

The specific nature of the binding is corroborated by the findings of experiments with LMM denatured by heat or by incubation at low pH, respectively (Table IV). The results show that denatured LMM does not bind actin in spite

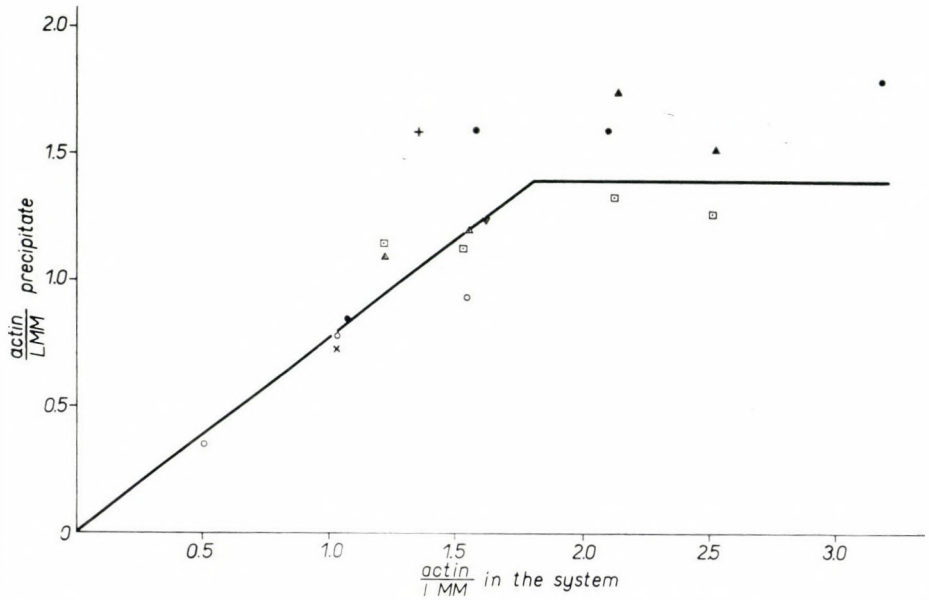

Fig. 1. Composition of acto-LMM precipitate in dependence of actin: LMM ratio in the total system. Summary of the pertaining data of experiments represented in Tables I., II., III., IV., and VII. Abscissa: actin: LMM ratio in the total system, ordinate: the same in the precipitate. ○: Table I/A; ●: Table I/B; △: Table II, by radioactivity; □: Table II, by protein measurement; +: Table III; ×: Table IV; △: Table VII

of being precipitated much like the undenaturated one. Similarly we did not find practically any binding with LMM Fr. 1 (Szent-Györgyi et al., 1960). In some further experiments we used LMM Fr. 1, because it is generally considered to be a more homogeneous purified form of LMM (Szent-Györgyi et al., 1960; Lowey and Cohen, 1962). LMM, on the other hand, may contain traces of unfragmented myosin impurities, due to the method of its preparation. The exclusion of the presence of myosin seemed essential from the point of view of our problem. Thus LMM Fr. 1, which cannot contain undenatured myosin because of the alcohol treatment used during its preparation, seemed to be an especially suitable material. Oddly enough we did not find any binding with our LMM Fr. 1 preparations (Table V).

The specificity of binding with regard to the other component could not be tested with the help of denaturation because denaturation results also in the

Table IV

Non-reactivity of actin with denatured LMM

LMM solution (in 0.25 M KCl) was heated to 100°C for 1 min. or was acidified to pH 4.1 by the addition of 0.1 volume of 0.1 M acetate buffer and neutralized again after 60 min. of incubation at 22°C. Amount of LMM (native or denatured) 5.77 mg, that of actin 5.70 mg

LMM	Amount of protein (mg) in the supernatant when prec. in the		Amount of actin in supernat. (mg)	Amount of actin precipitated (mg)	Amount of LMM precipitated (mg)
	absence of actin	presence of actin			
	(*a*)	(*b*)	(*c*) = (*b*) − (*a*)	(*d*) = 5.70 mg − (*b*)	(*e*) = 5.77 mg − (*a*)
Native	1.65	4.95	3.30	2.75	4.12
Heat denatured	no	6.00	6.00	(−0.30)	5.77
Denatured at pH = 4.1	0.60	5.92	5.32	(−0.22)	5.17

Table V

Non-reactivity of LMM Fr. 1. with actin

Amount of LMM Fr. 1. in the system 6.70 mg; unprecipitated 0.44 mg; precipitated (by difference) 6.26 mg

Amount of actin in the system (mg)	Amount of unprec. LMM Fr. 1. + actin (mg)	Amount of protein in supernatant (mg)	Amount of actin precipitated (mg)	Actin: LMM Fr. 1	
				in the total system	in the precipitate
a	*b* = 0.44 mg + *a*	*c*	*d* = *b* − *c*	*e*	*f*
2.00	2.44	1.66	0.78	0.32	0.12
3.80	4.24	3.34	0.90	0.61	0.14
5.65	6.09	5.00	1.09	0.90	0.17
7.90	8.34	6.45	1.89	1.26	0.30

depolymerization of actin. It seemed to be more interesting to test a protein quite foreign to muscle proteins. The experiment along these lines (Table VI) shows that if LMM is precipitated in the presence of hemoglobin no protein is being co-precipitated.

On the basis of all these experiments we have no doubt that a specific acto-LMM complex is formed at low ionic strength. In the following we give some preliminary characterization of acto-LMM.

According to the data presented in Table VII with increasing ionic strength acto-LMM goes into solution; at high ionic strength the complex dissociates. It is to be noted that the amount of actin precipitated diminishes more rapidly than the amount of LMM in the precipitate. The actin: LMM ratio decreases to an insignificant value at an ionic strength where nearly half of the LMM present

Table VI

Specificity of actin to bind LMM

To 5.95 mg LMM varying amounts of horse hemoglobin were added and LMM was precipitated by dilution and the samples were centrifuged as in the experiments with actin. The extinctions of the supernatants at 545 mμ were compared to those of hemoglobin solutions at the same dilutions

Amount of hemoglobin added (mg)	Extinction of the supernatant	Extinction of hemoglobin at identical dilutions
1.5	0.170	0.172
3.0	0.327	0.320
4.5	0.480	0.483
6.0	0.630	0.620

Table VII

Dissociation of acto-LMM by high ionic strength

Samples containing 1.95 mg LMM and 3.14 mg actin in 1.5 ml 0.25 M KCl were diluted with borax-borate buffer to 7.5 ml. Amounts of KCl were added to reach the ionic strength indicated

Ionic strength	Amount (mg) of protein in the supernatant with		Amount of (mg) LMM in supernatant + actin	Amount of precipitated (mg)		Actin : LMM in the precipitate
	LMM only	LMM + actin		Actin	LMM	
	a	b	c = 3.14 mg + a	$d = c - b$	e = 1.95 mg − a	$f = d/e$
0.05	0.68	2.25	3.82	1.57	1.27	1.24
0.11	0.75	3.08	3.89	0.81	1.20	0.68
0.15	1.35	4.35	4.49	0.14	0.60	0.23
0.25	No precipitate					

still remains precipitated. The effect of ionic strength to induce dissociation is not influenced by 1 mM of Mg or Ca. EDTA (1 mM) has no effect on complex formation.

According to preliminary experiments at an ionic strength of 0.15 μ ATP, ADP and ITP in 10^{-3} M concentration dissociate the complex but 10^{-4} M has no influence. These findings show that the acto-LMM complex is less sensitive to the action of nucleotidetriphosphates than actomyosin or acto-HMM. In 10^{-4} M concentration ATP or ITP considerably influence the viscosity response or the superprecipitation of actomyosin (see e.g. Maruyama and Gergely, 1962). As the effect of inorganic pyrophosphate is much like that of ATP it seems to us probable that the effects concerned are not specific but are due to polyvalent anions, though the question has not been investigated in more detail yet. It should be noted that phosphate ions do not induce dissociation. If the actin−LMM mixture is pre-

cipitated by dilution with M/150 phosphate buffer at pH 7.0, the actin precipitates just like with the borax-borate buffer used routinely.

On the basis of the above findings the question arises how the interaction between actin and HMM is influenced by the binding of LMM to actin. The elucidation of the question is made somewhat difficult by the fact, first mentioned by Leadbeater and Perry (1963) for HMM prepared with chymotrypsin that acto-HMM at low ionic strength is not a homogeneous solution. In spite of using much lower protein concentrations than in the experiments cited, if the acto-HMM solution which seems to be homogeneous is centrifuged at 20,000 g for 20 minutes most of the protein and ATP-ase present are sedimented. If increasing amounts of HMM are added to a constant amount of actin, the amount of HMM sedimenting (estimated by measuring ATP-ase activity in the supernatant) rapidly declines (see Table VIII). In these mixtures containing relatively high HMM concen-

Table VIII

Reaction of acto-LMM with HMM

Tests: to 4.6 mg actin + 6 mg LMM in 1.5 ml 0.25 M KCl, the amounts of HMM (in 0.02 M borax-borate) indicated were added, the mixtures were diluted to 7.5 ml with borax-borate buffer of pH 7.0 and centrifuged as described in "Methods". Control 1: the same mixtures, LMM omitted Control 2: HMM only. At corresponding dilutions Ca—ATP-ase activity in the tests and the control were measured with aliquots of 0.8 ml for 3 minutes. Activity expressed as extinction obtained in phosphorus determination, with the extinction of the blanks substracted

HMM added (mg)	ATP-ase activity		
	in the supernatant	in control 1.	in control 2.
none	0.05	—	—
3.35	0.04	0.04	0.16
6.70	0.06	0.05	0.32
10.50	0.05	0.07	0.41
13.40	0.33	0.39	0.50
16.75	0.52	0.45	0.63

trations the separation by centrifugation seems to be incomplete probably because of the high viscosity of the solution. We have to assume that besides the saturation of actin with HMM incomplete sedimentation plays a significant role, because paradoxically enough the total enzyme activity of the sediment is substantially less with more HMM present that when sedimented with the same amount of actin and less HMM. This behaviour of our acto-HMM preparations makes the conclusions somewhat uncertain but as the results presented in Table VIII show, the presence of a considerable amount of LMM is of no influence. It seems as though HMM would easily displace LMM in the complex. If increasing amounts of LMM are added to a constant amount of acto-HMM similar results are obtained: the presence of LMM does not influence the amount of HMM precipitated (Table IX).

Table IX

Reaction of acto-HMM with LMM

To 10.5 mg HMM and 4.48 mg actin the amounts of LMM indicated were added, the mixtures were made up to 1.5 ml and were 0.25 M in KCl, diluted to 7.5 ml with borax-borate buffer of pH 7.0, and centrifuged as described in "Methods". Ca—ATP-ase activity was measured with 0.8 ml aliquots for 7 minutes. Activity is expressed as the extinction obtained in phosphorus determination minus the extinction of appropriate blanks

Composition of the mixture centrifuged	ATP-ase activity in the supernatant
HMM only	0.39
HMM + actin	0.12
HMM + actin + 2.28 mg LMM	0.14
+ 4.56	0.17
+ 6.84	0.12

Results in accordance with these experiments were obtained when an effort was made to show the competition between LMM and HMM (or myosin) for actin. In these experiments no influence of LMM on the Mg activated ATP-ase of acto-HMM or acto-myosin was detected.

Discussion

It is to be noted first of all that the results of our experiments do not contradict the data of other authors. All the experiments showing the non-reactivity of the proteins concerned apply ultracentrifugation or viscosimetric techniques with proteins dissolved at high ionic strength (Mihályi and Szent-Györgyi, 1952; Gergely et al., 1952; Szent-Györgyi,* 1953). Neither did we find any complex formation at ionic strengths bringing about dissolution of LMM (in 0.15—0.20 M KCl) as judged by viscosimetry. We think that the experiments presented are not related to the findings of Kalamkarova and Nankina (1964) either, the only paper in which, on the basis of an increase in double refraction of flow of LMM solutions upon addition of actin an interaction between the two was established. These authors have suggested in a later work (Kalamkarova et al., 1965) that the reaction concerned is given by a certain protein fraction present in LMM preparations. This fraction was tentatively identified as the SH-containing protein separated from LMM by Lowey and Cohen (1962). As described in the "Methods", we obtained unchanged complex formation with an LMM, which was purified by ultracentrifugation in the presence of actin (at high ionic strength). Any protein

* He did not find any binding between actin and LMM by adding actin to LMM, precipitated in 0.05 M KCl, 0.005 M Mg and 1 mg/ml ATP. Our experiments suggest that this was due to the presence of ATP.

forming a complex with actin under these conditions should sediment together with the later.

A rather unexpected finding in this paper is the non-reactivity of LMM Fr. 1 with actin. In our laboratory another similar fact has been found in experiments on the Ca binding of muscle proteins: LMM Fr. 1 lacks the high-affinity binding sites found in LMM (Biró et al., to be published).

LMM Fr. 1. seems to be a native protein and structurally closely related to myosin and to LMM in spite of the alcohol treatment used for its preparation. This is shown by its antigenicity and by the fact that antisera prepared against LMM Fr. 1 will react with myosin (Holtzer et al., 1957). This indicates that there are identical protein structures in LMM Fr. 1 and myosin. In our opinion the two findings are not in contradiction: there are several instances where a protein looses one biological property as a result of some treatment while others remain. It is interesting to note that tropomyosin, another muscle protein which withstands alcohol treatment has been suggested recently (Ebashi and Ebashi, 1964), to exist in muscle in a form the original activity of which gets lost during the traditional isolation procedure.

Based on the fact of complex formation between actin and LMM interesting suggestions could be made concerning the molecular mechanism underlying the superprecipitation of actomyosin gel, which is by no means completely understood as yet. If the interaction of the two proteins rests uniquely on the binding between actin and the HMM part of myosin it is rather difficult to account for the fact that under the conditions of superprecipitation, which in the case of acto-HMM leads to dissociation actin does not go into solution. If, however, the LMM part of myosin would bind to actin, too, and this binding would be less sensitive to the effect of ATP to induce dissociation, as suggested by our experiments, this difficulty could be resolved by the assumption of a two-point attachment between the two interacting proteins. The suggestion of an actin-LMM interaction *in vivo* is, however, hardly acceptable on the basis of recent findings by Huxley (1963) on the architecture of the A-rods of myofibrils. According to these investigations it is highly probable that when the molecules are assembled to form the A-rods the "tail" of the myosin molecule, i.e. the LMM part aggregates side to side and in this arrangement the actin located in the I-filament cannot approach the LMM structures. Regarding these difficulties an eventual functional role of the actin-LMM interaction described here is regarded as a remote but not entirely negligible possibility.

Acknowledgement

We are indebted to Dr. E. Mühlrad for several interesting suggestions during discussions on the actual work in our laboratory.

References

Bárány, M., Biró, N. A., Molnár, J. and Straub, F. B. (1954) Acta. Physiol. Ac. Sci. Hung. *5* 369
Bárány, M., Finkelman, F. and Therattil-Antony, T. (1962) Arch. Biochem. Biophys. *98* 28
Drabikowski, W. and Gergely, J. (1962) J. Biol. Chem. *237* 3413
Ebashi, S. and Ebashi, F. (1964) J. Biochem. (Tokyo) *55* 604
Fiske, C. H. and SubbaRow, Y. (1925) J. Biol. Chem. *66* 375
Gergely, J., Gouvea, M. A. and Karibian, D. (1955) J. Biol. Chem. *212* 165
Gornall, A., Bardawill, C. J. and David, N. J. (1949) J. Biol. Chem. *177* 751
Holtzer, H., Marshall, J. M. and Finck, H. J. (1957) Biophys. Biochem. Cytol. *3* 705
Huxley, H. E. (1963) J. Mol. Biol. *7* 281
Kalamkarova, M. B. and Nankina, W. P. (1964) Biofizika *9* 383
Kalamkarova, M. B., Nankina, W. P. and Kofman, E. B. (1965) Biofizika *10* 166
Leadbeater, L. and Perry, S. V. (1963) Biochem J. *87* 233
Lowey, S. and Cohen, C. (1962) J. Mol. Biol. *4* 293
Maruyama, K., Gergely, J. (1962) J. Biol. Chem. *237* 100
Mihályi, E. and Szent-Györgyi, A. G. (1953) J. Biol. Chem. *201* 211
Mommaerts, W. F. H. M. (1952) J. Biol. Chem. *198* 445
Portzehl, H., Schramm, G. and Weber, H. H. (1950) Z. Naturforschung *5b* 61
Szent-Györgyi, A. G. (1953) Arch. Biochem. Biophys. *42* 305
Szent-Györgyi, A. G., Cohen, C. and Philpott, D. E. (1960) J. Mol. Biol. *2* 133

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 139—148 (1966)

The Mechanism of Ion Transport in Human Erythrocytes

I. The Role of 2.3-Diphosphoglyceric Acid in the Regulation of Potassium Transport

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(Received November 22, 1965)

There is a close correlation between 2.3-diphosphoglycerate metabolism and potassium transport. The rate of potassium outflow observed in the absence of adenosine triphosphate is determined to a great extent by the enzyme activity of 2.3-diphosphoglycerate-phosphatase. Whenever the enzyme is inhibited, or is under conditions characterized by lack of substrates available, an outflow of potassium of a considerable rate, exceeding 5 milliequivalents per liter erythrocytes per hour starts. The presence of calcium ions is a prerequisite of this phenomenon.

Introduction

The concentration of 2.3-diphosphoglyceric acid (2.3-DPGA)* is strikingly high in human erythrocytes, more than 50 per cent of the total acid soluble organic phosphate esters is present in the form of this compound (Rapoport, Guest, 1941). The physiological role of 2.3-DPGA is not known exactly, yet it has been postulated long ago that this phosphoorganic ester played a definite role in the maintenance of physiological equilibrium of ions (Guest, Rapoport, 1939). This hypothesis has been based among other facts on data of comparative physiology (Kerr, 1937). Mammals can be divided into two main groups with respect to their content in 2.3-DPGA:

1. Species belonging to the first group (e.g. man, pig, rabbit, rat) are characterized by high 2.3-DPGA content (4 to 6 μmoles per ml); potassium content of the erythrocytes of these species is also high.

2. The 2.3-DPGA content of animals which belong to the second group (e.g. cat, beef, goat) is hardly one tenth of the values characteristic for the first group; potassium content in the erythrocytes of these species is also low, ranging from one fifth to one tenth of the values of the first group.

Along these lines experiments have been carried out in order to investigate the connection between 2.3-DPGA content and potassium transport of human red blood cells. The effect of purine nucleosides (adenosine in the first place) has

* Abbreviations: 2.3-DPGA = 2.3-diphosphoglyceric acid, ATP = adenosinetriphosphate, 3-PGA = 3-phosphoglyceric acid, IA = iodoacetate.

been investigated in the presence of various glycolytic inhibitors. The results of these experiments have been reported in several papers (Gárdos, 1956, 1960, 1961). The conclusions of these investigations have pointed to a definite correlation between 2.3-DPGA metabolism of the erythrocytes and potassium *outflow*. The present paper was aimed at the further support and extension of these experimental results.

It has been shown that 2.3-DPGA in the erythrocytes can be split selectively in the presence of $NaHSO_3$, while glycolytic activity and ATP content are not influenced to a considerable extent (Mányai, Várady, 1956, 1958; Mányai, 1959). The mode of action of $NaHSO_3$ is still a debated question. In Mányai's opinion the activation of 2.3-DPGA-phosphatase (EC 3.1.3.13) is responsible for the effect in the first place. At the same time no activating effect of this type has been found by Sauer and Scholz (1965) who were working with a purified enzyme preparation. Anyhow working in the presence of $NaHSO_3$ has yielded, a method for the investigation of the effect of 2.3-DPGA on cation transport independent from glycolysis.

Methods

Fresh, defibrinated human blood was used in the *in vitro* experiments at 37°C. Samples contained 75 per cent of normal blood; substrates, inhibitors and activators were present in the remaining 25 volume per cent. Red blood cell ghosts were prepared according to the method of Straub (1953), Gárdos (1954).

The two methods used parallelly for the estimation of ATP were the following:

a) Ion exchange chromatography (Deutsch, Nilsson, 1953);

b) enzymatic test of Boehringer with the aid of phosphoglyceraldehyde-dehydrogenase and phosphoglycerate-kinase (Adam, 1963).

2.3-DPGA was estimated by ion exchange chromatography (Bartlett, 1959), lactic acid was determined according to Barker, Summerson (1941), inorganic phosphate according to Fiske, SubbaRow (1925) colorimetrically. Potassium was determined by flame photometry.

2.3-DPGA was prepared by the method of Greenwald (1925) and 3-phosphoglycerate (3-PGA) by the method of Tankó (1941).

Results

Effect of $NaHSO_3$ on the metabolism of human erythrocytes

The results are summarized in Table 1. It may be seen that $NaHSO_3$ (1.5×10^{-2} M final concentration) does not influence the ATP level of erythrocytes, lactic acid production remains unaffected, too. [The slight increase of lactate production is a consequence of a small increase in aldolase and phosphotriose-

Table 1

The effect of $NaHSO_3$ on carbohydrate metabolism in human erythrocytes

Time	Lactate		ATP		Inorg. P		2.3-DPGA	
	μmoles/ml blood							
	A	B	A	B	A	B	A	B
0^h	0.89	0.93	0.59	0.59	1.03	1.03	1.77	1.77
1^h	2.16	2.16	0.57	0.60	0.94	2.74		
2^h	3.22	3.50	0.50	0.57	0.87	4.03		
3^h	4.18	4.90	0.50	0.57	0.81	4.78	1.70	0.09

A: control; B: $NaHSO_3$, 1.5×10^{-2} M (final concentration).

isomerase activities (Meyerhof, Lohmann, 1934).] At the same time the amount of inorganic phosphate is increased markedly. This increase is due merely to the cleavage of 2.3-DPGA; 95 per cent of the 2.3-DPGA content of the erythrocytes is split in the presence of $NaHSO_3$ within 3 hours at 37°C.

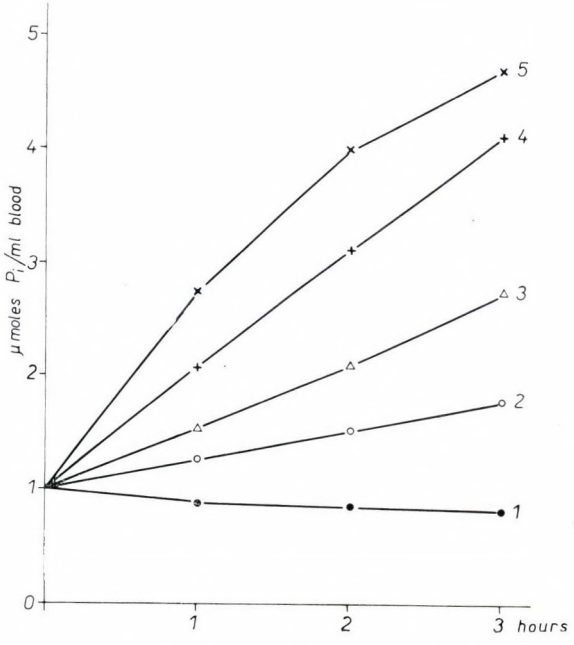

Fig. 1. The effect of increasing concentrations of $NaHSO_3$ on the kinetics of changes in inorganic phosphate (P_i) content of human blood. 1: control; 2: 3×10^{-3} M $NaHSO_3$; 3: 6×10^{-3} M $NaHSO_3$; 4: 1×10^{-2} M $NaHSO_3$; 5: 1.5×10^{-2} M $NaHSO_3$

This effect is dependent on the concentration of $NaHSO_3$. This is seen in Fig. 1, where the increase of the inorganic phosphate level is represented at different concentrations of $NaHSO_3$.

Connection between 2.3-DPGA metabolism and potassium transport in human erythrocytes

The outflow of potassium from the erythrocytes in the presence of 1×10^{-3} M IA starts after 1 hour incubation, the ATP content of the cells is decomposed almost completely by that time (Gárdos, Straub, 1957). The rate of potassium outflow is low in this case, 1.6 meqs per 1 erythrocytes per hour. When the experiments were continued for longer periods, it was found that a sudden raise in the rate of potassium outflow (5.4 meqs per 1 erythrocytes per hour), took place

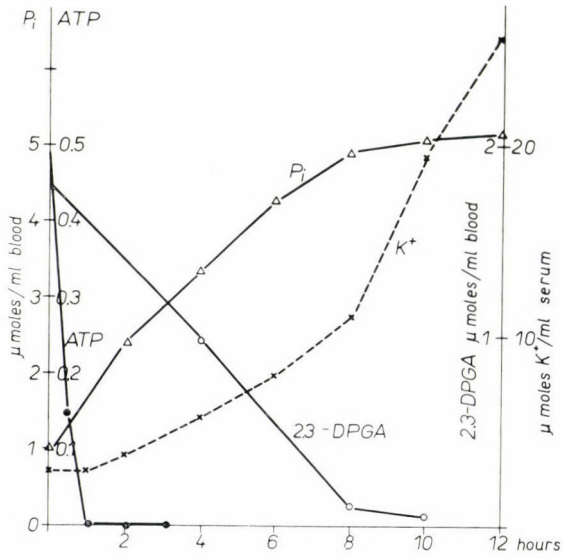

Fig. 2. The effect of IA (10^{-3} M) on the kinetics of changes in ATP, 2.3-DPGA and inorganic phosphate (P_i) content and potassium transport of human blood

after an incubation for 8 hours. The increase in inorganic phosphate and 2.3-DPGA content of the cells was measured in the same experiment and it was found that the beginning of this second, high rate potassium outflow period coincided with the time, when the 2.3-DPGA content decreased to 10 per cent of its original level (Fig. 2).

The breakdown of 2.3-DPGA occurring in the presence of IA, could be accelerated to an optional degree by varying the concentration of $NaHSO_3$. The analysis of the connection between 2.3-DPGA breakdown and potassium trans-

port led to an interesting result: it turned out that high rate potassium outflow started in each case at a given level of inorganic phosphate in the blood (5 μmoles per ml). This level was reached, when 90 per cent of 2.3-DPGA was already split (Fig. 3).

It has been reported in earlier papers (Gárdos, 1956, 1960, 1961) that a high rate potassium outflow was observed in the presence of IA + adenosine. This process could be inhibited by the addition of small amounts of $NaHSO_3$ (3×10^{-3} M).

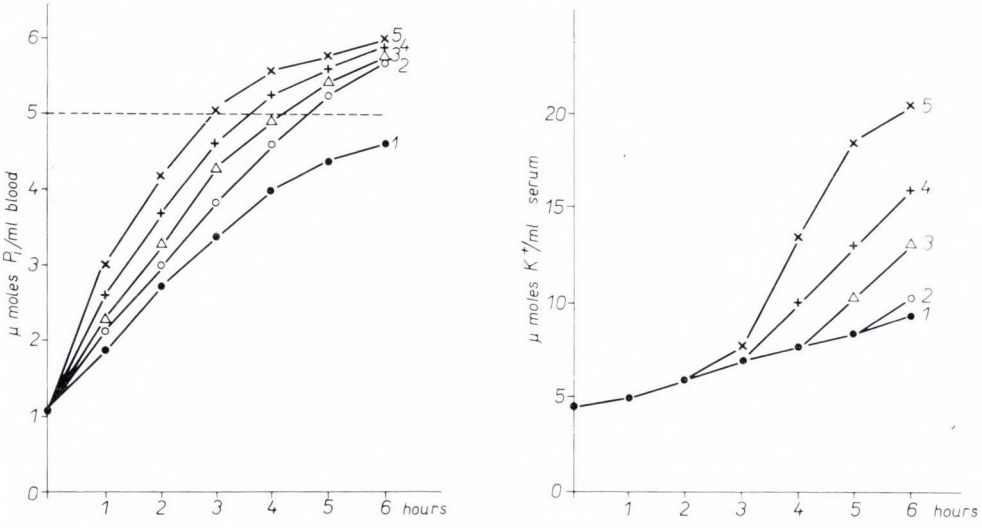

Fig. 3. The effect of increasing concentrations of $NaHSO_3$ on the kinetics of changes in inorganic phosphate (P_i) content and potassium transport of human blood in the presence of IA (10^{-3} M). For markings cf. Fig. 1

The rate of potassium outflow was reduced to 1.6 meqs per 1 erythrocytes per hour, i.e. to the same value which was measured in the presence of IA (10^{-3} M) alone. The inhibition of the rate of potassium outflow lasted for shorter periods when the concentration of $NaHSO_3$ was high: the duration of inhibition was inversely proportional to the concentration of $NaHSO_3$ (Fig. 4). The setting in of high rate potassium outflow coincided in all cases with an almost complete disappearance of 2.3-DPGA, but not with the vigorous breakdown of the same compound elicited by $NaHSO_3$.

It seems very probable that alterations in 2.3-DPGA metabolism play also an important role in the outflow of potassium caused by NaF. Namely, the effect of varying NaF concentrations in the presence of $NaHSO_3$ (1.5×10^{-2} M) on the level of blood inorganic phosphate and potassium content of serum was investigated simultaneously in 3 hours experiments at 37°C and the following patterns were found (Fig. 5): the breakdown of 2.3-DPGA induced by $NaHSO_3$ was inhibited almost completely in the presence of 8×10^{-3} M NaF, 50 per cent

inhibition of this process occurred at 2×10^{-3} M NaF. No outflow of potassium took place at all at this concentration, but it started at higher concentrations (above 6×10^{-3} M), its rate was increasing gradually, reaching a maximum value at the concentration where the 2.3-DPGA splitting activity was inhibited completely.

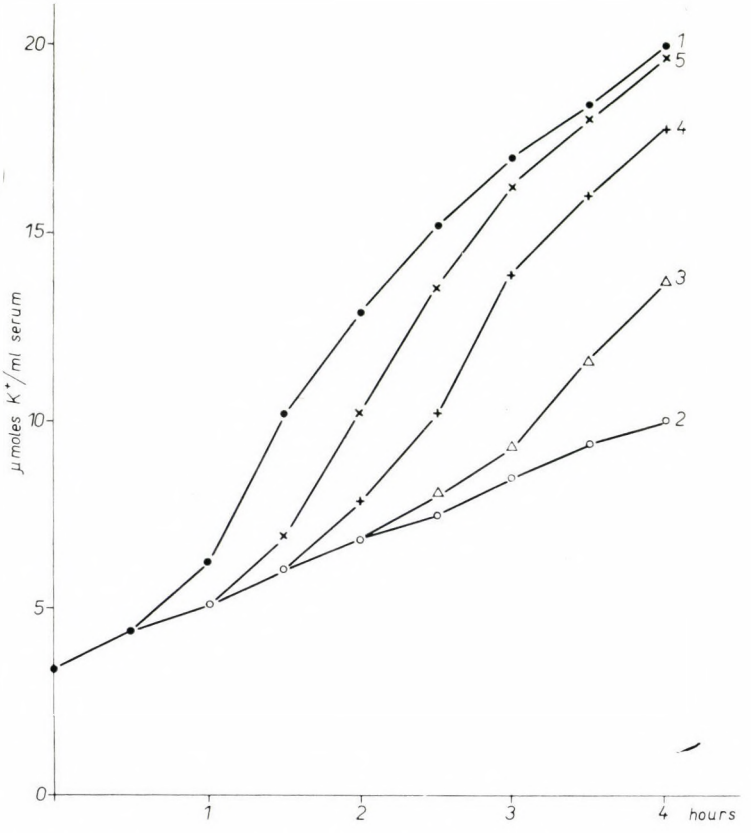

Fig. 4. The effect of increasing concentrations of $NaHSO_3$ on potassium transport of human blood in the presence of IA (10^{-3} M) + adenosine (10^{-2} M). For markings cf. Fig. 1

The rate of potassium outflow (1.6 meqs per 1 erythrocytes per hour) measured in the presence of IA could be increased by the addition of NaF to the reaction mixtures. Samples, which contained both 1×10^{-3} M IA and 4×10^{-3} M NaF at the same time were characterized by an extremely rapid loss of potassium. Although NaF at the concentration just mentioned caused alone only a slight outflow of potassium (1.6 meqs per 1 erythrocytes per hour), when used together with IA rate was nearly eight times higher (12.5 meqs per 1 erythrocytes per hour). The changes in inorganic phosphate content were determined in the same experi-

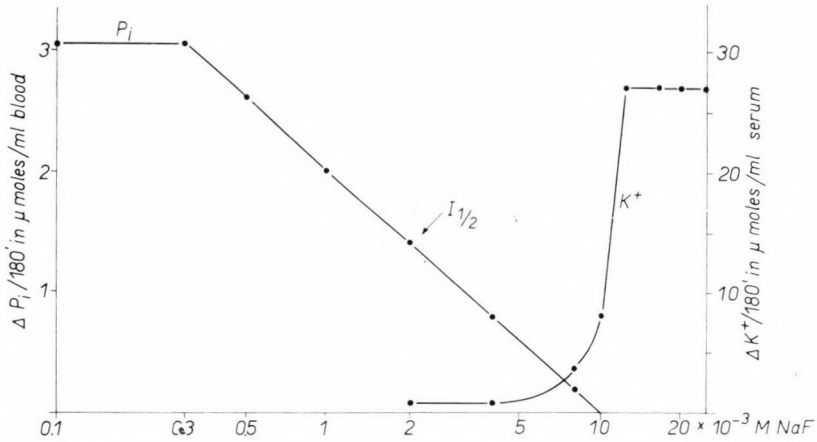

Fig. 5. The effect of NaF at different concentrations on changes in inorganic phosphate (P_i) and serum potassium content of human blood in the presence of $NaHSO_3$ (1.5×10^{-2} M). $I_{1/2}$: 50 per cent inhibition of the maximal activity

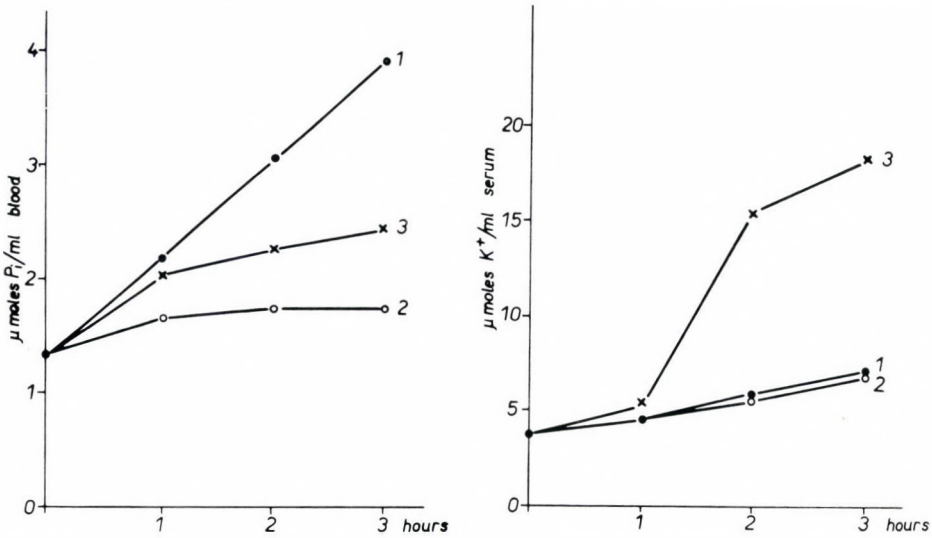

Fig. 6. The effect of IA and NaF on the kinetics of changes in inorganic phosphate (P_i) content and potassium transport of human blood. 1: 10^{-3} M IA; 2: 4×10^{-3} M NaF; 3: 10^{-3} M IA + 4×10^{-3} M NaF

ments and it became evident that NaF was blocking the decomposition of 2.3-DPGA induced by IA to a considerable extent (Fig. 6).

Blocking of 2.3-DPGA-phosphatase activity by 3-PGA is known (Rapoport, Luebering, 1951). Red blood cell ghosts, containing 4×10^{-3} M 3-PGA were pre-

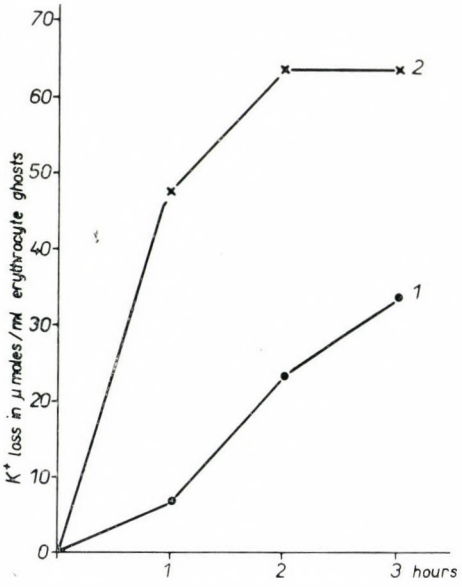

Fig. 7. The effect of 3-PGA on potassium transport of human erythrocyte ghosts treated with NaF. 1: 1.6×10^{-2} M NaF; 2: 1.6×10^{-2} M NaF + 4×10^{-3} M 3-PGA

pared, the rate of potassium outflow was studied in the presence of NaF. It was found that the rate of outflow was merkedly enhanced by 3-PGA. The corresponding values were: 11.7 meqs per 1 red blood cell ghost per hour without 3-PGA and 32.2 meqs per 1 red blood cell ghost per hour in the presence of 3-PGA (Fig. 7).

Discussion

The physiological function of 2.3-DPGA present in high concentrations in various mammalian species is widely discussed. The role of 2.3-DPGA as the coenzyme of phosphoglycerate-mutase is known (Sutherland et al., 1949). Participation of this compound in the regulatory processes of glycolysis as well as in the regulation of the intensity of the hexose monophosphate shunt is unquestionable, as the enzymes hexokinase, transaldolase and transketolase are blocked to a high degree even by physiological concentrations of 2.3-DPGA (Dische, 1941; Dische, Igals, 1963). Furthermore the energy production of glycolysis is influenced by 2.3-DPGA: the increase in the intensity of the 2.3-DPGA shunt is accompanied by a decrease in the intensity of the phosphoglycerate-kinase reaction. This means that one of the ATP synthesizing chemical steps is less efficient.

Nevertheless the significance of 2.3-DPGA, as a non-penetrating anion in the regulatory mechanism of cation transport has been recognized long ago (Guest, Rapoport, 1939). It was assumed that 2.3-DPGA is contributing to the

establishment of physiological Donnan equilibrium. Experiments carried out in this laboratory point to the fact, that the role of this compound is not explained in a satisfactory way by its polyvalent anionic character, but 2.3-DPGA linked enzyme reactions play a definite part in the regulation of cation transport.

Summing up the results of the experiments presented in this paper, factors regulating potassium transport of human erythrocytes may be divided into the following groups:

1. Anaerobic glycolysis and a definite level of ATP produced and maintained as a consequence of glycolysis serves as a fundamental prerequisite for the maintenance of physiological unequal potassium distribution (Straub, 1953; Gárdos, 1954).

2. The energy required for active potassium accumulation against the concentration gradient is provided by ATP (Gárdos, 1954). There is a close connection between the functioning of "transport ATPase" localized in the cell membrane and active cation transport (Post et al., 1960; Dunham, Glynn, 1961; Gárdos, 1964).

3. The inhibition of glycolysis, or the stopping of glycolytic processes by lack of substrates is followed by the disappearance of ATP (Mányai, Székely, 1954). Thus, active cation transport is stopped as well and a low rate potassium outflow (1.6 meqs per 1 erythrocytes per hour) is observed (Gárdos, Straub, 1957).

4. The rate of potassium outflow is regulated by 2.3-DPGA metabolism. Whenever the activity of 2.3-DPGA-phosphatase, an enzyme catalyzing the transformation of 2.3-DPGA to 3-PGA is blocked by inhibitors or by lack of substrate, the rate of potassium outflow increases above 5 meqs per 1 erythrocytes per hour. It is possible that the shift of the equilibrium of adenylyl-2.3-DPGA synthesis and decomposition, discovered by Hashimoto et al. (1961) may have some role in the regulation of ion transport on these occasions.

5. The rapid outflow of potassium produced by the blocking of 2.3-DPGA-phosphatase is observed exclusively in the presence of calcium ions. Whenever the experimental system is deprived of calcium ions (by the addition of chelating substances), high rate potassium outflow is prevented (Gárdos, 1958, 1958a, 1959, 1961a). The role of calcium ions in influencing potassium transport may have most probably a double character. It is based partly on a direct membrane effect (Lepke, Passow, 1960, 1960a; Lindemann, Passow, 1960, 1960a), and partly on the partial inhibition of 2.3-DPGA-phosphatase (Clarkson, Maizels, 1952).

Acknowledgements

Thanks are due to Professor F. B. Straub for valuable advices, and to Dr. S. Hollán for providing the possibilities for this investigation. Thanks are also due to Miss. E. Mészáros for skilful and precise technical assistance.

References

Adam, H. (1963) in Methods of Enzymatic Analysis, Bergmeyer, H. U. (editor), Academic Press, New York p. 539
Barker, S. B., Summerson, W. H. (1941) J. Biol. Chem. *138* 535
Bartlett, G. R. (1959) J. Biol. Chem. *234* 469
Clarkson, E. M., Maizels, M. (1952) J. Physiol. *116* 112
Deutsch, A., Nilsson, R. (1953) Acta Chem. Scand. *7* 1288
Dische, Z. (1941) Bull. Soc. Chim. Biol. *23* 1140
Dische, Z., Igals, D. (1963) Arch. Biochem. Biophys. *101* 489
Dunham, E. T., Glynn, I. M. (1961) J. Physiol. *156* 274
Fiske, C. H., SubbaRow, Y. (1925) J. Biol. Chem. *66* 375
Gárdos, G. (1954) Acta Physiol. Hung. *6* 191
Gárdos, G. (1956) Acta Physiol. Hung. *10* 185
Gárdos, G. (1958) Biochim. Biophys. Acta *30* 653
Gárdos, G. (1958a) Acta Physiol. Hung. *14* 1
Gárdos, G. (1959) Acta Physiol. Hung. *15* 121
Gárdos, G. (1960) Folia Haematol. *77* 138
Gárdos, G. (1961) Haematologia Hung. *1* 230
Gárdos, G. (1961a) in Membrane Transport and Metabolism, Kleinzeller, A., Kotyk, A. (editors), Academic Press, New York p. 553
Gárdos, G. (1964) Experientia *20* 387
Gárdos, G., Straub, F. B. (1957) Acta Physiol. Hung. *12* 1
Greenwald, I. (1925) J. Biol. Chem. *63* 339
Guest, G. M., Rapoport, S. (1939) Amer. J. Dis. Child. *58* 1072
Hashimoto, T., Ishii, Y., Tatibana, M., Yoshikawa, H. (1961) J. Biochem. (Japan) *50* 471
Kerr, S. E. (1937) J. Biol. Chem. *117* 227
Lepke, S., Passow, H. (1960) Arch. Ges. Physiol. *271* 389
Lepke, S., Passow, H. (1960a) Arch. Ges. Physiol. *271* 473
Lindemann, B., Passow, H. (1960) Arch. Ges. Physiol. *271* 488
Lindemann, B., Passow, H. (1960a) Arch. Ges. Physiol. *271* 497
Mányai, S. (1959) Folia Haematol. *76* 350
Mányai, S., Székely, M. (1954) Acta Physiol. Hung. *5* 7
Mányai, S., Várady, Zs. (1956) Biochim. Biophys. Acta *20* 594
Mányai, S., Várady, Zs. (1958) Acta Physiol. Hung. *14* 103
Meyerhof, O., Lohmann, K. (1934) Biochem. Z. *273* 413
Post, R. L., Merritt, C. R., Kinsolving, C. R., Albright, C. D. (1960) J. Biol. Chem. *235* 1796
Rapoport, S., Guest, G. M. (1941) J. Biol. Chem. *138* 269
Rapoport, S., Luebering, J. (1951) J. Biol. Chem. *189* 683
Sauer, G., Scholz, D. (1965) Folia Haematol. *83* 377
Straub, F. B. (1953) Acta Physiol. Hung. *4* 235
Sutherland, E. W., Posternak, T., Cori, C. F. (1949) J. Biol. Chem. *181* 153
Tankó, B. (1941) Magyar Kémiai Folyóirat *47* 162

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 149—157 (1966)

Factors Influencing the Basal Penicillinase Synthesis in Bacillus cereus

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(Received November 22, 1965)

The basal penicillinase synthesis in *B. cereus* was studied under various experimental conditions. In cells germinating from spores basal penicillinase synthesis begins immediately and proceeds at a constant rate. In growing cells the rate of synthesis is directly proportional to the increase in dry matter content. Various influences, such as incubation without carbon and nitrogen sources, chloramphenicol inhibition, etc. result in an increased basal penicillinase synthesis. The results are discussed from the point of view of the control of penicillinase synthesis.

Introduction

The majority of data on the biosynthesis of penicillinase refer to induced synthesis (Pollock, 1950, 1952, 1953; Torriani, 1956), as compared to that of other induced enzymes. The synthesis of basal penicillinase proceeds at a fairly high rate in cells of *B. cereus* (Pollock, 1956), but practically no vital information is available on its regulation. The aim of our experiments was to study the changes in basal synthesis during the life cycle of the bacterium and to investigate the environmental conditions which may affect basal synthesis.

Materials and Methods

The experiments were carried out with strain NRRL-B-569 of *B. cereus*. The nutrient medium consisted of 2.72 g of potassium dihydrogenphosphate, 0.35 g of magnesium sulphate, dried, 1.25 g of sodium citrate, 10.00 g of acid casein hydrolysate (Difco), 1.00 ml of tween 80, 2.50 mg of ferric ammonium citrate, 1.00 ml of trace element solution and distilled water to 1000.00 ml. It had a pH of 7.0 and was sterilized at 110°C. This nutrient medium was, as required, supplemented with 0.4 per cent glucose (w/v).

The trace element solution contained 0.10 mg of $ZnSO_4 \cdot 7H_2O$, 0.10 mg of $CoCl_2 \cdot 6H_2O$, 1.00 mg of $MnCl_2 \cdot 4H_2O$, 0.01 mg of $CuSO_4 \cdot 5H_2O$, 0.10 mg of $(NH_4)_2MoO_4$ and distilled water to 100.00 ml.

For incubation in a carbon and nitrogen free medium the following solution "M" was used to wash the cells: 1.25 g of sodium citrate, 0.35 g of magnesium

sulphate, dried, 2.00 g of sodium chloride, 2.72 g of potassium dihydrogen phosphate, 3.00 g of tris and distilled water to 1000.00 ml. The pH of the solution was 7.0.

B. cereus utilizes no citrate (Torriani, 1956). The method used for measuring penicillinase activity has been described in an earlier communication (Csányi, 1961).

Penicillinase is readily absorbed on glass surfaces (Kogut et al., 1956). We have found that this phenomenon makes enzyme determinations less reliable and used therefore for the incubation of bacterium cultures from which penicillinase activity was determined flasks coated with a 5 mm thick paraffin layer. The overall penicillinase content i.e. the sum of endo- and exopenicillinase was measured. The results are given in units, one unit being the quantity of enzyme which destroys 1 μM penicillin at 30°C in a 0.1 M phosphate buffer solution, pH 6.5, within one hour. Dry matter content of bacterium was measured photometrically in an Extinctiometer type photometer. The values of optical densities were calibrated for dry weight by direct gravimetric measurements.

Experimental Results

The basal penicillinase synthesis of bacterium cells growing from spores. – First, changes in basal penicillinase synthesis during the life cycle of *B. cereus* were studied. Spores were prepared by repeated washings of the centrifuged sediment of a 72 hour old bacterium culture with sterile distilled water and by heat treatment of the spores at 60°C for one hour in order to destroy vegetative cells. The heat treated spores were washed again several times, taken up in the nutrient medium and changes in dry matter content and penicillinase synthesis were followed at 37°C under constant shaking. The results are plotted in Fig. 1. It may be seen that basal penicillinase synthesis begins immediately in the germinating bacterium cells and proceeds at a constant rate during the lag phase without an increase in overall dry substance content. From the beginning of growth the rise in dry substance content and the rate of penicillinase synthesis proceed parallel.

In the following the influence of various growing conditions on basal enzyme synthesis was studied.

In these experiments usually cells in the logarithmic phase of growth were used. By applying the following method for their production well reproducible results were obtained:

Pre-incubation: the nutrient medium was inoculated with a spore suspension and the culture incubated without shaking at 37°C for 15 hours. Under these conditions the spores germinated and the culture started to grow slowly. At this stage 0.4 per cent (w/v) glucose was added and the culture was shaken. The initial value of the dry matter content varied between 0.01 and 0.03 mg per ml and in about 60 minutes the culture entered the logarithmic phase.

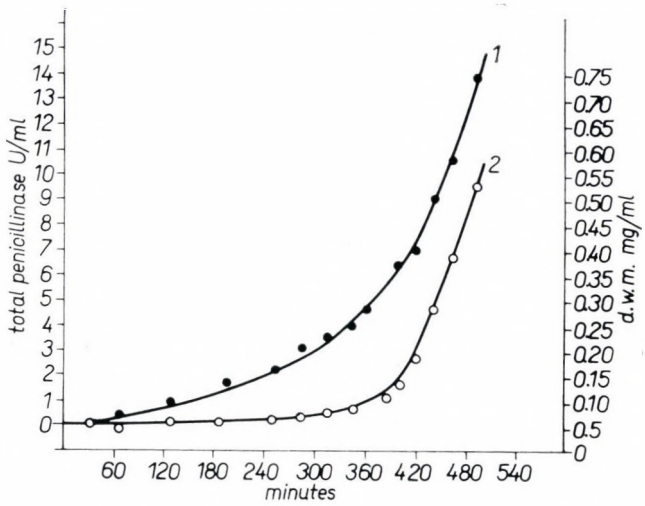

Fig. 1. Penicillinase production and growth of germinating bacterial cells. Curve 1, penicillinase synthesis. Curve 2, growth

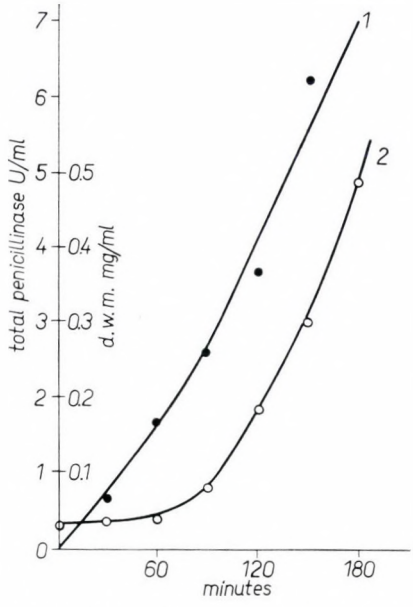

Fig. 2. Penicillinase synthesis and growth of pre-incubated cells. Curve 1, penicillinase synthesis. Curve 2, growth

Changes in the basal penicillinase synthesis of the cells during and after pre-incubation were then examined (Fig. 2). Essentially similar results were obtained as with cells without pre-incubation. Basal penicillinase synthesis pro-

ceeds at a uniform rate even when the increase in dry substance content is still very slow, but later the ratio between the two processes reaches a constant value which corresponds to the value measured with cells grown without pre-incubation.

The experiments were repeated without the addition of glucose. In this case growth was slower after the beginning of shaking, but the rate of increase in dry matter content and of penicillinase synthesis was similar to that in the previous experiment.

In the following experiments pre-incubated cells were used.

Changes in basal penicillinase synthesis after interfering with normal growth. In experiments on penicillinase synthesis the washing of cells to remove the inducer or the penicillinase produced is an often applied operation. Usually a

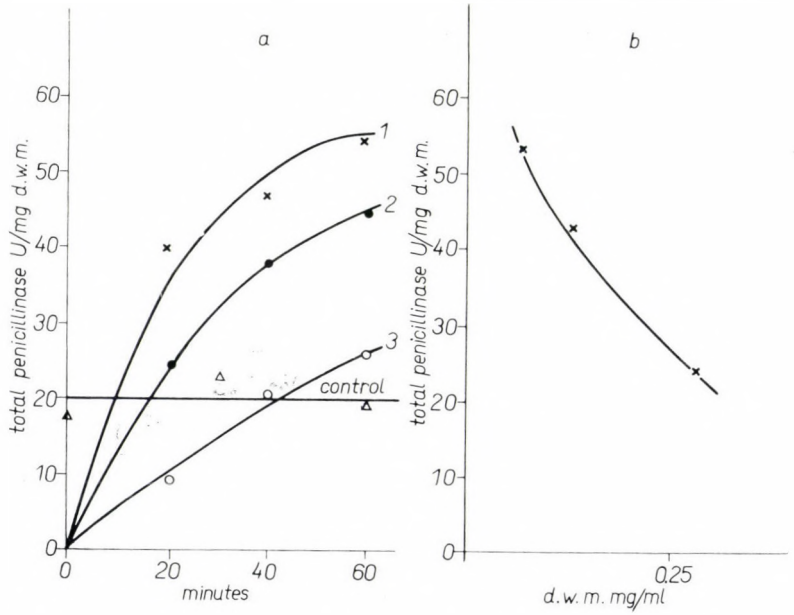

Fig. 3. The differential values of penicillinase synthesis of cells washed with solution "M". *a)* Changes of the differential values versus time. The dry substance content of the initial culture: *1.* 0.075 mg per ml. *2.* 0.125 mg per ml. *3.* 0.250 mg per ml. In all three cases the dry substance contents of the cultures were adjusted to 0.10 mg per ml after washing. Changes of the differential value of the initial culture during the logarithmic phase (the dry matter content of cells rose from 0.05 mg per ml to 0.20 mg per ml) are shown. Control test. *b)* Differential values measured after 60 minutes versus the dry matter content of the initial culture

neutral salt solution with the same osmotic pressure as the nutrient is used. We have used solution "M" for washing the cells. Because of a lack of nutrient the growth of the cells stops during washing and growth re-starts only when the cells are taken up again in the nutrient. The influence of this temporary inhibition of cell growth on the basal penicillinase synthesis of the cells was then examined.

In the experiment shown in Fig. 3. aliquots of cells with increasing dry substance contents were taken from a culture at different time intervals and centrifuged off. The cells were then washed twice with solution "M" and taken up in the nutrient medium. Fig. 3a shows the differential values of penicillinase production of these samples as a function of time.

[In the study of penicillinase synthesis Torriani (1956) was the first to use the differential value $p = \frac{dZ}{dX}$, where dX = the increase in the amount of enzyme produced in U/ml and dZ = increase in dry matter content in mg per ml.]

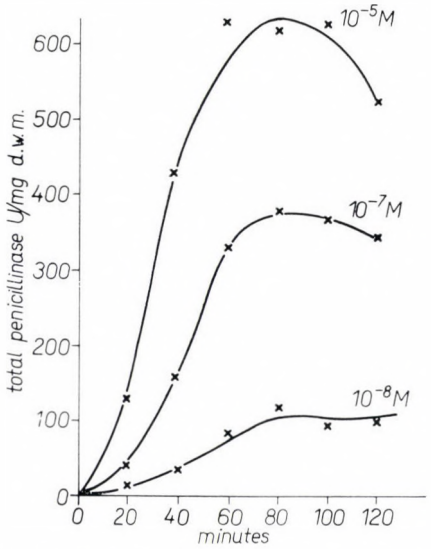

Fig. 4. Differential values of penicillinase synthesis of logarithmically growing cells induced by penicillin versus time. The figures on the curves indicate penicillin concentrations

The differential value as a function of time reflects with high accuracy the relationship between penicillinase synthesis and the synthesis of the overall cell substance. If these two values increase parallel, that is to say if the newly formed cell substance produces penicillinase at the same rate as the old one, the change of the differential value as a function of time will give a characteristic saturation curve. The height of the plateau of the saturation curve is a reliable basis of comparison. Fig. 3b shows the values measured at the 60th minute of the experiment as a function of the dry substance content of the initial culture.

The differential values enable us to calculate both the percentage of the synthetizing capacity of the cell upon activation by washing and the proportion of this value to the values of normal basal synthesis.

The maximum penicillinase synthetizing capacity which can be achieved by induction with penicillin was arbitrarily chosen as the overall synthetizing ca-

pacity of the bacteria. Logarithmically growing cells were induced with penicillin of different concentrations. The differential values are plotted against time in Fig. 4. Maximal inducibility of cells is reached at a penicillin concentration of 10^{-6} *M* (Torriani, 1956). In experiments where the concentration of penicillin was still higher by an order of magnitude, the differential value of penicillinase synthesis was found to be 615 U/mg dry material at saturation. If this value is taken as the maximum inducing capacity, it will be evident that washing the cells

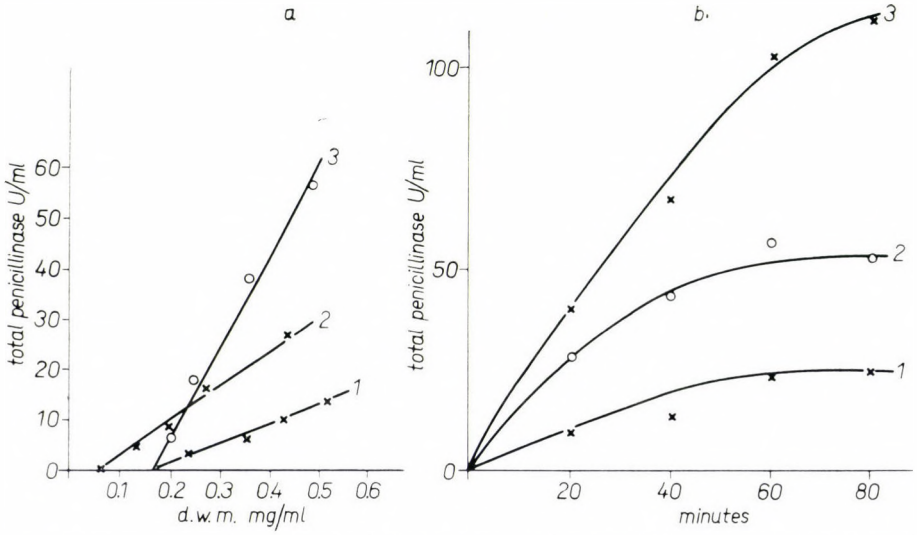

Fig. 5. The influence of chloramphenicol treatment on basal penicillinase synthesis. 1. control cells, 2. incubated in solution "M" for 30 minutes, 3. incubated with chloramphenicol (100 mg per ml) for 30 minutes. *a)* penicillinase synthesis represented isometrically, *b)* the differential values of synthesis versus time

with solution "M" will liberate about nine per cent of the overall synthetizing capacity, while the value for basal synthesis is only three per cent.

Changes in basal enzyme synthesis upon inhibition with chloramphenicol. The regular process of cell growth may be disturbed also by the addition of chloramphenicol. To logarithmically growing cells 100 mg per ml chloramphenicol was added and incubation was continued for another 30 minutes. To remove the chloramphenicol the culture was washed once with solution "M" and taken up in the nutrient medium. One control consisted of cells which were incubated in solution "M" instead of treating them with cells without any treatment except for transferring them into a fresh nutrient medium served as a second control.

The penicillinase production of the three samples treated differently is shown isometrically in Fig. 5a.

It may be seen that even incubation in solution "M" is sufficient to induce increased enzyme production. This is in agreement with the earlier findings on

washing the cells with solution "M". Inhibition with chloramphenicol results in a significant penicillinase synthesis.

The changes in time of the differential values are shown in Fig. 5b. Calculations from the saturation values indicate that upon inhibition with chloramphenicol 19 per cent of the capacity of the synthetizing system is being activated, though it should be mentioned that a fairly great fluctuation of this value was found from one experiment to the other.

Discussion

Our experimental results seem to indicate a special regulation of the basal penicillinase synthesis in *B. cereus*. Approximately 30 minutes after the introduction into the nutrient medium the spores begin to germinate (Fitz – James, 1955) and basal penicillinase synthesis begins practically simultaneously with germination and proceeds linearly during the entire lag phase, though, probably because of the blocking of various control systems, the synthesis of the bulk of enzymes has not started yet (with the exception of enzymes participating in the lag phase).

After the lag phase the cells begin to grow logarithmically and basal penicillinase synthesis proceeds synchronously with this growth. The pattern is the same with pre-incubated cells. These probably facts point to the special position occupied by the "centres" responsible for penicillinase synthesis in the regulating system of the cell and show that the activity of these centres is controlled by a separate mechanism which is independent of the general regulating system of the cell.

This assumption is supported by the fact that changes in environmental conditions, such as washing, incubation without carbon and nitrogen source, greatly enhance basal penicillinase synthesis. It is easy to imagine that the reason for this effect being so pronounced is that the general control system of cellular metabolism is unable to cope with these changes because of the independent nature of penicillinase regulation. The problem of increased basal enzyme synthesis after chloramphenicol treatment should be discussed in greater detail, especially as a somewhat similar phenomenon has been reported with other proteins (Gallant, Stapleton, 1964). Gallant et al. have studied the regulation of the alkaline phosphatase synthesis in *E. coli*. They have demonstrated that alkaline phosphatase is being synthetized in spite of the presence of inorganic phosphate if the cells are incubated under phosphate repression in the presence of chloramphenicol which is then removed. The authors assume that chloramphenicol inhibits the synthesis of an aporepressor of protein nature.

It is very tempting to interprete the effect of chloramphenicol in our experiments in an analogous way. Inhibition by chloramphenicol of the synthesis of a protein like penicillinase repressor may result in a temporary repressor deficiency and an increased penicillinase synthesis. (In the case of both alkaline phosphatase and penicillinase it has to be assumed that the repressor protein is metabolically unstable.

However obvious this explanation may seem we must bear in mind the nature and complexicity of this phenomenon. Chloramphenicol inhibits the synthesis of all cellular proteins, while other processes – respiration, nucleic acid synthesis, etc. – proceed undisturbed for some time. Under these conditions sooner or later on the control systems of the cell will be affected, too.

Neither is the possibility excluded that in the case of both alkaline phosphatase and penicillinase the effect of chloramphenicol is due to the inhibition of repressor synthesis. With the present preliminary results available, this is only one of the many possible explanations. This is especially true for penicillinase synthesis where – as shown by the experiments described – influences considerably milder than the blocking of overall protein synthesis have also produced similar phenomena.

The natural inducer – penicillin – blocks the synthesis of the cell wall. It is quite conceivable that the various treatments, including that with chloramphenicol, also act on some of the processes which lead to the synthesis of the cell wall, the only difference being that penicillin is more effective that the other factors.

In the phenomena described so far the regulation of basal penicillinase synthesis is remarkably labile. All influences which disturb the normal course of growth induce penicillinase syntheses of longer or shorter duration. The regulatory systems of induced enzyme synthesis are generally assumed to act on the genes at the site of messenger RNA synthesis. In such cases, however, fluctuations similar to those reported in this paper are to be found with respect to basal enzyme synthesis.

In control systems in which the inhibition is presumably on the genes synthesis is far more strictly controlled, as only single structural genes are present in the cell and these can be kept under control by the slightest excess of repressor. When the supposed repressor molecules act directly on the ribosomes, presumably larger quantities of the repressor will necessary to give the same effect. Should a process which ensures a high repressor level be disturbed, even a temporary reduction in the repressor level will be sufficient to induce a temporary synthesis of the given protein.

It may be suggested that with penicillinase, too, the centre of regulation is in the ribosomes. This hypothesis is supported by the facts which indicate that penicillinase regulation is independent of general cell regulation.

According to the above hypothesis the cells contain a messenger fraction which is always ready to direct penicillinase synthesis and is generally greatly inhibited (the degree of inhibition can be estimated by comparing maximum inducing capacity and basal synthesis). Under various influences, however, this messenger fraction is temporarily liberated and initiates an increased penicillinase synthesis.

The hypothesis outlined above is, of course, mere speculation and has to be substantiated or revised by further experimental results.

Acknowledgement

Thanks are due to Prof. F. B. Straub and Dr. P. Zabos for their valuable help in the planning and evaluation of the experiments, and Miss Gy. Prágai for her excellent technical assistance.

References

Csányi, V. (1961) Acta Physiol. Hung. *18* 261
Fitz-James, P. C. (1955) Can. J. Microbiol. *1* 525
Gallant, J., Stapleton, R. (1964) J. Mol. Biol. *8* 431
Kogut, M., Pollock, M. R., Tridgell, E. J. (1956) Biochem. J. *62* 391
Pollock, M. R. (1950) Brit. J. Exp. Path. *31* 739
Pollock, M. R. (1952) Brit. J. Exp. Path. *33* 587
Pollock, M. R. (1953) Brit. J. Exp. Path. *34* 251
Pollock, M. R. (1956) J. Gen. Microbiol. *14* 90
Torriani (1956) Biochem. Biophys. Acta, *19* 224

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 159–167 (1966)

Ca Uptake by the Sarcoplasmatic Reticular Fraction

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(Received November 23, 1965)

A method is described for studying the uptake of Ca by the sarcoplasmatic reticular fraction of rabbit skeletal muscle. The method involves putting of the sarcoplasmatic reticular fraction to a cellulose column and subsequent passing through the column of an incubation mixture which contains ATP and $^{45}CaCl_2$. Under standard conditions, the sarcoplasmatic reticular fraction is retained in the upper 5–6 mm layer of a column of 8 to 9 mm diameter and 50 mm length throughout the experiment. Because the incubation mixture is applied to the column under continous flow, the concentrations of ATP, oxalate, $^{45}CaCl_2$, and $MgCl_2$ in the solution are well defined and constant.

It is shown that the sarcoplasmatic reticular fraction and ^{45}Ca are retained in the same region of the column. Optimum binding of ^{45}Ca is found under the following conditions: 3–5 mM ATP, 5–6 mM oxalate, 0.12–0.24 mM $^{45}CaCl_2$ and 5–10 mM $MgCl_2$. Under these conditions 2.5–3.0 μmoles ^{45}Ca are bound per mg protein when the concentration of $^{45}CaCl_2$ in the solution is 0.12 mM.

Introduction

The importance of the sarcoplasmatic reticular fraction (SRF) of the muscle fiber in the regulation of intracellular Ca level has been demonstrated in a number of recent publications (Ebashi 1960; Hasselbach, Makinose 1961; Ebashi, Lipman 1962; Weber et al. 1963; Martonosi, Feretos 1964). Application of ^{45}Ca, separation of SRF by fractionation in the ultracentrifuge and Milipore filtration are the main experimental methods which have been used in such studies (Ebashi, Lipman 1962; Martonosi, Feretos 1964).

It is a common feature of the procedures mentioned above that the concentration of ATP is decreasing during the course of Ca uptake and that the concentration of the hydrolysis products of ATP is simultaneously increasing. In our improved method there is no change in ATP concentration during the course of Ca uptake, and the reaction products are being continuously removed.

Methods

The sarcoplasmatic reticular fraction was obtained by the method of Nagai et al. (1960). The 8,000–25,000 × g microsomal fraction was used in all experiments. Protein content of SRF was determined by the micro–Kjeldahl method.

Analytical grade reagents were used. Water of $2-3\times10^6$ Ohm resistance obtained by the use of a column of ion-exchange resin was used for making the solutions. $^{45}CaCl_2$ applied with a specific activity of 99 μC/0.12 mM was obtained from the Isotope Institute of the National Committee of Atomic Energy. Dowex 50 resin was used for the removal of Ca from the commercial samples of ATP obtained from Reanal (Seidel, Gergely 1963).

Results

SRF was given to a cellulose column and the incubation mixture was continuously flown through it in order to secure a constant concentration of ATP, and the removal of the hydrolysis products of ATP, respectively. Considering the size of SRF particles their movement in the column is much slower than that of the incubation mixture.

Binding of ^{45}Ca by the cellulose column. First we had to determine whether the cellulose column itself would bind ^{45}Ca under the conditions of the planned uptake experiments. Exactly 0.7 g powdered cellulose (Whatman, chromato-

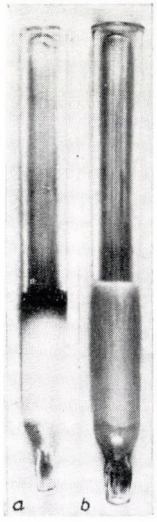

Fig. 1. Localization of SRF on a cellulose column with acidic fuchsin. The SRF sample contains 1.0 mg protein. For further details see text

graphic grade) was suspended in deionized water and packed into Rasotherm glass tube of an inner diameter of 8 mm. The cellulose column was supported by a washed cotton plug, and the height of the column was 50 mm (Fig. 1). The packed column was washed first with deionized water and then three times with 5 ml portions of 0.01 M Tris/maleate buffer, pH 6.8 which contained 0.055 M

KCl, 0.005 M $MgCl_2$ and 0.005 M potassium oxalate (washing solution). Subsequently the column was washed with 5 ml of a Ca-free incubation mixture of the following composition: 0.05 M KCl, 0.005 M $MgCl_2$, 0.005 M potassium oxalate and 0.005 M ATP in 0.01 M Tris/maleate buffer, pH 6.8. At this stage the flow rate was adjusted to one drop per 10 sec. with a 3 cm high layer of solution over the column surface. As soon as the Ca free incubation mixture was soaked into the column 5 ml of the ^{45}Ca containing incubation mixture (0.12 μM/ml $CaCl_2$) was added. This was followed by the addition of 5 plus 4 ml isotopefree incubation mixture. The flow rate was 5 ml per 8 min. The first 3 ml fraction collected after the addition of the ^{45}Ca containing solution was discarded because it did not contain any radioactivity and apparently corresponded to the hold-up volume of the column. Thereafter 1.3 ml fractions were collected, of which 2×0.5 ml samples were withdrawn for radioactivity counting. The samples were dried under infrared lamps into aluminium planchettes, and counted with an end-window GM-counter. Total recovery of radioactivity in the collected fractions was calculated to determine whether any ^{45}Ca had been adsorbed to the cellulose.

Table 1 shows that practically no binding of ^{45}Ca was found under these conditions. Determination of the radioactivity in the ash of the columns after combustion yielded the same result.

Table 1

Recovery of ^{45}Ca from the cellulose column

Column number	Applied	Recovered	$\frac{\mu M\ ^{45}Ca\ recovered}{\mu M\ ^{45}Ca\ applied} \times 100$
	$\mu M\ ^{45}Ca$		
1.	0.600	0.594	99.0%
2.	0.600	0.603	100.5%
3.	0.600	0.600	100.0%
4.	0.600	0.590	98.3%

Combustion and counting of the radioactivity of the columns were carried out in the following way. The column was frozen in dry ice. Subsequently the glass tube was gently heated and the solid, frozen cellulose was pushed out with a glass rod. The cellulose was cut into 10 disks, of equal length and each disk was separately transferred to an aluminium planchette. The samples were dried and then combusted at 250°C. In order to complete the combustion and to secure a layer of uniform thickness, 3 drops of concentrated HNO_3 were added to each planchette. The planchettes were returned to the oven for the completion of combustion and for the complete evaporation of the acid. The dry samples were counted with an end-window GM-counter.

Localization of SRF on the cellulose column. For studying the differential rate of movement in the column of SRF fragments, soluble proteins, and the com-

ponents of the incubation mixture, the following experiment was set up. A 0.5 ml aliquot of an SRF suspension which contained 2 mg protein per ml was added to the washed column. This was followed by adding Ca free, ^{45}Ca containing, and then again two aliquots of the Ca free incubation mixture (5 + 5 + 5 + 4 ml) exactly as above. The *fractions were collected immediately* after applying the SRF to the column. A total of fourteen 1.3 ml fractions were collected. No protein was found with sulfosalicylic acid in any fraction, and this demonstrates that the SRF particles were retained by the column.

In order to localize the SRF on the column, the bottom of the glass tubes was closed, and then the columns were transferred to a water bath of 95°C. The columns were kept vertically in the bath for 20 minutes. The denatured protein was stained with acidic fuchsin (Dévényi, Gergely 1963).

It can be seen in Fig. 1. that SRF was retained in the uppermost 5–6 mm layer of the column. When 0.5 ml of the supernatant of SRF which contained soluble proteins was substituted for SRF, there was a high concentration of protein in fractions 3 and 4 of the effluent and no protein was found on the column (Fig. 1b).

Uptake of ^{45}Ca by SRF on a cellulose column. The column was loaded with 0.5 ml of a SRF suspension containing 0.15–0.25 mg protein. Thereafter, the same procedure was followed as previously described (Binding of ^{45}Ca by the cellulose column). Ca uptake was measured by determining the Ca content of 1.3 ml effluent fractions. In control experiments, that is without protein or with a denatured protein sample, the distribution of ^{45}Ca in the fractions was described by a bell-shaped curve. When SRF was present the shape of the curve was determined by the amount of SRF and by the composition of the incubation mixture (oxalate, ATP, and so on).

The solid line in Fig. 2 shows the result of a control experiment, while the broken line shows the results obtained when less SRF was used than necessary to bind the total quantity of ^{45}Ca applied. It is possible to calculate the amount of ^{45}Ca taken up by SRF from the difference between the two areas circumscribed by the individual curves. Instead, we have computed the amount of ^{45}Ca taken up, by adding up separately the Ca contents of the fractions obtained in the experiments with denatured and native SRF, respectively, and by establishing the difference between the two sums. The value thus obtained was expressed as μM Ca bound/mg protein.

From the results shown in Fig. 2 the uptake of ^{45}Ca by rabbit SRF is 2.8 μM ^{45}Ca/mg protein.

Localization of ^{45}Ca on the column in the presence of SRF. Subsequent to an uptake experiment the cellulose column was out into ten segments each 5–6 mm long and the ^{45}Ca content of each disk was determined after combustion. Table 2 shows the Ca content of the cellulose segments when native SRF, and heat-denatured SRF were used, respectively. It is seen that whenever native SRF was used, most of the ^{45}Ca retained by the column was present in the first segment which also contained the SRF-protein. In the experiment shown in Table 2 the amount

of SRF was such as to bind almost quantitatively the 0.6 μM of ^{45}Ca loaded on the column. In the following experiments in which the effect of various factors on Ca uptake was to be studied the amount of SRF was lower and a part of the

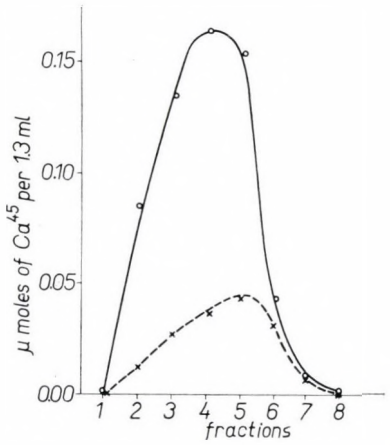

Fig. 2. Uptake of Ca by native SRF (×--------×) and by heat-denatured SRF (○———○). Incubation mixture: 50 mM KCl, 5 mM ATP, 5 mM $MgCl_2$, 5 mM oxalate 10 mM Tris/maleate buffer, pH 6.8, and 0.12 mM $^{45}CaCl_2$. Amount of SRF = 0.155 mg protein. For further details see text

applied ^{45}Ca was recovered in the effluent. Table 3 shows the results of an experiment in which only a part of the applied 0.6 μM ^{45}Ca was bound by SRF on the column. In this experiment the radioactivity of both the cellulose segments and the effluent fractions was determined.

It is seen from Table 3 that about 95 per cent of the applied ^{45}Ca is recovered in the column plus the effluent. It follows that Ca uptake can be reliably determined by analyzing the effluent, only and this is favourable because handling of

Table 2

Localization of ^{45}Ca on the cellulose column in the presence of native and heat-denatured SRF, respectively

Treatment	Column Segment										^{45}Ca bound μM
	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	
	μM ^{45}Ca										
Native SRF*	0.550	0.035	—	—	—	—	—	—	—	—	0.585
Native SRF	0.530	0.052	—	—	—	—	—	—	—	—	0.582
Heat-denatured	—	—	—	—	—	—	—	—	—	—	
Heat-denatured	—	—	—	—	—	—	—	—	—	—	

* 0.6 μM ^{45}Ca was applied to all four columns. The sign — stands for <0.0002 μM Ca.

Table 3

The distribution of ^{45}Ca among the column

Treatment		Number of segment			
		1.	2.	3.	4
Native SRF	*a*:	0.4677	0.0156	—	—
	b:	0.0066	0.0066	0.0113	0.0160
Native SRF	*a*:	0.1510	0.0505	—	—
	b:	0.0067	0.0128	0.0679	0.1096
Heat-denatured	*a*:	—	—	—	—
	b:	0.0094	0.0859	0.1325	0.1654

the column segments is a tedious operation and also presents a number of sources of error.

The above studies resulted in a convenient and reliable method which was subsequently used to study the effect of various factors on the uptake of Ca^{++} by SRF of rabbit skeletal muscle.

Effect of ATP on Ca uptake. Fig. 3. shows that maximum effect is obtained with 3–6 mM ATP. Ca uptake is lowered by lowering the ATP-level to 2 mM.

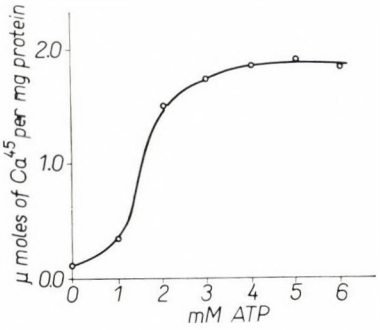

Fig. 3. Effect of ATP on Ca uptake by SRF. ATP concentration is shown on the abscissa, the other components of the incubation mixture as in Fig. 2. Amount of SRF = 0.26 mg protein

Effect of oxalate on Ca uptake. It is seen from Fig. 4 that there is a steep increase in Ca uptake when oxalate concentration is increased up to 4 mM. No further effect is obtained when the oxalate concentration is increased to 6 mM.

Effect of Ca concentration on Ca uptake. The uptake of Ca by SRF is a linear function of $CaCl_2$ concentration up to about 0.24 mM (Fig. 5). The curve starts to level off above 0.24 mM and the maximum value is reached with about

segments (a), and effluent fractions (b)

or fraction						Sum total μM ^{45}Ca	Per cent recovery
5.	6.	7.	8.	9.	10.		
μM ^{45}Ca							
—	—	—	—	—	—	0.5783	96.40
0.0244	0.0141	0.0094	0.0066	—	—		
—	—	—	—	—	—	0.5693	94.90
0.0955	0.0410	0.0222	0.0121	—	—		
—	—	—	—	—	—	0.6022	100.36
0.1551	0.0432	0.0094	0.0013	—	—		

a column segments
b effluent fractions (1.3 ml)

0.36 mM. No further change was found in the range of 0.36 to 0.48 mM. Ca concentration could not be increased above 0.48 mM because of the precipitation of Ca-oxalate. Maximum rate of Ca uptake by SRF was calculated from the results obtained with 0.36 mM $CaCl_2$. The figure obtained was 0.72 μM ^{45}Ca/mg protein/min. The total volume of the ^{45}Ca containing incubation mixture (5 ml) went through the column in 8 minutes. This period of time can be regarded to be the time of incubation of SRF with $CaCl_2$.

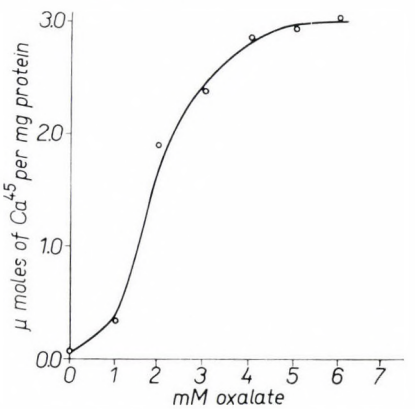

Fig. 4. Effect of oxalate on Ca uptake by SRF. Oxalate concentration is shown on the abscissa, the other components of the incubation mixture as in Fig. 2. Amount of SRF = 0.16 mg protein

Effect of Mg^{++} on Ca uptake. Fig. 6 shows that there is a linear increase in Ca uptake with increasing concentrations of $MgCl_2$ up to about 5 mM. There is a further slight increase in uptake between 5 and 10 mM Mg^{++}, and higher Mg^{++} concentrations are markedly inhibitory.

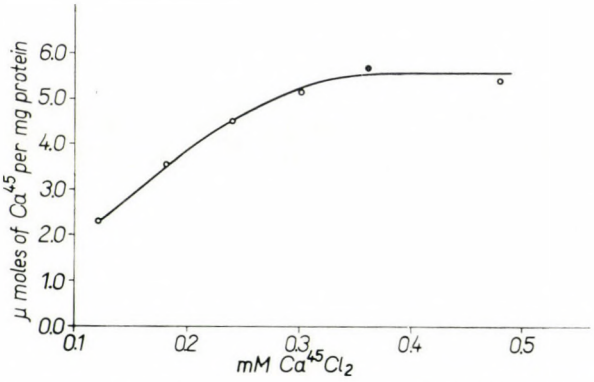

Fig. 5. Effect of Ca concentration on Ca uptake by SRF. $^{45}CaCl_2$ concentration is shown on the abscissa, the other components of the incubation mixture as in Fig. 2. Amount of SRF = 0.25 mg protein

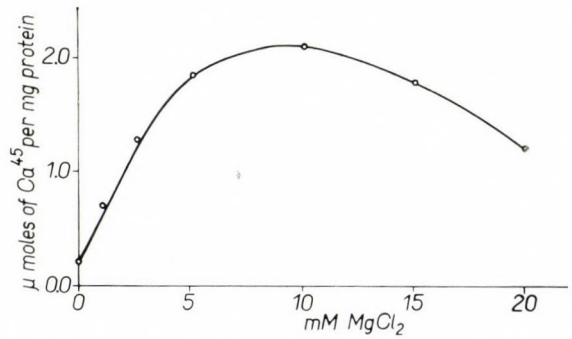

Fig. 6. Effect of Mg^{++} on Ca uptake by SRF. Concentration of $MgCl_2$ is shown on the abscissa, the other components of the incubation mixture as in Fig. 2. Amount of SRF = 0.23 mg protein

Discussion

Whenever physiological processes are studied in model experiments, the experimental conditions should simulate as much as possible the conditions in the living cell. This is what we wanted to achieve with the described method. The cellulose column retained SRF and it was possible to pass the immobilized SRF with the incubation mixture under continuous flow. In this way, concentration of ATP could be kept constant. This was also achieved by others (Ebashi, Lipman 1962) by the use of an ATP regenerating system consisting of phosphoenol pyruvate and pyruvate kinase. However, when our method is applied Ca concentration is also maintained at a constant level. Naturally, the relative constancy of Ca level is strictly valid only for the uppermost layer of the column and there is a progressive dilution of Ca in lower layers (Fig. 2).

Fig. 1 and Table 2 demonstrate that ^{45}Ca is retained by SRF. Under the given conditions 2.5–3.0 μM ^{45}Ca/mg protein was the amount of Ca taken up by SRF from 5 ml 0.12 mM $^{45}CaCl_2$ solution. This figure is similar to or slightly higher than the corresponding figures of Martonosi and Feretos (1964) and Takauji et al. (1965). If the external concentration of $^{45}CaCl_2$ is increased to 0.36 mM, the corresponding figure is 5.7 μM ^{45}Ca/mg protein, and 0.72 μM ^{45}Ca/mg protein/minute is the figure obtained for the rate of uptake. In some of our experiments, markedly lower values were obtained. This was certainly due to the use of aged SRF preparations. Ageing of SRF preparations upon storage at 0°C results in a loss of their capacity to take up Ca and in a change of their ATP-ase activity, as reported Ebashi, Lipman (1962) and Martonosi, Feretos (1964). Recently (Samaha, Gergely 1965) the same phenomenon has been observed with SRF from human muscle.

It was shown that 3–5 mM ATP (Fig. 3), 5–6 mM oxalate (Fig. 4), 0.12–0.24 mM $^{45}CaCl_2$ (Fig. 5) and 5–10 mM $MgCl_2$ (Fig. 6) present the optimum conditions for Ca uptake by SRF when the described method is used.

It is noteworthy that some Ca was also taken up in the absence of Mg^{++}. Similar observations were described by Martonosi, Feretos (1964) and Fanburg, Gergely (1965).

In its presented form, our method is not perfectly suitable for kinetic studies. Under the standard conditions of our experiments (5 ml effluent in 8 minutes) reproducible results are obtained. However, there are systematic changes in the flow rate as the level of the solution on the column is changing. It should be added that if needed our method can be adopted to studies which involve a constant flow rate by stabilizing perfusion pressure in the usual way.

References

Dévényi, T., Gergely, J. (1963) Aminosavak, peptidek, fehérjék. Medicina, Budapest
Ebashi, S. (1960) J. Biochem. (Tokyo) *48* 150
Ebashi, S., Lipman, F. (1962) J. Cell. Biol. *14* 289
Fanburg, B., Gergely, J. (1965) J. Biol. Chem. *240* 2721
Hasselbach, W., Makinose, M. (1961) Biochem. J. *333* 518
Martonosi, A., Feretos, R. (1964) J. Biol. Chem. *239* 648
Nagai, T., Makinose, M., Hasselbach, W. (1960) Biochim. Biophys. Acta *43* 223
Samaha, F. J., Gergely, J. (1965) J. clin. Invest. *44* 1425
Seidel, J. C., Gergely, J. (1963) J. Biol. Chem. *238* 3648
Takauji, M., Nakano, I., Taniguchi, M. (1965) Jap. J. Physiol. *15* 188
Weber, A., Herz, R., Reiss, I. (1963) J. Gen. Physiol. *46* 679

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 169—177 (1966)

Enzyme Functions of Glucose 6-Phosphatase

Correlation between the Activities of Liver Glucose 6-phosphatase, Inorganic Pyrophosphatase, Pyrophosphate-glucose Phosphotransferase and Ontogenesis, and the Changes of these Enzyme Activities in Diabetes and in Various other Physiological States

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(Received December 7, 1965)

Changes in the glucose 6-P-ase,* inorganic pyrophosphatase (PP_i-ase) and pyrophosphate-glucose phosphotransferase (P-transferase) activities of liver were studied in different physiological conditions and during ontogenesis.

It was found that dextrin maltose diet results in a parallel decrease in all three activities, while a diet rich in fructose induces a parallel rise. The parallel rise of PP_i-ase and P-transferase activities with the increase in glucose 6-P-ase activity was demonstrated in diabetes. Insulin induces a marked decrease in three activities.

In the early stage of ontogenesis not only glucose 6-P-ase, but also PP_i-ase and P-transferase activities are missing from the liver of mammal (rat) embryos. All three functions appear simultaneously in the days immediately preceding birth, after which they increase parallel to each other up to a value which is considerably higher than that in adults. Later all three activities decrease again in a parallel way. Contrary to mammals, the presence of all three activities can be demonstrated in the liver of fowl (chicken) embryos much before birth.

These findings are considered to prove the assumption that liver glucose 6-P-ase, PP_i-ase and P-transferase activities are three functions of one and the same enzyme and are controlled by a common gene.

The PP_i-ase and P-transferase activities of glucose 6-P-ase (EC. 3.1.3.9) in the liver have been described in recent years by Nordlie and Arion (1964), Arion and Nordlie (1964), Stetten (1964) and Stetten and Taft (1964). The three functions of the enzyme may be expressed by the following equations:

$$\text{Glucose 6-P} + H_2O \rightarrow \text{glucose} + P_i$$

$$PP_i + H_2O \rightarrow 2\,P_i$$

$$PP_i + \text{glucose} \rightarrow \text{glucose 6-P} + P_i$$

It has been assumed that all three activities are displayed by the same enzyme. This is supported by the findings that the three functions are localized

* *Abbreviations:* glucose 6-P-ase = glucose 6-phosphatase (G-6-P-ase); P-transferase = pyrophosphate-glucose phosphotransferase; PP_i-ase = inorganic pyrophosphatase; glucose 6-P = glucose 6-phosphate; PP_i = inorganic pyrophosphate; P_i = inorganic orthophosphate; EDTA = ethylenediamine tetraacetate.

in the microsomal fraction of the liver and that during fractionation or inactivation the proportions between the three activities remain constant. A further evidence was provided by the preliminary observations of Stetten and Taft (1964) according to which PP_i-ase and P-transferase activities increase parallel to glucose 6-P-ase activity, and that in diabetic animals insulin induces a decrease in the activity of both glucose 6-P-ase and P-transferase (Nordlie, Arion, 1965).

Illingworth and Cori (1965) also attributed microsomal glucose 6-P-ase and PP_i-ase activities to the same enzyme. They have demonstrated an extensive reduction in liver PP_i-ase activity in that form of Gierke's disease which is accompanied by a genetically determined glucose 6-P-ase deficiency.

There is a considerable change in liver glucose 6-P-ase activity upon various physiological influences. We have demonstrated (Bot et al., 1965) that a dextrin i.e. glucose rich diet reduces, while feeding of fructose and fat increases – just like starving and adrenaline – glucose 6-P-ase activity.

In the present paper we shall present some data which will show that upon the influences mentioned above the activities of PP_i-ase and P-transferase increase or decrease parallel to glucose 6-P-ase activity. The study of the three enzyme effects seemed to be of particular interest with respect to ontogenesis. In addition, the eventual PP_i-ase and P-transferase activities of the liver of mammal embryos which have no glucose-6-P-ase activity was also examined. The finding that the liver of chicken embryos – contrary to the liver of mammals – exhibits beside glucose 6-P-ase activity also the other two enzyme activities is a further evidence that the three activities are controlled by the same gene.

Methods

Female white rats of a body weight of 150 to 200 g were used in the experiments. The diets consisted in 24 hours starvation after which the animals were given for 48 hours ad libitum only dextrin maltose or fructose. (The baby food Dexmaltan was given as dextrin maltose. It has the following composition: 31 per cent maltose, 68 per cent dextrin, 1 per cent NaCl, 0.1 per cent $CaCO_3$). Alloxane diabetes was induced by a single intraperitoneal injection of alloxane monohydrate (150 mg per kg body weight). The blood sugar level of the diabetic animals was over 300 mg per cent after 48 to 96 hours. Insulin treatment consisted in the subcutaneous injection of 4 times 5 U Zn-protamine-insulin every 12 hours. The animals were prepared 12 hours after the last injection.

The animals were stunned by a hit at the nape, decapitized and bled. The liver was kept in dry ice till further use. A 1 : 10 homogenate was prepared from part of the liver with a 0.001 M EDTA solution in a glass homogenizer and the glucose 6-P-ase, PP_i-ase and P-transferase activities of this homogenate were measured.

Glucose 6-P-ase activity was measured by the modified method of Cori (Cori, Cori, 1952) which involves the determination of the P_i liberated from

glucose 6-P. The incubation mixture had the following composition: 0.04 M citrate buffer (pH 6.0), 0.025 M glucose 6-P and homogenate corresponding to 20 mg of liver, in a total volume of 1 ml. Incubation for 15 minutes at 30°C and pH 6.0 was followed by the addition of 5 ml of 3 per cent $HClO_4$ to stop the reaction. P_i was determined in the supernatant solution.

PP_i activity was followed by measuring the P_i liberated from PP_i. The composition of the incubation mixture was the following: 0.04 M citrate buffer (pH 6.0), 0.1 M PP_i and homogenate corresponding to 20 mg of liver, in a total volume of 1 ml. Incubation at pH 6.0 for 15 minutes at 30°C was followed by the addition of $HClO_4$ to stop the reaction. The P_i formed from PP_i was determined in the supernatant solution.

The activity of P-transferase was measured in the same incubation mixture to which 0.4 M glucose had been added. P-transferase splits off one of the phosphates, of PP_i and transfers the other one to glucose to form glucose 6-P. The quantity of glucose 6-P formed by the action of P-transferase was calculated from the difference of P_i released from PP_i with and without glucose. In the presence of glucose less P_i is split off the PP_i, and this decrease in P_i (in μmole) is equivalent to the quantity of P_i transferred to the glucose. Thus

$$\text{P-transferase activity} = P_i \text{ (without glucose)} - P_i \text{ (with glucose)}.$$

Under the given conditions (pH 6.0, no Mg) aspecific pyrophosphatase and phosphotransferase display only a negligible activity (Stetten, 1964). On a few occasions the quantity of glucose 6-P formed by the action of P-transferase was measured directly with glucose 6-P-dehydrogenase. The results obtained in this way agreed within the limits of experimental errors with the indirectly calculated values.

The assay of P_i was carried out by the slightly modified method of Taussky and Shorr (1953). In the high concentrations used PP_i interferes with the photometric determination of P_i, because it inhibits, the development of the P-molybdenum blue colour, on the one hand and undergoes hydrolysis in the acid medium on the other. If, however, the reagent which contains $FeSO_4$ contains also a sufficient amount of Fe^{3+} ions, then the Fe^{3+} ions form a complex with PP_i and thus block the disturbing effect of the latter. Therefore, we used in our experiments instead of a freshly prepared reagent one which was allowed to stand for 6 to 8 days, and were able by strictly adhering to the 10 minutes development time to determine P_i accurately even in the presence of PP_i.

The enzyme activities have been expressed in μmole of substrate transformed in one minute per 1 g of liver and 100 g of body weight, respectively.

Results and Discussion

The effect of dextrin maltose and fructose diets

In the first part of our experiments the effect of various diets on the three types of enzyme activities was examined. Earlier experiments (Bot et al., 1965) have shown that a diet with high glucose content reduces the glucose 6-P-ase activity of rat liver, while a diet rich in fructose has the opposite effect. It remained an open question whether P-transferase and PP_i-ase activities will also undergo similar changes. Table 1 shows the effect of dextrin maltose and fructose diets on glucose 6-P-ase, PP_i-ase and P-transferase activities.

Table 1

Effect of Dexmaltan and fructose diets on glucose 6-P-ase, PP_i-ase and P-transferase activities of rat liver

The rats were kept on a mixed diet, starved for 24 hours before the beginning of the experiment, then fed with ad libitum dextrin maltose and fructose, respectively. They were deprived of food 4 hours to be killed by decapitation and bleeding. The liver was kept in dry ice until used. Preparation of the homogenates and measuring of enzyme activities were performed by the methods described

Diet	No	Substrate converted µmole per minute per g liver			Substrate converted µmole per minute per 100 g body weight		
		G-6-P-ase	PPi-ase	P-transferase	G-6-P-ase	PPi-ase	P-transferase
Dextrin maltose	1	9.4	14.8	7.7	26.7	40.2	21.2
	2	10.9	12.9	7.1	59.0	62.2	34.2
	3	14.2	15.8	9.7	47.3	52.7	32.3
	4	11.6	17.7	9.0	46.0	70.3	35.7
	5	14.2	19.0	9.0	40.9	66.7	31.6
	Mv.	12.1 ± 2.0*	16.0 ± 2.4	8.5 ± 1.1	44.0 ± 11.7	58.4 ± 12.3	31.0 ± 5.7
Fructose	1	16.9	20.0	14.2	63.2	74.7	53.1
	2	15.5	20.0	14.8	73.2	94.4	70.0
	3	16.1	20.3	11.6	53.2	67.1	38.3
	4	15.5	18.1	10.3	67.0	78.3	44.5
	Mv.	16.0 ± 0.7	19.6 ± 1.0	12.7 ± 2.1	64.1 ± 8.4	78.6 ± 11.5	51.5 ± 13.7

* ± standard deviation

It can be seen from the data in Table 1 that PP_i-ase activity is somewhat higher and P-transferase activity somewhat lower than glucose 6-P-ase activity. After feeding dextrin maltose to the animals P-transferase activity is about half of the PP_i-ase activity. Under the effect of fructose diet PP_i-ase and P-transferase activities increase parallel to the activity of glucose 6-P-ase. This effect of the fructose diet is manifest in both the values which refer to 1 g of liver and to 100 g of body weight.

The effect of alloxane diabetes and of insulin

Alloxane diabetes is accompanied by a considerable increase in the glucose 6-P-ase activity of liver. If the three functions of the liver microsomes can be attributed to the same enzyme then there should be a similar increase in the activities of PP_i-ase and P-transferase as well. Table 2 shows the enzyme activities in the liver of alloxane diabetic rats.

Table 2

Liver glucose 6-P-ase, PP_i-ase and P-transferase activities of alloxane diabetic rats

Alloxane treatment: 150 mg per kg body weight intraperitoneally, 48 to 96 hours later decapitation, bleeding. Procedure and measurements of enzyme activities like in the experiments of Table 1

No	Substrate converted μmole per minute per g liver			Substrate converted μmole per minute per 100 g body weight		
	G-6-P-ase	PP_i-ase	P-transferase	G-6-P-ase	PP_i-ase	P-transferase
1	16.1	28.7	18.7	—	—	—
2	27.1	38.4	25.2	—	—	—
3	29.0	43.9	31.2	—	—	—
4	19.4	29.0	23.9	80.4	120.0	99.1
5	17.4	21.0	14.2	54.8	66.1	44.7
6	26.5	39.7	34.2	94.1	141.0	121.5
Mv.	22.6 ± 5.6	33.5 ± 8.6	24.6 ± 7.5	76.4 ± 19.9	109.0 ± 38.6	88.4 ± 39.5
Normal Mv.*	12.1 ± 2.0	16.0 ± 2.4	8.5 ± 1.1	44.0 ± 11.7	58.4 ± 12.3	31.0 ± 5.7

* mean values of animals kept on dextrin maltose diet (see Table 1).

It can be seen from Table 2 that in alloxane diabetic animals glucose 6-P-ase activity has nearly doubled and that there is a parallel increase in the activities of PP_i-ase and P-transferase. It is remarkable that the activity increase of P-transferase is considerably higher than that of the other two enzymes.

The high glucose 6-P-ase activity of diabetic animals can be normalized with insulin treatment. Experiments not to be described here on a few diabetic animals have shown that insulin treatment reduces not only glucose 6-P-ase activity, but also the activities of PP_i-ase and of P-transferase. The effect of insulin treatment on the three types of enzyme activities was studied in greater detail on normal animals. A single insulin injection is known to reduce glucose 6-P-ase activity (Hawkins et al., 1959). We have studied the effect of insulin on the three types of enzyme activities by administering a single injection or alternatively by applying a continuous insulin treatment (Table 3).

It can be seen from Table 3 that when insulin is given to normal animals beside a reduction in glucose 6-P-ase activity there will also be a considerable decrease in the activities of PP_i-ase and P-transferase. A single insulin injection

reduces activity only in comparison to the value of animals on fructose diet, while four injections decrease the activity also relative to the values obtained with glucose diet. These data indicate a parallelism in the reduction of the three enzyme functions in insulin treated animals.

Table 3

Effect of insulin on the liver glucose 6-P-ase, PP_i-ase and P-transferase activities of normal animals

Insulin treatment: *a)* 5 U insulin subcutaneously, mixed diet, decapitation 20 hours after the administration of insulin.
b) 4 times 5 U insulin, subcutaneously at 12-hour intervals. Mixed diet. Decapitation 12 hours after the 4th insulin injection.
Procedure and measurements of enzyme activities like in the experiments of Table 1.

No	Insulin dose *a.)*	Substrate converted μmole per minute per g liver			Substrate converted μmole per minute per 100 g body weight		
		G-6-P-ase	PP_i-ase	P-transferase	G-6-P-ase	PP_i-ase	P-transferase
1	5 E	10.3	11.0	5.2	55.7	59.5	28.1
2	5 E	10.3	13.0	5.2	55.4	60.7	28.0
3	5 E	10.3	12.9	7.7	55.3	69.2	41.3
4	5 E	14.2	17.1	9.7	69.7	84.0	47.6
Mv.		11.3 ± 1.9	13.1 ± 2.8	7.0 ± 2.2	59.0 ± 7.1	68.4 ± 12.2	38.2 ± 10.1
	b)						
1	4×5 E	4.5	6.8	3.2	27.3	41.3	19.4
2	4×5 E	6.4	6.8	1.9	34.6	40.0	11.2
3	4×5 E	6.4	9.9	4.5	39.5	61.1	27.8
4	4×5 E	7.1	9.9	4.5	48.0	67.0	30.4
5	4×5 E	9.7	11.9	7.7	59.1	72.6	47.0
6	4×5 E	11.6	13.9	7.7	66.7	77.9	44.3
Mv.		7.3 ± 2.6	9.9 ± 3.6	4.9 ± 2.4	45.9 ± 15.0	60.0 ± 16.0	30.0 ± 13.8
Mv.	I. Normal	16.0 ± 0.7	19.6 ± 1.0	12.7 ± 2.1	64.1 ± 8.4	78.6 ± 11.5	51.5 ± 13.7
Mv.	II. Normal	12.1 ± 2.0	16.0 ± 2.4	8.5 ± 1.1	44.0 ± 11.7	58.4 ± 12.3	31.0 ± 5.7

I. Normal: means of the values of animals on fructose diet.
II. Normal: means of the values of animals on dextrin maltose diet.

The appearance of glucose 6-P-ase, PP_i-ase and P-transferase activities in the course of ontogenesis

There is a great difference in glucose 6-P-ase formation during the ontogenesis of mammals and fowls. Glucose 6-P-ase is not present in the liver of mammal embryos where it will appear but shortly before birth (Nemeth, 1954; Bot et al., 1960). After birth the activity gradually increases and reaches on the third day about twice that observed in adults. In the next two to three months the activity

drops to the level found in adult (Dawkins, 1963). On the other hand, the liver of fowls contains already prior to hatching significant quantities of glucose 6-P-ase. In order to decide whether the three types of activities may be attributed to the same enzyme we have investigated the eventual presence of PP_i-ase and P-transferase activities in the liver of mammal embryos. A further question was whether these appear only after birth and if so whether their increase and decrease during

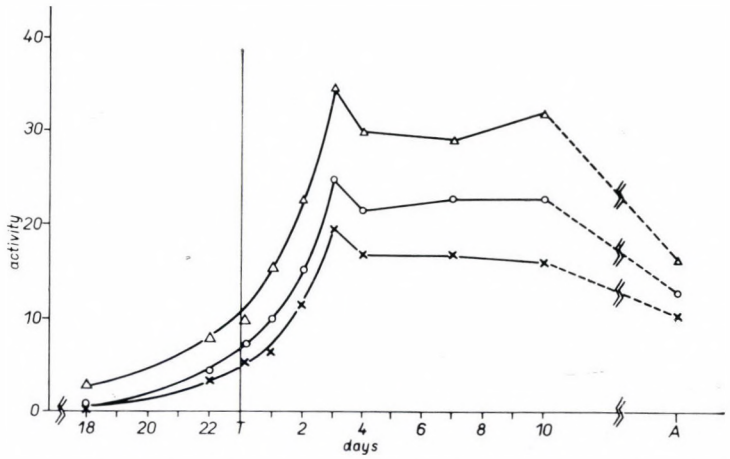

Fig. 1. Changes in the glucose 6-P-ase, PP_i-ase and P-transferase activities of rat liver during ontogenesis. ○—○ glucose 6-P-ase. △ — △ PP_i-ase. × — × P-transferase. T: day of birth. A: value found in adults. Activity: substrate converted in μmole per g liver per minute

ontogenesis is parallel to those of glucose 6-P-ase, and whether the liver of fowl embryos displays all three activities. The results of these experiments are shown in Fig. 1.

As may be seen from Fig. 1 – similarly to glucose 6-P-ase – hardly any PP_i-ase and P-transferase can be demonstrated in the liver of 18 days old rat embryos. On the last day of pregnancy all three activities are already quite marked, and after birth there is a parallel rapid rise of all three. The activities reach their maximum on the third day after birth when the activities of PP_i-ase and P-transferase are also considerably higher than in adults. Further changes in PP_i-ase and P-transferase activities are also parallel to those constantly decreasing of glucose 6-P-ase. The activities of all three enzymes are decreased but even on the 10^{th} day they are still higher than in adult animals. According to the present conception the appearance of glucose 6-P-ase at birth is a *de novo* enzyme synthesis. Therefore the parallel appearance of the three functions examined may be attributed to the formation of one and the same enzyme protein.

The results obtained during the ontogenesis of rat – namely that the three enzyme functions may be attributed to the formation of a single enzyme protein have been confirmed by experiments carried out on fowls. We have demonstrated in an earlier communication (Bot et al., 1960) that the liver of the chicken embryo

contains already at a very early embryonal stage considerable quantities of glucose 6-P-ase. Contrary to mammals, the blood sugar production of fowls has to be ensured right from the beginning by the liver of the embryo. Thus, the embryo needs under all circumstances for the production of its blood sugar the activity of glucose 6-P-ase. If the microsomally bound glucose 6-P-ase enzyme possesses in addition also PP_i-ase and P-transferase activities, then these activities must also be present in the liver of the chicken embryo. PP_i-ase and P-transferase activities were examined in the liver of 17 days old chicken embryos, and it was found that all three enzyme activities were present in significant quantities in the liver already at this early stage (Table 4).

Table 4

Liver glucose 6-P-ase, PP_i-ase and P-transferase activities of 17 days old chicken embryos

The embryos were taken from eggs which had been incubated for 17 days and frozen in liquid air. The liver was excised and stored in dry ice. Homogenate was prepared each time from the livers of two embryos. Enzyme activities were measured as described in the text

No	Substrate converted μmole per minute per g liver		
	Glucose 6-P-ase	PP_i-ase	P-transferase
1— 2	9.0	11.6	9.7
3— 4	9.0	12.3	9.7
5— 6	9.7	14.2	11.6
7— 8	11.0	12.9	11.0
9—10	12.9	18.4	14.2
Mv.	10.3 ± 1.7	13.9 ± 2.7	11.2 ± 1.9

It can be seen from Table 4 that the liver of the chicken embryos exhibits not only glucose 6-P-ase, but also PP_i-ase and P-transferase activities.

The results obtained with the embryos of mammals and fowls indicate that glucose 6-P-ase, PP_i-ase and P-transferase activities appear simultaneously and remain coupled during ontogenesis. In the liver of young rat embryos all three are missing, and all three appear at the same time at birth after which they follow a parallel increase. The liver of chicken embryos exhibits all three activities already before hatching. These ontogenetic data agree with physiological experiences which indicate that the appearance of all three functions may be attributed to the formation of a single enzyme protein.

References

Arion, W. J., Nordlie, R. C. (1964) J. Biol. Chem. *239* 2752
Bot, G., Andrássy, K., Kovács, E. (1960) Acta Physiol. Hung. *17* 337
Bot, G., Andrássy, K., Porcsalmy, I., Vereb, G. (1965) Acta Physiol. Hung. *26* 297
Cori, G. T., Cori, C. F. (1952) J. Biol. Chem. *199* 661
Dawkins, M. J. R. (1963) Quart. J. Exp. Physiol. *48* 265

Hawkins, R. D., Ashworth, M. A., Schachter, H., Haist, R. R. (1959) New Engl. J. Med. *261* 434
Illingworth, B., Cori, C. F. (1965) Biochem. Res. Commun. *19* 10
Nemeth, A. M. (1954) J. Biol. Chem. *208* 773
Nordlie, R. C., Arion, W. J. (1964) J. Biol. Chem. *239* 1680
Nordlie, R. C., Arion, W. J. (1964) J. Biol. Chem. *240* 2155
Stetten, M. R. (1964) J. Biol. Chem. *239* 3576
Stetten, M. R., Taft, H. L. (1964) J. Biol. Chem. *239* 4041
Taussky, H. H., Shorr, E. (1953) J. Biol. Chem. *202* 675

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 179—186 (1966)

Agar Gel Diffusion Tests on Bacteriophages

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(Received January 26, 1966)

A new method has been developed for measuring diffusion coefficients of the T2 and T7 phages of E. coli B in gel at different agar concentrations. The diffusion coefficient measured in free liquid was determined by the help of a regression equation, calculated with the least square method; this value is in good agreement with the value of the diffusion coefficient calculated on the basis of phage adsorption. The size of the spherical T7 phage particles was calculated from the value of the diffusion coefficient, and it was found to agree with that found in the literature. The resistance of the agar gel at different concentrations to the diffusion of phage particles was also determined. It was found that this diffusion resistance depends very much on the size of the diffusing phage particles and on the concentration of the agar gel.

Introduction

To determine the approximate shape and size of macromolecules the diffusion method is prefered to other suitable physical methods by several authors (Hercik, 1959; Setlow, Pollard, 1962). Although this method yields only rough estimates about the size of the investigated particles it is often used, because it does not require preparations of extreme purity; and so informations more characteristic for the native state of the particles can be obtained.

Among the methods used for the determination of diffusion coefficients until now only those have been employed in practice in which the experimental conditions make possible to get a sharp initial boundary.

This type of boundary was used by Polson (Polson, 1943; Setlow, Pollard, 1962) to determine the diffusion of macromolecules in free liquids. The drawback of the method lies in the fact that it is difficult to carry out, and that convectional flow cannot be excluded totally.

Another procedure is to separate the liquid containing the molecules and the pure solvent by a porous membrane (Ackers, Steere, 1962; Steere, Ackers, 1962). The drawback of the method is that the membrane is difficult to prepare; for the calibration very homogeneous preparations of macromolecules with a known diffusion coefficient are needed.

The above mentioned drawbacks are eliminated by the method employed by us. Originally this method has been developed for the determination of the

diffusion coefficients of inorganic and organic small molecules (Tamás et al., 1959; Tamás, Rontó, 1963). According to this method a certain amount of agar is added to the ion containing solution. The mixture is slightly heated and poured into a glass tube with a known inner radius. After stiffening the agar gel is blown out from the tube and cut into pieces of about 1 g each. One of them is used for the experiment. Agar gel is easy to handle. Its advantage is, that the agar concentration in the gel does not influence or hardly influences the value of diffusion constant. The concentration gradient $\frac{\partial c}{\partial x}$ was ∞ between the inner side of the agar gel and the outside liquid at $t = 0$ time. The diffusion coefficient (D) is calculated from the following equation:

$$\log \frac{c_1}{c_2} = \frac{5.78\, D}{2.30\, r^2} [t_2 - t_1]$$

where c_1 and c_2 are the ion concentrations of the agar-gel at time t_1 and t_2, respectively, and r is the radius of the gel-cylinder.

The aim of our present investigations was to try whether the well proved method for small molecules will be suitable for determining the diffusion coefficients of bacteriophages and consequently of other biologically important macromolecules. In addition to determine the accurary of the measurements we also wanted to establish the extent to which the agar-gel influences the free diffusion of such giant molecules and to see how the value of the diffusion resistance depends on the concentration of the gel. To decide the latter question the adsorption constants of the phages were measured, and then the diffusion coefficients for free liquid were determined by the Smoluchowski coagulation theory (Kruyt, 1952; Schlesinger, 1933). The data thus obtained were then compared.

Method

The method developed by us for the determination of the diffusion coefficients of small molecules (Tamás et al., 1959; Tamás, Rontó, 1963) was used in the present investigation in the following way: the T7 and T2 phages of E. coli B were added in the amounts required to sterile agar dissolved in solution M9 of 1, 1,8 and 3 per cent, respectively. Thereafter the gel was poured into glass tubes. The number of phages in the gel of a constant geometry and a weight of 1 g, was determined and considered as the initial concentration (c_0). After that the passive migration of the phages towards the 10 ml sterile M9 solution changed at given intervals was examined. The number of the phages which diffused into the solvent was determined by the Gratia-titration. The determination of the kinetics of phage adsorption was done by the Adams method (Adams, 1959); samples were taken

from the mixture of phages and their host-bacteria at 0 time and thereafter at given intervals and the titre of the non-adsorbed phages was determined in each sample.

Results

1. First the diffusion of phage T7 in 1.8 per cent agar gel was examined. The results of a typical measurement are illustrated in Fig. 1. The logarithm of the phage concentration in the gel (ordinate) is plotted against the time of diffusion

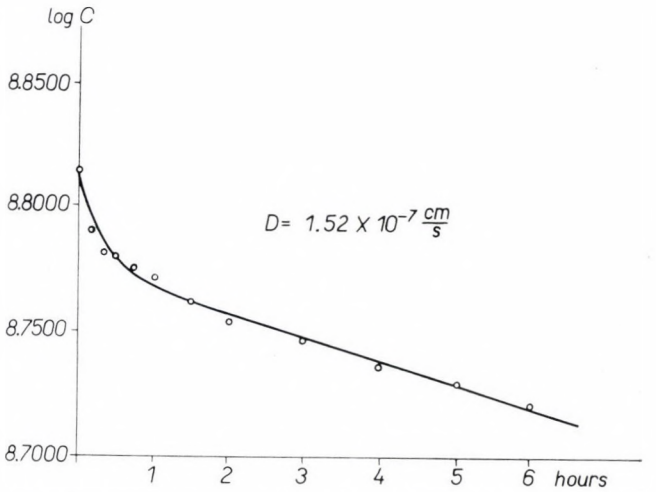

Fig. 1. Diffusion of T7 phages in 1.8 per cent agar gel. The logarithm of the phage concentration in the gel (ordinate) is plotted against the time of diffusion (abscisse)

(abscisse). As it can be seen from the Figure 12 points were used to draw the curve. Each point represents the average of 2 titer determinations (the standard deviation is ± 10 per cent).

On the basis of the determination of 28 diffusion constants in the above mentioned way the diffusion coefficient of phage T7 in a 1.8 per cent agar gel was found to be

$$[1.54 \pm 0.11] \cdot 10^{-7}\ \mathrm{cm^2/s}.$$

The diffusion coefficient of the T2 phages was also determined at the same agar concentration, and taking the average of 16 measurements the value was:

$$D = [0.608 \pm 0.04] \cdot 10^{-7}\ \mathrm{cm^2/s}.$$

The diffusion constants of the T7 and T2 phages were compared by the help of the t test, and it was found that $P \ll 0.01$. Thus the difference between the two series is strongly significant.

2. Next, the resistance of agar gels at different concentrations to the diffusion of phage macromolecules was investigated. The diffusion of the T7 phages was measured in 1, 1.8 and 3 per cent, respectively, agar gels in the above discussed manner. The average of 41 measurements was calculated by the equation

$$y = 2.11 \cdot 10^{-7} - 0.33 \cdot 10^{-7}x$$

with the least square method. Fig. 2 illustrates the regression line obtained. The values of the diffusion coefficients (ordinate) were plotted against the percentage values of the agar concentrations employed (abscisse). The correlation coefficient

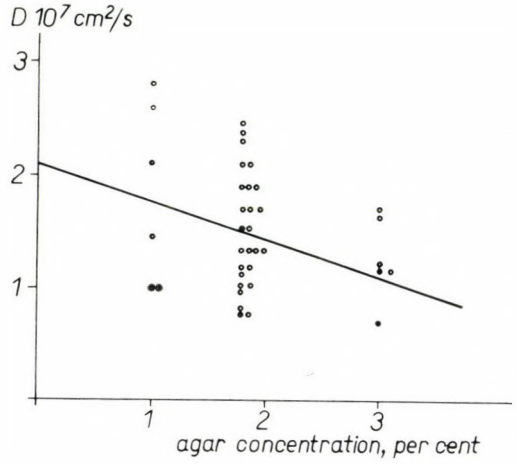

Fig. 2. The value of the diffusion coefficient of T7 phages is plotted against the agar concentration. The dots in the figure represent values of individual measurements

was found by calculation to be 0.35. To establish the significance of the regression coefficient ($0.33 \cdot 10^{-7}$) the t test was used. The value P was found to be 0.01.

As the relation between the diffusion coefficient and agar concentration in the investigated range may be approximated by a linear connection and as there exists no specific interaction between the gel and the diffusing phage particles, the value of D belonging to the agar concentration of zero per cent obtained by the equation of the resulting straight line is: $D = 2.11 \cdot 10^{-7}$ cm^2/s, according to the linear equation obtained. This value can be considered as the diffusion coefficient of T7 phage in free liquid. The data of the next chapter will support this procedure.

For the diffusion coefficient of the T2 phages in free liquid we obtained the value of $D = 0.955 \cdot 10^{-7}$ cm^2/s using the same method as with the T7 phages. It should be mentioned that the measurement carried out on the T2 phages have only informative character.

3. The other way to determine the free diffusion consists in employing the Smoluchowski – Collins – Kimball reaction theory (Kruyt, 1952; Schlesinger, 1933; Setlow, Pollard, 1962) on the coagulation of submicroscopic particles.

The following equation was used (Schlesinger, 1933):

$$D = \frac{k}{4 \pi R}$$

where k is the adsorption velocity constant characteristic for the interaction of phage and bacterium, R is the sphere-equivalent radius of bacterium. R was taken as $4.3 \cdot 10^{-5}$ cm according to Schlesinger's data (1933); k was determined

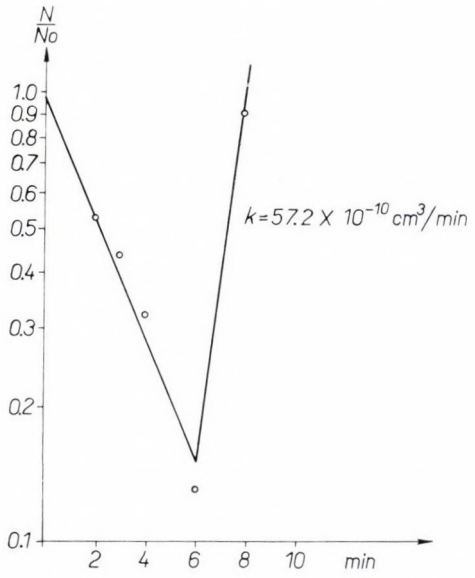

Fig. 3. The adsorption curve of phage T7 (a typical experiment). The quotient of the free phage titre (N) and the phage titre (N_0) at 0 minute (ordinate) is plotted against the incubation period in minutes (abscisse). The adsorption reaches its maximum in the 6th minute

by measuring the adsorption kinetics (Fig. 3). On the average of 10 measurements the adsorption velocity constant was found to be $70.7 \pm 4.8 \cdot 10^{-10}$ cm^3/min, the value of the diffusion coefficient computed from it is: $2.2 \cdot 10^{-7}$ cm^2/s.

It should be noted that while employing the Smoluchowski process several simplifications had to be done; for example it had to be assumed that each contact leads to adsorption at the same time, the sphere-equivalent radius of the bacterium, etc. Even so the agreement is very good between the diffusion coefficient calculated in free liquid in the above manner, and the diffusion coefficient obtained by extrapolation in Fig. 2.

4. The size of the spherical T7 phage particles was also calculated from the value of the diffusion coefficient by the help of the Einstein formula. The diameter of the phage was found to be 34 nm which is in good agreement with the literary data determined by different methods.

Discussion

Comparing our results with those in the literature, we found a good agreement. Measurements were made by Polson and Sheppard (Hercik, 1959; Polson, 1943) on T3 phage which has a similar size as T7. They found the diffusion constant to be 1 to $5.3 \cdot 10^{-7}$ cm^2/s depending on the concentration of phage particles. The diffusion constant with a phage concentration of 10^9 particles/ml if compared to that measured in free liquid ($2.1 \cdot 10^{-7}$ cm^2/s) agrees with these values.

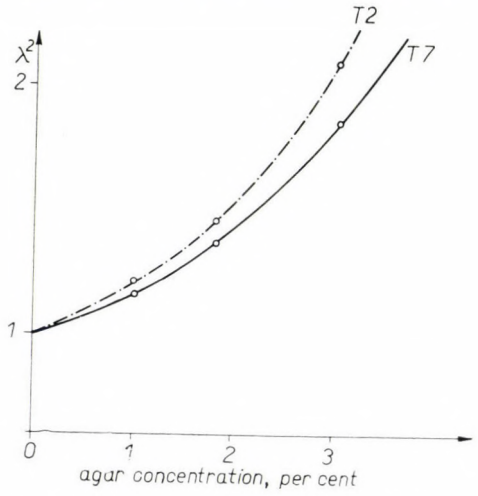

Fig. 4. The diffusion resistance plotted against the gel concentration with T2 and T7 phages

In connection with Steere and Ackers' data (Steere, Ackers, 1962; Ackers, Steere, 1962) on the diffusion coefficient measured by investigating the migration of particles across agar gel membrane, the following remark should be made: Although they were working with biological macromolecules other than phages, and consequently there is no opportunity for direct comparison, the diffusion coefficient given by them is small as compared to the other diffusion constant values found in the literature. It is possible that these different results are to be explained by the effect of particle adsorption on the agar gel membrane.

The resistance λ^2 of the agar gel at different concentrations (where λ^2 is the factor which shows how many times the diffusion coefficient is smaller at a given gel concentration than in free liquid) to the diffusing T2 and T7 phage particles

is illustrated in Fig. 4. The Figure reveals that diffusion resistance is greater to the larger T2 phage, even at a similar gel concentration, than against the T7 phage. The diffusion resistance which is characteristic for the gel structure depends very much on the size of the phage particles, as expected. Consequently it has to be determined with each macromolecule separately.

It must be noted that the λ^2 values measured by our method are much lower than the values given by Ackers and Steere (1962) for the resistance of 1, 1.8 and 3 per cent agar gels, respectively. These authors reported the reciprocal of λ^2 in their communication cited above; their data compared to ours are illustrated in Table 1.

Table 1

Comparison of diffusion resistances

Particles used	Concentration of agar		
	1 per cent	1.8 per cent	3 per cent
SBMV*	1.7	4	20
T7	1.19	1.39	1.88
T2	1.22	1.47	2.12

* Steere, Ackers' data

The great difference between the diffusion resistances given by the two different methods can be explained by the difference in preparing the agar – gel membrane and the gel cylinder and consequently by the difference in their structure. The size and shape of the diffusing SBMV particles, however, are nearly equal to those of our T7 phage particles.

On the basis of the results discussed above we may state that our agar gel diffusion method can be applied very well besides measuring the λ^2 also for the determination of diffusion coefficients of bacteriophages and of similar macromolecules in free liquid. Our experiments have proved that a 1.8 per cent agar gel can be handled easiest (with most favorable mechanical properties) and that the results obtained at this concentration have the least deviation. It seems to be advisable therefore to use this concentration also in other experiments to determine diffusion coefficients because this concentration assures the best reproducible conditions. Finally, the advantage of our method over the earlier ones is its being quick and simple. Its accuracy and adequacy can be compared well that of the procedures used for such purposes until now. Adsorption on agar gel is not probable owing to the comparatively high values of the diffusion coefficients.

Acknowledgement

Authors would like to express their thanks to M. Rideg and N. Naményi for their help in the accomplishment of the experiments.

References

Ackers, G. K., Steere, R. L. (1962) Biochim. Biophys. Acta *59* 137
Adams, M. H. (1959) Bacteriphages. Intersci. Publ. Inc. New York
Benezech, Chr. (1958) Physico-Chimie Biologique et Medicale, Paris
Hercik, F. (1959) Biophysik der Bakteriophagen. VEB Dtsch. Verl. d. Wiss. Berlin
Kruyt, H. R. (1952) Colloid Science. Els. Publ. Comp. Amsterdam
Polson, A. (1943) Nature *154* 823
Polson, A. (1958) Biochim. Biophys. Acta *29* 426
Schlesinger, M. (1933) Z. Hyg. *114* 149
Setlow, R. B., Pollard E. C. (1962) Molecular Biophysics. Addison-Weseley Publ. Comp. Reading, Mass.
Steere, R. L., Ackers, G. K. (1962) Nature *196* 475
Tamás Gy., Rontó Gy., Tarján I. (1959) Magy. Fiz. Folyóirat *7* 407
Tamás Gy., Rontó Gy. (1963) Magy. Fiz. Folyóirat *11* 513

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 187—196 (1966)

Determination of Extrathyroidal Iodine by Neutron-Activation Analysis

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(Received January 28, 1966)

1. Neutron-activation analysis was used for the determination of stable iodine in biological samples. The aim of the experiments was to create better conditions for the determination of specific activity values for isotope-kinetic studies, eliminating the discrepancy between the sensitivities in measurements of active and inactive iodine, respectively.

2. In samples of extrathyroidal rat tissues iodine was estimated with an accuracy of 10^{-9} g. This sensitivity can be enhanced by increasing the neutron flux and by partial automatization.

3. The chemical separation of the activated iodine was completed after the irradiation. The gamma spectrometrical evaluation was greatly promoted by the fact, that the I-128 isotope of the sample was prepared radiochemically pure for the measurements by the separation of cuprous iodide by precipitation.

4. A method of isotopic double labeling was accomplished: iodine content of such samples was determined by activation in which the acute incorporations of other radio-iodine isotopes (I-131 and I-125) were measured previously.

5. To characterize the efficiency of the method, the concept of specific sensitivity was introduced.

Introduction

Concerning the movement of iodine the extrathyroidal iodine pool plays an important role in the organism. The detection of iodine concentrations less than 40 μg per cent (3—4 orders of magnitude smaller than that of the thyroid gland) is necessary. In the case of small samples this can be achieved only if it is possible to increase the sensitivity of the analysis. The activation analysis of iodine has an important advantage in sensitivity over other tracer elements, and over other methods of iodine analysis. This is shown in Fig. 1 on a logarithmic scale. As far as trace elements are concerned the data of Fukai and Meinke (Fukai and Meinke, 1959) as well as Lenihan (Lenihan, 1964) were used. To compare the different analytical methods of iodine determination with our own procedures data of a few fundamental and review articles were used (I.U.P.A.C., 1963; Lambert, 1959; Leipert, 1937; Spitzy, 1958; Taurog, 1946). The figure shows that the average sensitivity (I_{act}) obtained by the activation of the samples surpasses that of the best classical iodine analysis by one order of magnitude, but it is smaller by one order of magnitude than the optimum sensitivity (I'_{act}) of our activation method.

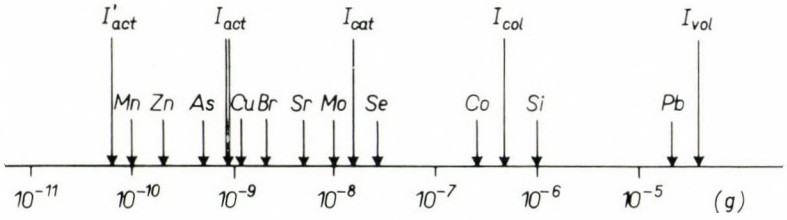

Fig. 1. The lowest limit (g) of the detection of biologically important trace elements by activation analysis as compared to different iodine analysis methods

I_{act} = sensitivity reached under our present radiometrical and yield conditions,
I'_{act} = maximum sensitivity attainable under our present activation conditions,
I_{cat} = sensitivity of catalytic iodine analytic procedures
I_{col} = sensitivity of colorimetric iodine analytic procedures,
I_{vol} = sensitivity of volumetric and gravimetric iodine analyses

Principles of Iodine Activation Analysis

Concerning the general theoretical aspects of activation and its possibilities in our country only one of our review papers (Kertész, 1966) is refered to.

If biological samples containing stable iodine are placed into the flux of thermal neutrons, radioactive iodine isotope will be produced according to the reaction $^{127}I(n, \gamma)\,^{128}I$. The amount of irradiated elements can be determined by the induced activity. Fig. 2 shows the relations between the atomic number (Z)

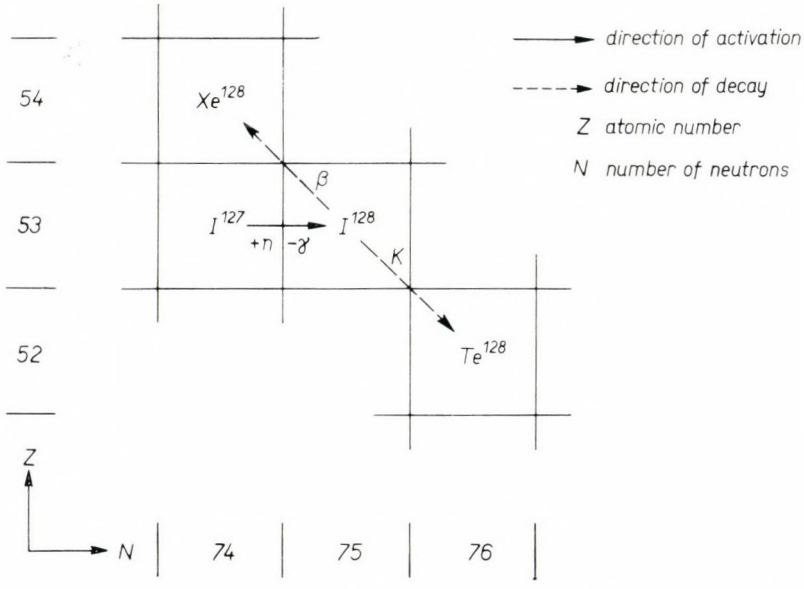

Fig. 2. Activation of iodine by thermal neutrons and the decay of the radioactive iodine isotope produced

and the number of neutrons (N) in the nuclei of the element to be activated (target-element or precursor isotope = ^{127}I), of the product (^{128}I) and of the end products (^{128}Xe and ^{128}Te), respectively. This shows that the product used will be converted into ^{128}Xe mostly by negative beta decay and into ^{128}Te by electron capture in a smaller percentage. A gamma radiation of 450 keV energy takes place most probably following the beta decay of the nucleus.

Other reactions can also be chosen besides the above mentioned ones, according to the possibilities and demands. The product of these is I-126 with more advantageous radiation characteristics, and consequently with properties more favourable for measurements. Unfortunately, due to the disadvantageous parameters of the irradiation, the sensitivity will be lower than with the thermal neutron activation used by us.

Materials and Methods

Albino rat tissues with low iodine content (plasma, stomach wall, muscle, liver) and rats' food were used in our experiments. The samples were taken from experimental material previously treated with I-131, and I-125, respectively. One

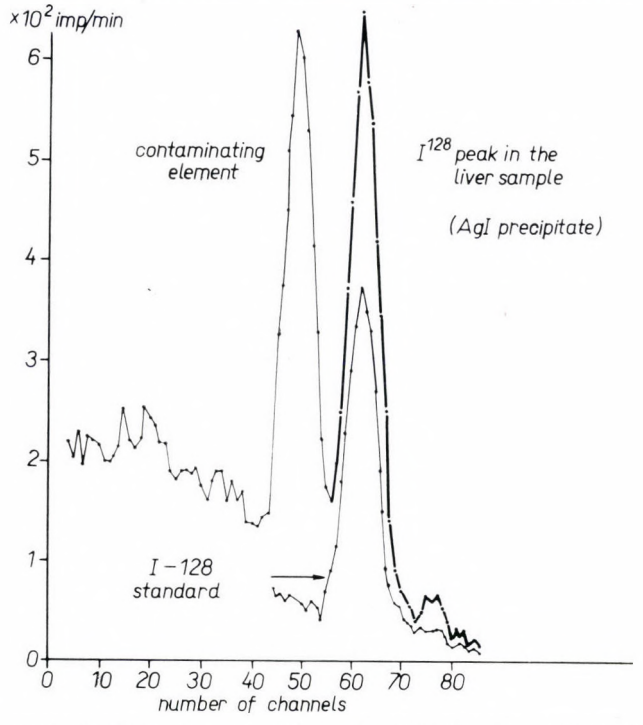

Fig. 3. Gamma peak of 450 keV energy of the activated iodine in rat liver, after separation by AgI precipitation. The interfering peak, appearing in the 49th channel also in the sample originated from a contaminating element compared to the pure iodine standard

part of the rats was kept on a normal diet, the other part on iodine deficient food. Completing the tracer experiment the inactive iodine content of the samples used for radioactive counting in vitro was determined by activation analysis. The material to be irradiated was stored until the decay of I-131, and I-125, respectively. The samples were combusted before irradiation. To test whether or not any iodine loss occurred during combustion samples were taken from the stomach wall of rats treated with radioactive iodine, and were combusted. No significant decrease in the activity was observed during the process. It should be noted that the radioactive iodine added to the homogenate of the inactive sample in vitro shows a loss of 29 – 50 per cent.

The irradiations were performed in the Hungarian experimental reactor (Central Research Institute for Physics of the Hungarian Academy of Sciences, Budapest) with a neutron flux of 10^{13} n/cm^{2}sec for 2 hours.

Table 1 gives a survey of the procedure of the iodine activation analysis. The data in the table are supplemented by the following.

Table 1

Sketch of the procedure used in the neutron activation analysis of iodine

Procedure	Details	Notes (loss and source of error)
1. inactive sample	tissues or food of albino rats with low iodine content standard NH_4I	the frequency of the natural occurrence of the target element is 100 per cent
2. preparation	drying incineration at 300°C for 2 hours	there is no significant loss of iodine
3. irradiation	neutron flux 10^{13}n/cm^2.sec irradiation time: 2 hours nuclear reaction: $^{127}I(n, \gamma)^{128}I$	activation to 95 per cent of saturation the half life of the end product is 25 minutes
4. chemical separation	liquefying with NaOH extraction with CCl_4 separation of the CuI precipitate	the average yield of iodine is 40 per cent; the radiochemical purity of the end-product is almost 100 per cent; the time elapsed from the end of irradiation till the counting is on the average 1.5 hour
5. counting of radioactivity	γ-spectrometrical of counting of the 450 keV radiation of I-128 checking the speed of decay	efficiency of counting is about 25 per cent; background radiation; statistical errors

The analysis was carried out by irradiating the iodine standard simultaneously. Iodine was separated from the other activated carriers of the sample after irradiation. Thus, we were able to add inactive iodine as a carrier to the sample

and to the standard to avoid adsorption and co-precipitation, to estimate the yield, and to disregard the iodine contamination of the reagents as well (Kellershon et al., 1961).

The sample was taken from the radiation silica vessel to the Ni vessel and it was liquefied with NaOH at 600°C for 5 minutes. The alkaline liquid was then dissolved in distilled water and mixed with a KI-carrier solution containing 5 mg

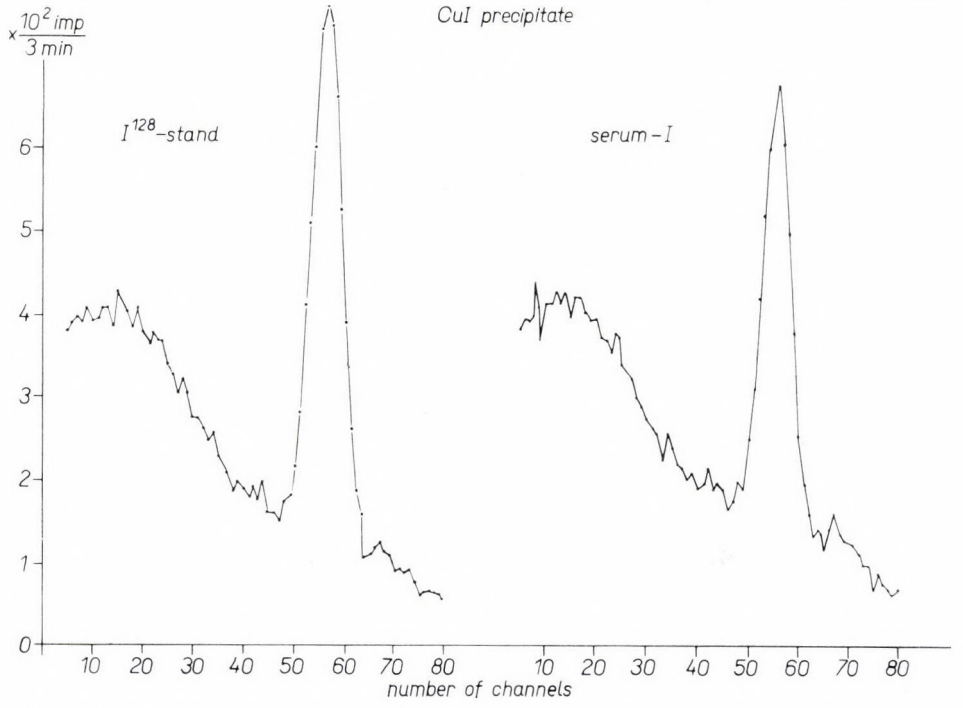

Fig. 4. Activated iodine content of rat serum in radiochemically pure state after separation by CuI precipitation

iodide. After acidification with 6n HCl the iodide was oxidized to iodine with KNO_2, and extracted three times with 10 ml CCl_4. The reextraction was done three times with 10 ml of n NaOH. After the mild acidification of the solution (pH $\sim$ 6) CuI precipitate was separated with CuCl. Our experiences have shown that applying CuI precipitates (Figs. 4 and 5), the product can be obtained in a higher radiochemical purity than in the form of the more generally applied AgI precipitate (Fig. 3). AgI precipitate is known to have a stronger adsorption capacity. The NH_4I standard was diluted after irradiation and an aliquot was taken out from the solution. The standard was also separated in the form of CuI precipitate in the presence of carrier.

During the purification the average yield of the iodine was 40–45 per cent.

The radioactive measurements were carried out on the basis of the gamma peak of I-128 of 450 keV energy. For the counting a NaI(Tl))-crystal (3 × 3 inches), and a 128-channel amplitude analyser of type KFKI-Y-52-R were used. The peak hights of the samples and standards were determined in the usual way, i.e. on

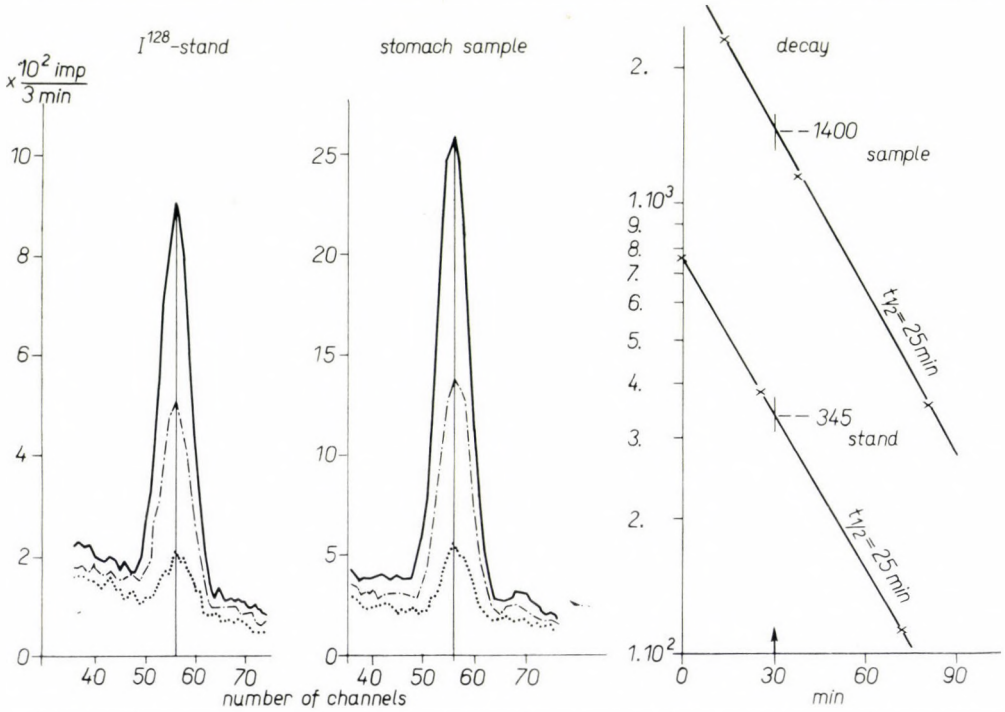

Fig. 5. The amount of activated iodine in the rat stomach wall as compared to the irradiated iodine standard

the basis of the number of impulses obtained at the bisecting vertical line of the peak width, after deducing the background. Repeating the measurements several times the decreasing values of the peak-heights were plotted against the time on a semi-logarithmic scale. It was checked whether the decrease of activity goes parallel to the half-life of I-128 (Fig. 5). Since the percentage yield and the iodine concentration of the standard were known the iodine content of the sample could be estimated by counting the number of impulses of both the sample and the standard at the same time, e.g. thirty minutes after the starting of the experiment.

Results

After the preliminary experiments to create adequate conditions for the activation and chemical separation 20 samples and the same number of standards were irradiated. Routine determinations could not be performed because of the

labourous procedure involved in the analysis and the inefficiency of automatization. Instead, individual samples were taken and analyzed from a normal, and from an iodine deficient animal. The same experiment was then repeated once more. The data in Table 2 therefore show the mean values of two parallel activation

Table 2

Quantities of iodine (μg) found in some important extrathyroidal tissues (1 g wet weight) and in the food (1 g dry weight) of the albino rat

	With activation analysis	With catalytic procedure
On normal diet		
serum	0.058	0.05_2
serum-PBI	0.024	0.02_7
stomach wall	3.469	0.32_6
skeletal muscle	0.370	
liver	1.330	
dry food	1.315	1.40_0
On iodine deficient diet		
serum	0.028	0.02_9
serum-PBI	0.026	0.02_5
stomach wall	1.273	0.23_2
dry food	0.141	0.13_6

analyses. The data involve both the biological fluctuations of the specimens and the experimental error. Because of this and because of the limited number of measurements we do not give full details about the accuracy of the method, we only mention (see Table 1, point 4 and 5) the sources of errors: the weights of precipitates determined for the calculation of the chemical yield, the statistical errors in counting the activities of the samples and of the standards and consequently the evaluation of the peak heights. If taken together, they result in an experimental error of about 5 per cent. On the other hand, the biological fluctuation is very significant in tissues with low iodine concentrations. According to some references (Van Zyl, cit. Long, 1961) a scattering of date higher than 50 per cent can be expected in the case of rat serum-PBI.

Table 2 illustrates the iodine concentrations of a few types of samples as determined by the classical catalytic microanalysis in a ceric-arsenite system. The material was taken from the same animals and from the same food which were used for the activation analysis. Significant difference in the results of the two different determinations were observed only with normal, and iodine deficient rat stomachs.

It should be mentioned that both the normal and the iodine deficient total iodine serum, and also serum PBI values refer to the same serum by pairs and can be related to each other: concerning rats kept on normal mixture of food this is $\frac{\text{PBI}}{\text{serum total iodine}} = 0.41_{(\text{act. anal.})}$ and $0.51_{(\text{catalyt.})}$, respectively; this quotient

concerning animals insufficient of iodine is $0.93_{(act.\ anal.)}$ and $0.86_{(catalyt.)}$, respectively. It must be added that quotient gained by the catalytical way are more inaccurate, because their factors are values estimated only in the third decimal number.

The iodine concentrations shown in the Table make it clear that the activation analysis has a great advantage especially in the determination of the iodine content of the serum, of the serum-PBI and of iodine deficient food samples, because of the limited total amount of the removable and measurable samples (Smith et al., 1964).

Discussion

The sensitivity of the neutron activation analysis

Recent experiments – beyond the demand for the determination of low extrathyroidal iodine concentrations – served the purpose of determining adequately the stable isotope of the organism in iodine turnover experiments in which iodine isotopes are incorporated. For this purpose the sensitivity must be increased to a great extent.

The counting of the iodine standards revealed that the lowest iodine amount detectable is $G_{\min} = 9.8 \pm 2.3_6 \cdot 10^{-10}$ g, depending on the conditions during counting (neutron flux, the time elapsed from the irradiation to the counting, the chemical yield, the efficiency of counting). By improving the conditions of forwarding the sample (dispatch-tube) and those of the yield (Table 1, point 4) the sensitivity can be expected to rise by one order of magnitude.

If radioactive counting conditions (counting geometry, background radiation, etc.) can be chosen in such way that an activity of 0.5 mμCi can still be detected, the amount of the target element can be calculated (Kertész, 1966; Spencer et al., 1958): $G_{\min} = 7.10^{-11}$ g I-127. Owing to different limiting factors this value, as we have seen, is overestimated, but by *raising the neutron flux* the calculated limit of sensitivity can still be increased. This would be necessary to achieve the above mentioned purpose, if we realize that the same 0.5 mμCi I-131 represents only $4.05 \cdot 10^{-15}$ g iodine atoms in a tracer carrier free state. This calculated sensitivity of the activation analysis is almost four orders of magnitude lower than that of the carrier free tracer method, presuming that there is no stable iodine in the labelled system or it did not get into equilibrium with it.

$8.6 \cdot 10^{-18}$ g of iodine will be converted into the radioisotope I-128 from the amount $G_{\min}$ of stable iodine, i.e., at the end of the activation $\frac{8.6 \cdot 10^{-18}}{7 \cdot 10^{-11}} \times 10^6 =$ $= 0.123$ labelled atoms will belong to each million of I-127 atoms. According to this conception the optimum standard, the index number of sensitivity of the *activation labeling* is 0.123 ppm.

It seems advisable to introduce the concept of *specific sensitivity* depending on the substance of the biological sample. That smallest quantity of iodine is meant by this, which can be detected from the maximum mass ($m_{\max}$ in gramms)

of the sample in question that could be taken for determination. Its value is $\frac{G_{min}}{m_{max}}$ and it is in proportion with the minimum specific activity which can be induced in the sample. The success of attaining the calculated sensitivity depends on this ratio and its performability. The specific sensitivity of the analysis with respect to rat stomach ($m_{max} = 1.1$ g) under our experimental conditions is $\frac{G_{min}}{1.1} = 8.9 \cdot 10^{-11}$ g I/g stomach. The hypothetical iodine content of the rat hypophysis (0.01 g) could be determined only at a higher concentration than the specific sensitivity of the $\frac{G_{min}}{0.01} = 9.8 \cdot 10^{-8}$ g I/g hypophysis.

Acknowledgement

F. Ditrói assistant prof. (Dept. of Gynaecology, University Medical School, Debrecen) performed the colorimetric determination of iodine catalyzing the oxidation of the ceric-arzenite system in some samples for the purpose of comparison. We would like to express our thanks for his valuable help.

References

Fukai, R., Meinke, W. W. (1959) Limn. and Oceanogr. *4* 398
Hoppe. Seyler/Thierfelder (1955) Hdb. der physiol. und path. chem. Analyse. III/1., Springer-Verlag, Berlin, 136, 248
I.U.P.A.C. — Publ. (1963) Tables of Spectrophotometric Determination of Elements. Butterworth Publ. Co., London
Kellershon, C., Comar, D., Le Poes, C. (1961) J.A.R.I. *12* 87
Kertész, L. (1966) Kísérletes Orvostudomány. (In the press)
Lambert, J. J. (1959) Anal. Chem. *23* 1251
Leipert, T. (1937) Biochem. Ztschr. *270* 448
Long, C. (1961) Biochemist's Handbook. E. and F. N. Spon Lt. London
Smith, E. M., Mozley, J. M., Wagner, H. N. (1964) J. Nucl. Med. *5* 828
Spencer, R. P., Mitchell, T. G., King, E. R. (1958) J.A.R.I. *3* 104
Spitzy, H., Rease, M., Skrube, H. (1958) Mikrochimica Acta, 488.
Taurog, A., Chaikoff, I. L. (1946) J. Biol. Chem. *163* 313
Proceedings of Symposium on Radioactivation Analysis, 1960, Butterworth Press, London.
L'analyse par radioactivation et ses applications aux sciences biologiques. 3° Congrès International de Biologie de Saclay, 1964 Presses Universitaires de France, Paris.
Symposium on Radioisotope Sample Measurement: Techniques in Medicine and Biology, 1965, IAEA, Vienna

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 197—202 (1966)

Separation of Isotopes by Thermodiffusion

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(Received November 15, 1965)

Potassium and calcium isotopes were separated in a liquid system by the help of thermodiffusion. The efficiency of the separation was determined by the separation factor (q) which is given by the ratio of the values of the activity and specific activity of the solution samples taken from the lower and upper reserve volumes of the thermodiffusion tube. In most of the experiments $q > 1$. The experiments were evaluated by the t test, in the case of $P = 0.05$ the separation for potassium and calcium isotopes is significant.

Introduction

The phenomenon of thermodiffusion has been known for a long time. It was described by Ludwig (1856) and Soret (1879, 1881) for liquids, and theoretically Enskog (1911, 1911a, 1912) and Chapman (1920) worked it out for gases. For the separation of isotopes it was employed first by Clusius and Dickel (1938, 1940) who succeeded to separate Cl-35 and Cl-37 from each other in the gas form. The separation of Zn-64, Zn-66 and Zn-68 isotopes in a liquid system was done by Korsching and Wirtz (1939).

It is known in biology that living systems are able to produce and maintain very significant concentration differences. Moreover, the fact should be taken into consideration that some ions are more concentrated in the urine than others, for example. For the explanation of these phenomena experiments have been done to separate the substances of different atomic weights (Ernst and Makarész, 1954). The separation of potassium and calcium isotopes was investigated in a liquid system in model experiments.

Method

A three-walled glass vessel was used for the experiments (Fig. 1), and a reserve volume was formed on the lower and upper sides of the tube (Korsching and Wirtz, 1940). At the beginning of the experiment the narrow slit of the thermodiffusion tube was filled with the solution to be investigated, that is, with $K^{42}Cl$,

or 0.1 M KCl and $CaCl_2$ pro anal. solution labelled with $Ca^{45}Cl_2$. The temperature gradient at the two sides of the slit was established by continuously heating the inner side of the tube with a steam of hot water (T_2) and cooling the outer

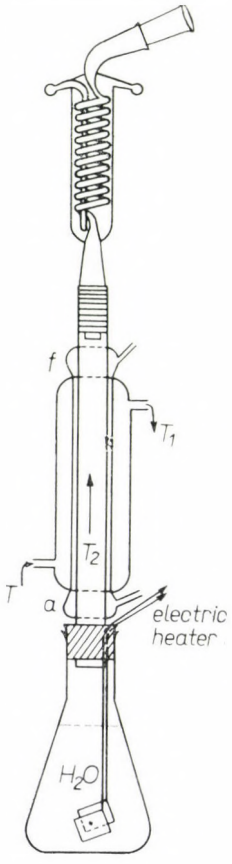

Fig. 1. Thermodiffusion apparatus for the separation of potassium and calcium isotopes. T = temperature of the cooling water flowing in, T_1 = temperature of the cooling water flowing out, T_2 = temperature of the vapour of the boiling water ($\sim$ 100°C); l = lower reserve volume; u = upper reserve volume

side with flowing tap water (T_1). To ensure the constant boiling of the distilled water, capacity heaters of about 200 watts were placed into it, with about 30×50 mm thick nickel sheets, about 5–6 mm far from each other. In such a way the experiments could be continued night and day. To avoid the evaporation loss of the water owing to the continuous boiling, the thermodiffusion tube was fitted with a cooling coil and this ensured the condensing of the vapour and its flowing back to the heating flask.

A thermometer hanging into the inner steam space was used to record the T_2 and the temperatures of the inflowing and outflowing water. The temperature difference $T_2 - T_1 = \Delta T$ was about 80°C. After the experimental period the concentrations of the solution samples taken from the lower and upper reserve volumes were determined by a Zeiss flamephotometer. The activities of the solutions, after drying, were counted with an end window Geiger–Müller tube and expressed as counts/minute. The efficiency of separation is indicated by the value of the separation factor (q) which is the quotient of the concentration, activity, and specific activity of the solutions in the lower (l) and upper (u) part or the reserve volume, respectively. That is:

$$q_{cc} = \frac{\text{mg/ml}_l}{\text{mg/ml}_u}$$

$$q_a = \frac{\text{Imp/ml}_l}{\text{Imp/ml}_u}$$

$$q_{sa} = \frac{\text{spec. akt}_l}{\text{spec. akt}_u} = \frac{q_l}{q_{cc}}$$

The value of q shows the efficiency of the separation.

Good thermodiffusion efficiency can be reached by the correct: *a)* distance of the narrow separating slit, *b)* length of the tube, *c)* concentration, *d)* time of separation, and *e)* ΔT.

a) The width of the working space d is 0.2–0.3 mm.

b) The length os the tube is ~20 cm.

c) 0.1 M solution is used for the time being.

d) The time of separation is 2–5.5 hours in the case of KCl and 1–8 hours in the case of $CaCl_2$.

e) $\Delta T = T_2 - T_1$. T_1 is the mean temperature of the water flowing in and out.

f) Reserve volumes are ~18 ml; working volume ~2 ml.

Samples were taken every hour or more often with a syringe fitted with an injection needle. The experiments were performed with thermodiffusion tubes found as best by us, and all other factors were kept constant, save the time of separation. The most favourable experimental time is not known yet. The amount of the energy consumed can be estimated from the difference in temperature and in the amount between the inflowing and outflowing cooling water.

Results

The following Tables illustrate the results of the thermodiffusion experiments with different isotopes. The second column of Table 1 shows that in a 0.1 M KCl solution a separation with a factor of about 1.04 to 1.05 could be

Table 1

Thermodiffusion of a pro anal. KCl solution labelled with $K^{42}Cl$

Experimental time (hours)	$q_{cc} = \frac{cc_l}{cc_u}$	$q_a = \frac{\text{Imp.}_l}{\text{Imp.}_u}$	$q_{S\,a} = \frac{\text{spec act.}_l}{\text{spec. act}_u}$
	1.09	1.10	1.01
	1.10	1.03	0.93
	1.04	1.06	1.01
	1.04	1.10	1.06
	1.02	1.09	1.07
	1.04	1.08	1.04
	1.02	1.08	1.06
	1.02	1.08	1.06
	1.02	1.08	1.06
	1.03	1.08	1.05
	1.04	1.06	1.02
5.5	1.04	1.11	1.07
	1.05	1.11	1.05
	1.04	1.10	1.06
	1.05	1.09	1.04
	1.05	1.10	1.05
	1.05	1.10	1.05
	1.06	1.09	1.02
	1.04	1.09	1.05
	1.05	1.07	1.02
	1.04	1.05	1.01
	1.05	1.07	1.02
	1.05	1.09	1.04
	1.05	1.06	1.01

achieved. It is important to note that the third column shows a *separation factor about twice as great for the radioactive isotope* K-42. 1 ml of a 0.1 M KCl solution contains $6.10^{23} \cdot 0.1 \cdot 0.001 = 6.10^{19}$ K^+ ions; the average specific activity of the used preparation containing radioactive K-42 is ~ 1 mC/gK; the 1 ml solution contains about 10^{-3} part of it, i.e. 1 μC, i.e. $\frac{45{,}000}{0.69} \cdot 3.7 \cdot 10^4 \cdot 2.3 \cdot 10^9$ K-42. Thus, the solution containing practically 10^{10} times less K^+-42 gave a separation factor twice as great in an experiment lasting for a few hours. This indicates that the described very simple thermodiffusion apparatus is sensitive enough to separate K-42 electively at a temperature gradient of about 80°C/0.2 mm.

Table 2 supports the above finding. It shows a value of ~ 1.08 for K-42 in contrast to the separation factor of ~ 1.05 for 0.1 M KCl.

The above conclusion is confirmed by the results in Table 3, according to which the thermodiffusion experiments performed with $CaCl_2$ under the same conditions (8 hours) show a remarkably high value of $1.18 - 1.19$ for Ca-45, in contrast to the average separation factor of $1.05 - 1.06$ for a $CaCl_2$ solution. It is true on the other hand, that all the experiments with shorter periods show much smaller differences.

Table 2

The average values of the separation factors of a 0.1 M KCl solution labelled with $K^{42}Cl$, lasting for 2.0, 3.0, 4.0, 4.5, 5.0 and 5.5 hours, respectively

Experimental time (hours)	$\bar{q}_{cc}$	$\bar{q}_a$	$\bar{q}_{s.a.}$
2.0	1.01	1.04	1.02
3.0	1.05	1.08	1.03
4.0	1.06	1.09	1.03
4.5	1.05	1.07	1.02
5.0	1.06	1.11	1.04
5.5	1.05	1.09	1.04

Table 3

Thermodiffusion of a pro anal. 0.1 M $CaCl_2$ solution labelled with $Ca^{45}Cl_2$

Experimental time (hours)	$q_{cc} = \frac{cc_l}{cc_u}$	$q_a = \frac{\text{Imp.}_l}{\text{Imp.}_u}$	$q_{s.a.} = \frac{\text{spec. ac}_l}{\text{spec. act}_u}$
	1.04	1.17	1.12
	1.06	1.19	1.12
	1.06	1.23	1.17
	1.06	1.18	1.11
	1.06	1.22	1.16
	1.01	1.08	1.07
	1.07	1.22	1.14
	1.05	1.20	1.14
	1.08	1.23	1.14
	1.07	1.14	1.07

Table 4

Average values of the separation factors of a 0.1 M $CaCl_2$ solution labelled with $Ca^{45}Cl_2$, lasting for 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0 and 8.0 hours, respectively

Experimental time (hours)	$\bar{q}_{cc}$	$\bar{q}_a$	$\bar{q}_{s.a}$
1.0	1.06	1.07	1.01
2.0	1.06	1.05	0.99
2.5	1.05	1.07	1.01
3.0	1.05	1.07	1.01
3.5	1.04	1.07	1.03
4.0	1.04	1.05	1.01
4.5	1.04	1.05	1.01
5.0	1.09	1.00	0.96
6.0	1.07	1.04	0.97
8.0	1.06	1.19	1.12

As it can be seen from Tables 2 and 4 the separation time of the pro anal. KCl solution labelled with $K^{42}Cl$ was 2.0, 3.0, 4.0, 4.5, 5.0 and 5.5 hours, respectively the separation time of the pro anal. $CaCl_2$ solution labelled with $Ca^{45}Cl_2$ was 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0 and 8.0 hours, respectively. According to the experimental data the optimum separation time for KCl is 5.5 hours, and for $CaCl_2$ 8 hours. The maximum separation time was 22 hours for KCl and 72 hours for $CaCl_2$.

The experiments were evaluated by the t test. For 122 experiments with KCl the value of t was 6.62 and for 120 experiments with $CaCl_2$ this value was 3.08. In both cases specific activities were used for the calculated ions. According to the experimental results the separation is significant in the case of $P = 0.05$.

Discussion

The isotopes of potassium and calcium were separated from each other in a liquid system by thermodiffusion (Mányi, 1963; Varga-Mányi, 1964). On the basis of both our experimental results and the literary data (Ernst, 1941; Ernst and Makarész, 1954) it can be supposed that in certain cells and organs the temperature gradient, owing to metabolism, may cause thermodiffusion. On the other hand, if the living cell is able to differenciate the isotopes, the problem of the precise evaluation of tracer experiments in biological systems can be raised.

Acknowledgement

Author wishes to express her gratitude to Prof. E. Ernst for the proposal of the problem and for his help in accomplishing it.

References

Chapman, S. (1920) Philos. Trans. Roy. Soc. *217* 115
Clusius, K., Dickel, G. (1938) Naturwiss. *26* 546
Clusius, K., Dickel, G. (1940) Naturwiss. *28* 711
Enskog, D. (1911) Phys. Zeitschr. *12* 56
Enskog, D. (1911a) Phys. Zeitschr. *12* 533
Enskog, D. (1912) Ann. der Physik. *38* 731
Ernst, E., Makarész, D. (1954) Acta Physiol. Hung. *6*, 125
Ernst, E. (1941) Thermoosmose und Thermodiffusion. Szeged
Ernst, J. Bevezetés a biofizikába (in press)
Fleischmann, R., Jensen, H. (1942) Erg. exakt. Naturw. *20* 121
Jost, W. (1957) Diffusion. Darmstadt. P. 158—168
Korsching, H., Wirtz, K. (1939) Naturwiss. *27* 367
Korsching, H., Wirtz, K. (1940) Ber. Deutsch. Chem. Ges. *73* 249
Ludwig, C. (1856) Sitzungsber. Akad. Wiss. Wien. Math. Naturw. Kl. *20* 539
Mányi, P. (1963) Bull. of Hungarian Biophysical Society, p. 41
Soret, Ch. (1879) Arch. Genève *3* 48
Soret, Ch. (1881) Ann. der Chemie und der Physik *5* 22
Varga-Mányi, P. (1964) Abstracts of Hungarian Biophysical Congress, Budapest, p. 54

Acta Biochim. et Biophys. Acad. Sci. Hung, Vol. 1, pp. 203—210 (1966)

Thermoosmosis in Hen's Egg

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(Received January 5, 1966)

According to literary data the osmotic pressure of the yolk surpasses that of the white of hen's egg by 2.0 atmospheres for months. The results presented in this paper have shown that: 1. The value of Q_{10} of the swelling of intact yolks placed into distilled water is 1.7. 2. The temperature of the yolk measured with a thermocouple is a few hundredth degrees higher than that of the white. 3. Yolks heated by diathermy and kept in different streaming solutions (0.5 n Ringer, n Ringer solution, and Ringer solution used for mammalian tissues) exhibited a temperature a few tenth centigrades higher than that of the solution. The water uptake of these yolks was significantly lower than that of the non-heated controls. These data suggest that thermoosmosis plays a role in the mechanism of maintaining the above mentioned concentration difference.

Introduction

Thermoosmotic experiments performed on models have been reported in the earlier communications of our Institute (Ernst, 1936; Ernst and Homola, 1952). According to later data (Vető, 1963) in plant tissues water flowed from warmer sites to colder ones. These results and some theoretical considerations support the working hypothesis that thermoosmosis might play an important role in the biological mobilization of fluids. For the further examination of the problem the hen's egg seemed to be an appropriate model because it shows another phenomenon which is closely related to ours. Several authors have stated that the freezing point depression of the yolk is about $-0.60°C$, while that of the white is only about $-0.45°C$ (Straub and Hoogerduyn, 1929; Meyerhof, 1931; Smith, Shepherd, 1931; Needham et al., 1932; Hale and Hardy, 1933; Smith, 1934; Moran and Hale, 1936; Orru and Brutti, 1940; Orel, 1960; Mészáros, 1960). The concentration difference corresponding to the freezing point depression disappears only in a few weeks or in 1—2 months. Grollman (1931) denied this difference but repeated measurements performed with other methods (e.g. vapour pressure measurements) confirmed the above-mentioned difference (Bateman, 1932; Johlin, 1933; Baldes, 1934; Johlin, 1935). This would mean an osmotic pressure difference of about 2.0 atm, which can be explained by the operation of thermoosmosis.

The aim of the experiments was to investigate the possible temperature difference between the yolk and its surrounding medium, and its effect on the water content of the yolk. Osborne (1931) called attention to the usefulness of intact yolks for the demonstration of osmotic processes.

Methods

1. Since the activation energy of water uptake yields useful informations concerning its mechanism the water uptake of intact yolks from distilled water at 25°C and 35°C during 1 hour was measured, i.e. the temperature coefficient of the water uptake was determined. Hen's eggs kept at room temperature for a few weeks were used. After breaking them the yolks were carefully separated from the whites. They were weighed in a plexi-glass container made for this purpose. After standing in distilled water for an hour at a given temperature, they were taken out, the container was quickly blotted, and they were weighed again. The water uptake of the yolk was expressed as the percentage of the original weight.

2. The next question was, whether or not the temperature difference between the egg yolk and egg white can be demonstrated in vivo. The measurements were carried out with 3 pairs of series-connected thermocouples made from copper constantan; the sensitivity of the thermometer apparatus connected to the galvanometer was 0.0015°C/mm/m. The 1 – 2 weeks old eggs to be measured were kept at the place of measurement at least for 24 hours to get into temperature equilibrium with their surroundings and to eliminate any rough temperature differences. A little hole was driven in the egg shell both in the middle and at the peaked end of it, through which the "measuring heads" of the insulated thermocouples were inserted into the middle of the yolk and into the white. The measurement was performed in a thermos which had a double wall, and was sealed with a thick cork. After placing the egg and the thermocouples into the thermos and after sealing it, 30 minutes elapsed (Fig. 1a). According to our experiences this is enough for the equalization of the possible artificial heat effects. (These effects can be caused by the heat of the hands.) 6 readings were taken during the following 30 minutes in every 5 minutes (Fig. 1b). The mean value of these 6 readings was taken as the actual temperature difference in the given egg. [In many cases ΔT was measured without changing the set-up also after 2 – 3 hours, and in most of the cases even then the yolk was warmer, although owing to the disturbance of the structure, the yolk and the white started to mix (Fig. 1c).] Temperature was measured also in hard-boiled and cooled eggs as a control exactly the same way as described above.

3. The next problem was, whether thermoosmotic effect could be demonstrated by raising artificially the temperature of the yolk above that of the medium. According to Biancani (1933), the white warms up only a little (1°C) if subjected to ultrasonic treatment, whereas the yolk shows a significant temperature increase

(11°C) as compared to the oil bath. Jellinek's method (Jellinek, 1933) was followed by us. He found that the yolk of the hen's egg irradiated with ultrashort waves of a wave length of 3 m was 2–3°C warmer than the white. The intact yolks separated from the whites placed in solutions of different compositions. The plexi containers with the yolks were placed between the electrodes of the diathermy apparatus and solution was streaming continuously through the containers. The

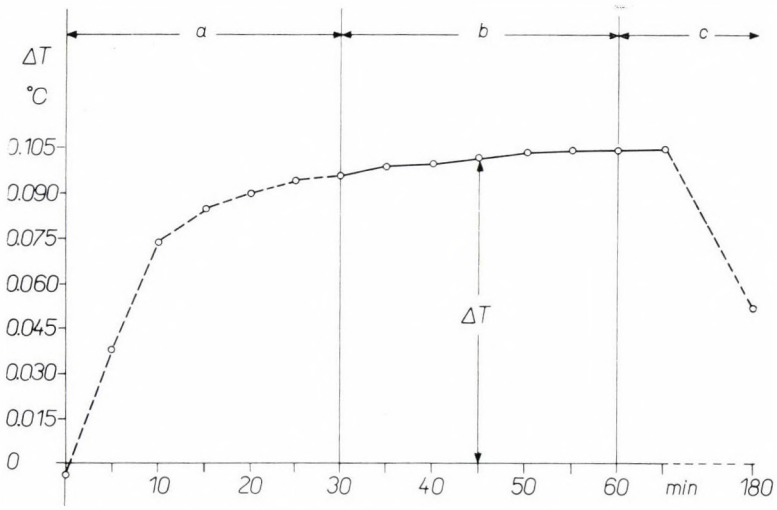

Fig. 1. Temperature difference (ΔT) between the yolk and white of fresh eggs. *a)* Changes before measurement, *b)* The measured difference (the yolk is warmer); *c)* Changes after measurement (shortened abscissa)

purpose of this was to keep the temperature of the solution stable during diathermy. Thus the temperature varied from 25 to 30°C, depending on the speed of the streaming. First of all ΔT was measured between the yolk and the solution. Thermocouples were used for this purpose, by placing one end to the yolk and the other into the solution. To avoid any possible errors caused by the asymmetry of the thermocouple the ends were interchanged several times. We found that the yolk gets warmer than the solution. ΔT has given the highest value (15°C) in a 9.5 per cent sucrose solution among the solutions used; the difference became gradually smaller in the following order: distilled water (10°C), urea 0.3 M (7°C), 0.5 n Ringer solution (1°C), n Ringer solution (0.5°C), Ringer solution used for mammalian tissues (0.2°C). It seems that the yolk irradiated in non-electrolite solution shows a higher tendency for a selective warming up, than in electrolite solution. After having established the ΔT in such a way, the actual experiments were started. Without measuring ΔT the yolks were weighed and irradiated continuously for 1 hour in one of the above-mentioned solutions. After 1 hour the yolks were taken out from the solutions together with the containers. The solution remaining in the container was quickly blotted with filter paper,

and the change of weight was measured. They were replaced into a streaming solution at the same temperature for another hour but without irradiation. The control measurements were carried out in the same way, but the other way round, without irradiation in the 1st hour, and irradiated in the 2nd hour.

Results

1. The water uptake of the yolks on the basis of 33 measurements was 5.4 ± 5.5 per cent of the original weight at 25°C during 1 hour (Fig. 2a) and 9.2 ± 8.3 per cent as determined from 36 measurements at 35°C (Fig. 2b). The difference evaluated both by the *t*-test, and by the "DD"-test – as there was an

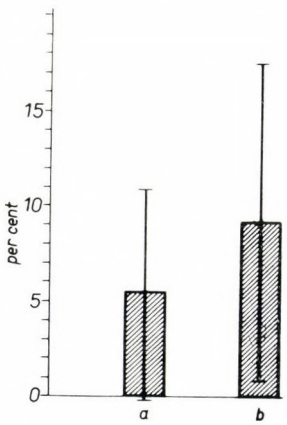

Fig. 2. Water uptake by yolks at different temperatures
a) Out of 33 weighings at 25°C: 5.4 ± 5.5 per cent/1 hour
b) Out of 36 weighings at 35°C: 9.2 ± 8.3 per cent/1 hour

asymmetric, perhaps Poisson distribution (3 data were left out at random from the experiments performed at 35°C) was significant at $P = 0.02$. Thus, the van't Hoff coefficient is $Q_{10} = 9.2/5.4 = 1.7$ supposing that water uptake is an exponential function of the temperature. From this, if the activation energy (E) can be calculated according to Arrhenius, the parallel heat of transfer (Q^*) according to Spanner (Spanner, 1954) will be:

$$E \approx Q^* \approx \frac{RT^2}{10} \ln (1.034 \cdot Q_{10}) = \frac{1.986 \cdot 303^2}{10} \ln (1.034 \cdot 1.7)$$

$$E \approx Q^* \approx 10300 \text{ cal/mol}$$

2. The yolks were warmer by +0.062 ± 0.070°C than the whites according to the measurements carried out in 27 fresh eggs (Fig. 3a). In the control hard

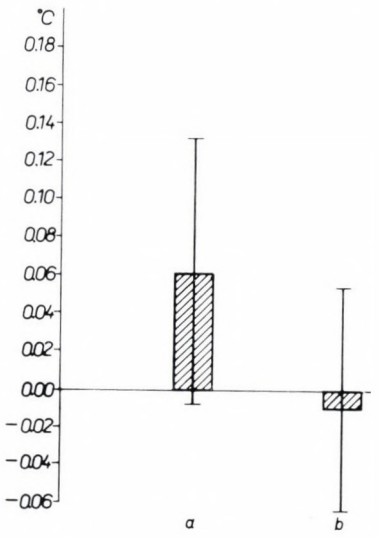

Fig. 3. Temperature difference between the yolk and white of fresh and hard boiled eggs
a) According to 27 measurements the yolks are warmer in fresh eggs: $+0.062 \pm 0.070$°C.
b) According to 23 measurements there is no temperature difference in hard boiled eggs, or rather the difference is: -0.009 ± 0.054°C

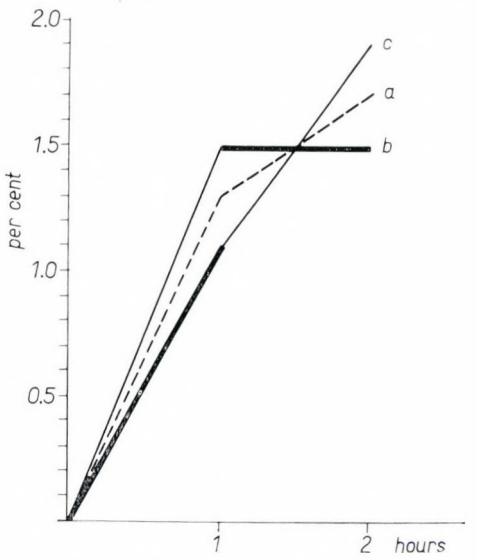

Fig. 4. Change in the weight of yolks in modified Ringer solutions irradiated (thick line) and not irradiated (thin line). *a)* The mean of all experiments. *b)* Not irradiated in the first hour, irradiated in the second hour. *c)* Irradiated in the first hour, not irradiated in the second hour

boiled eggs there was no temperature difference, (23 measurements) that is to say, the value obtained was $-0.009 \pm 0.054°C$ (Fig. 3b). The difference is significant at $P = 0.01$.

3. The average values for the water uptake by the yolks irradiated, and not irradiated in 0.5 n Ringer solution, n Ringer solution and Ringer solution used for mammalian tissues, can be seen in Fig. 4. The dotted line "a" of the curve shows the mean value of all the measurements (80). Curve "b" illustrates the change of weight of the yolks (40 measurements) which were not irradiated in the first hour (thin line) and irradiated in the second hour (thick line). Curve "c" is the reverse: it shows the water uptake of the yolks (40 measurements) which were irradiated in the first hour (thick line), and not irradiated in the second hour (thin line). This Figure indicates that diathermy leading to a temperature difference of 0.2 to 1.0°C decreases the rate of water uptake. Statistical, qualitative evaluations have also been made with the help of the χ^2-test (to see whether there was any effect or there was none) (Table 1). Each experiment was evaluated separately

Table 1

χ^2 contingency table to illustrate the effect of diathermy

1st hour	2nd hour	+	−	Total
Irradiated	Non-irradiated	31	9	40
Non-irradiated	Irradiated	10	30	40
	Total	41	39	80

to establish whether the quantity of the water taken up in the second hour in relation to the uptake in the first hour was greater (positive) or smaller (negative) than the same ratio deduced from the main curve "a". The contingency table shows that 75 per cent of the yolks which were not irradiated in the second hour took up more water in the second hour than the average, or rather, 75 per cent of the yolks which were irradiated in the second hour took up less water than the average. The effect of diathermy, i.e. the effect of the temperature difference is significant at $P = 0.001$, because

$$\chi^2 = \frac{11^2}{20} + \frac{11^2}{20} + \frac{10^2}{20} + \frac{10^2}{20} = 22 .$$

A doubt may arise concerning the results. The irradiated yolk is warmer than the non-irradiated control one, and consequently its evaporation is larger during the few minutes before weighing. Three different experiments were done to solve this problem, and the results showed that there was no evaporation effect.

Although the experiments in distilled water and in urea solution were performed exactly in the same way as in the Ringer solution, and a temperature difference of about $7-10°C$ was obtained yet there was no thermoosmosis effect. The diathermy effect is very significant in the experiments made in sucrose solution and seems to be in favour of thermoosmosis, it still cannot be considered as a proof because of the high value of ΔT (about $15-20°C$) which might cause evaporation.

Thus, according to the results performed in Ringer solutions the speed of the water uptake decreases if the yolk is warmer. This most probably indicates the operation of thermoosmosis. This effect could not be demonstrated in some other solutions, presumably because of the damaging effect of these solutions.

Discussion

Let us calculate by a simple approach the degree of the temperature difference ΔT needed to bring about the same change of vapour pressure as that between the yolk and white owing to the concentration difference, as described in the literature. From the difference of the osmosis pressure of 2.0 atm the logarithm of the relation of the vapour pressures can be calculated as

$$\ln \frac{p_0}{p_1} = \frac{\Delta Pv}{RT} = \frac{2{\cdot}18}{82{\cdot}300} = 0.00146.$$

From this according to Ernst and Homola (1952):

$$\Delta T = \frac{RT^2 \ln p_0/p_1}{H} = \frac{2{\cdot}90000 \cdot 0.00146}{10450} = 0.025\,°C$$

temperature difference can be calculated, where H is the molecular heat of vaporization of water at 27 °C. Thus, the value of ΔT (0.062 °C) measured by us in the egg agrees in terms of order of magnitude with the ΔT calculated above. Namely, the yolk is a few hundredth degrees warmer than the white, and this ΔT can ensure the vapour pressure equilibrium in spite of the concentration difference. The experimental results point to the possibility of thermoosmosis, and at the same time an explanation is given for the special case of the eggs as described in the introduction.

Moreover, if we accept the value of heat of transfer (Q^*) as calculated according to Spanner (1954), and if we introduce some simplifications (there is no difference in the hydrostatic pressure; the membrane is ideally semipermeable; the system has only 2 components and there are no chemical reactions) our case can be interpreted by the method used in the thermodynamics of irreversible processes (De Groot, 1952; Gyarmati, 1960). The problem is much more complicated in reality, because the egg is not a binary anelectrolite solution. Furthermore, biolog-

ical substances are generally swollen systems (e.g. Ernst, 1963) in which the state of water can be influenced by water-binding accompanied by swelling, too.

There is a further problem: Where does the energy come from which maintains the temperature difference, and how great is the loss of heat in the egg. The energy is supplied presumably by glycolysis, as proved by Needham et al. (1932). Further conclusions could have been drawn if the heat production could be measured experimentally, and if the decrease of the total calorie of the egg during storing were known.

The membrane in which the process takes place could be the "membrana vitellina" in the first place, but the possibility arises that the membrane of the "yolk spheres" or "Dotterkugeln" plays a role. These spheres can be found in the fine structure of the yolk-emulsion and are about $2-60\ \mu$ in diameter. It has been demonstrated by Grodzinski (1951) that they react osmotically finer than the erythrocytes.

References

Baldes, E. J. (1934) Proc. Roy. Soc. Lond. B. *114* 436
Bateman, J. B. (1932) J. of Exper. Biol. *9* 322
Biancani (1933) C. R. Acad. Sci. Paris *197* 1963
De Groot, S. R. (1952) Thermodinamics of Irreversible Processes. North Holland Publishing Co., Amsterdam p. 62
Ernst, E. (1936) Ber. ges. Physiol. exp. Pharm. *94* 658
Ernst, E. (1963) Biophysics of the Striated Muscle. Publishing House of the Hungarian Academy of Sciences, Budapest, p. 101
Ernst, E., Homola, L. (1952) Acta Physiol. Acad. Sci. Hung. *3* 487
Grodzinski, Z. (1951) Biol. Rev., Cambridge Philos. Soc. *26* 253
Grollman, A. (1931) Biochem. Z. *238* 408
Gyarmati, I. (1960) Bevezetés az irreverzibilis termodinamikába. University Publishing firm, Budapest, p. 125
Hale, H. P., Hardy, W. (1933) Proc. Roy. Soc. Lond. B. *112* 473
Jellinek, A. (1933) C. R. Acad. Sci. Paris *196* 1149
Johlin, J. M. (1933) J. Gen. Physiol. *16* 605
Johlin, J. M. (1935) J. Gen. Physiol. *18* 481
Meyerhof, O. (1931) Biochem. Z. *242* 244
Mészáros, B. (1960) Acta Univ. Debreceniensis, Hungary, *6* 195
Moran, T., Hale, H. P. (1936) J. of Exper. Biol. *13* 35
Needham, J., Smith, M., Shepherd, J., Stephenson, M., Needham, D. (1932) C. R. Soc. Biol. Paris *109* 688
Orel, V. (1960) A tojás és feldolgozása. Technical Publishing firm. Budapest
Orru, A., Brutti, N. (1940) Atti Accad. Italia, VII 619
Osborne, W. A. (1931) Austral. J. Exper. Biol. a. Med. Sci. *8* 239
Smith, A. J. M. (1934) J. of Exper. Biol. *11*, 228
Smith, M., Shepherd, J. (1931) J. of Exper. Biol. *8* 293
Spanner, D. C. (1954) Symposia of Soc. for exper. Biol. VIII. Activ Transport a. Secretion, Univ. Press, Cambridge, p. 76
Straub, J., Hoogerduyn, M. J. J. (1929) Rec. Trav. chim. Pays-Bas *48* 49
Vető, F. (1963) Acta Physiol. Acad. Sci. Hung. *24* 119

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 211—212 (1966)

Thermosmosis in Biology

(Short notice)

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(Received January 17, 1966)

A paper dealing with "thermal migration in biological transport" (Davies, 1965) mentions the "equivocal discussion" carried on by Burck, Mitchell and myself (1961). Davies "considering an osmometer in which water alone is present" came to the conclusion, by a mere mathematical demonstration, that "thermal migration can make only a trivial contribution to biological transport".

I should like to comment on these statements.

1. During the discussion quoted above (1961) Burck said: "As Prof. Ernst pointed out, if thermosmosis plays a role in water transport there should be an extrusion of water from the part at higher temperature to that at lower temperature. According to my results I would say that this does not happen" (p. 595). In contradiction to that Burck emphasized in his introductory lecture: "We showed that slices of inflamed tissue lose water during 45 minutes" (p. 583). Thus if any statement concerning thermosmosis could be made on the basis of experiments performed on inflamed tissue-slices, it is not a negative one as Burck pointed out in the discussion, but a positive one, according to Burck's lecture upon his experiments.

2. According to Davies (1965) his conclusion that "thermal migration" was insignificant in biology has been foreshadowed by Mitchell in the discussion. In contrast to that Mitchell said (p. 595): "It is not true that you can predict which way water will move through a membrane under a thermal gradient without knowing the properties of the membrane . . ." Thus Mitchell's comment did not contain the statement claimed by Davies.

3. In the discussion (p. 596) Ernst emphasized that he agreed with Prof. Mitchell's statement quoted above, but at the same time he laid emphasis on his results according to which a very significant fluid migration could be measured in model experiments on thermosmosis (Ernst and Homola, 1952).

I first delivered a short contribution to thermosmosis at the Congress of the Hungarian Physiol. Soc. in 1936. Since then a large number of papers and books have been published, recently Läuger's paper (1965) on the same topic. Although they contain very many uncertainties and contradictions, none of these publications disputes the possibility of thermosmosis through a membrane due to a temperature gradient. When experimental facts are denied on the basis of mathematical deri-

vations,* one is reminded of the great German philosopher Hegel scolding, in 1801, the astronomers looking for new planets over the known 7, though this number had been proved as the only possible one (Neptunus, 45 years later).

References

Davies, M. (1965): Biophysical J *5* 651
Burck, H. C., Ernst, E. and Mitchell, P. (1961) in: Transport and Membrane Metabolism (A. Kleinzeller and A. Kotyk, editors) London, Acad. Press, p. 595
Ernst, E. und Homola, L. (1952): Acta Physiol. Acad. Sci. Hung. *3* 487
Ernst, E. (1936): Ber. Ges. Physiol. *94* 658
Läuger, P. (1965): Z. Naturforschg. *20a* 1386

* The mathematical assertions of the Pythagorean school are not necessarily the only aspects of mathematical mysticism.

Printed in Hungary

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

A kézirat nyomdába érkezett: 1966. IV. 30. — Terjedelem: 8,75 (A5) ív, 37 ábra

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13. VIII. 1966 Index: 26.030

VOLUME 1
NUMBER 3
1966

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)

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 213—218 (1966)

Structural Changes Inducing Functional Disorders in Haemoglobin M Variants

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(First received November 23, 1965 and in revised form February 19, 1966)

A previous communication from this laboratory characterized *Hb M Kiskunhalas* with the symbol $\alpha_2^{58\ Tyr}\ \beta_2^A$, which is the same as that of *Hb M Boston*. Attention is drawn to the fact that in all known cases in which the substitution of a His residue resulted in a *Hb M*, Tyr was the substituting amino acid. The possible role of the aromatic side chain of the Tyr residue and the importance of the position of the mutation in maintaining the protein conformation is discussed.

Due to a genetically determined amino acid substitution, *Hb M* variants can no longer perform the fundamental task of haemoglobin: they cannot bind molecular oxygen (Gerald, Efron, 1961). This property of the *Hb M* mutants makes them especially suitable models for studying the correlations between molecular structure and biological function.

In previous communications (Hollán, Szelényi, 1962, 1963; Hollán et al., 1965) we have described a *Hb M* variant, *Hb M Kiskunhalas*, and identified the mutant as $\alpha_2^{58\ Tyr}\ \beta_3^A$. In this paper we report some tentative conclusions which resulted from a comparison of this abnormal haemoglobin to other members of the same group. It would appear that there are certain similarities in these mutants with respect to the amino acid substitution on the one hand, and the resulting change in the physico chemical properties of the mutant haemoglobin on the other.

Among the *Hb M* variants the His-Tyr replacement is dominant (*Hb M Boston*, *Hb M Saskatoon*, *Hb M Iwate*, *Hb M Hyde Park*) with the exception of *Hb M Milwaukee I* in which there is a Val-Glu replacement. This type of substitution does not occur in any other group of haemoglobin mutants (Table 1).

As early as 1961 it has been suggested by Gerald, that in the case of those *Hb M* mutants in which one of the essential histidines is substituted by a tyrosine residue, the acidic OH of tyrosine forms a stable complex with the ferric-ion of heme. The resulting methaemoglobin cannot be reduced, and therefore *Hb M* is not suitable for the reversible binding of oxygen. Therefore, the tyrosine which replaces histidine is responsible for the functional disturbance. However, this rea-

The usual abbreviations for the amino acid are used. Capital letters preceding the abbreviations denote helices.

Table 1

A survey of mutant haemoglobins based on the identity of the substituted amino acid

Location of the mutation	Substituted amino acid	Substituent amino acid	Mutant Hb	Reference
α 116	Glu	Lys	O Indonesia	Baglioni, Lehmann (1962)
β 6	Glu	Lys	C	Hunt, Ingram (1960)
β 7	Glu	Lys	C Georgetown	Pierce et al. (1963)
β 26	Glu	Lys	E	Hunt, Ingram (1961)
β 121	Glu	Lys	O Arabia	Baglioni, Lehmann (1962)
α 30	Glu	Glm	G Honolulu	Swenson et al. (1962)
β 121	Glu	Glm	D Punjab	Baglioni (1962 a)
β 6	Glu	Val	S	Ingram (1959)
β 7	Glu	Gly	G San Jose	Hill et al. (1960)
β 22 or 26	Glu	Ala	G Coushatta	Schneider, Barnett (1964)
β 43	Glu	Ala	G Galvestone	Bowman et al. (1964)
β 47	Asp	Gly	L Ferrara	Huehns, Shooter (1965)
α 79	Asp	Aspm	G Accra	Lehmann et al. (1964)
β 58	*His*	*Tyr*	*M Boston*	Gerald (1958)
α 87	*His*	*Tyr*	*M Iwate*	Shibata et al. (1960)
β 63	*His*	*Tyr*	*M Saskatoon*	Gerald, Efron (1961)
β 92	*His*	*Tyr*	*M Hyde Park*	Heller et al. (1966)
β 63	His	Arg	Zürich	Muller, Kingma (1961)
β 143	His	Asp or Glu	Kenwood	Bayracki et al. (1964)
α 15	Gly	Asp	J Oxford	Liddel et al. (1964)
α 22	Gly	Asp	J Medellin	Gottlieb et al. (1964)
α 57	Gly	Asp	Norfolk	Baglioni (1962 b)
β 16	Gly	Asp	J Baltimore	Baglioni, Weatherhall (1963)
β 67	*Val*	*Glu*	*M-Milwaukee-I*	Gerald, Efron (1961)
α 54	Glm	Glu	Mexico	Jones et al. (1963)
α 54	Glm	Arg	Shimonoseki	Hanada, Rucknagel (1963)
α 68	Aspm	Lys	Philadelphia	Baglioni, Ingram (1961)
α 16	Lys	Glu	I	Murayama, Ingram (1959) Beale, Lehmann (1965)
β 61	Lys	Aspm	Hikary	Shibata et al. (1964)

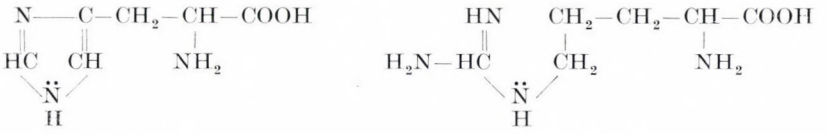
soning would equally apply to any other acidic amino acid, and does not account for the exclusive occurrence of tyrosine replacing histidine. Attention should be called to the fact that both tyrosine and histidine are aromatic amino acids. It is further suggested that the π-electron sextet of the imidazole ring of His plays some role in the maintenance of the conformation of a certain part of the protein molecule, and that the π-electron sextet of the benzene ring of Tyr may play the same role. The counteracting partner of the π-electron sextet may be some other amino acid residue in the polypeptide chain, or the delocalized π-electrons

of the porphyrin ring. Our suggestion is supported by the fact that in spite of the functional disorder, there is no difference in the solubility, alkali and acid resistance between *Hb M* variants and *Hb A*.

Recent X-ray crystallographic results by Perutz (1965) substantiate the view that aromatic side chains play an important role in the maintenance of protein conformation (e.g. the interaction between CD1 Phe and C7 α-Tyr, and β-Phe, respectively).

The suggested role of the π-electron sextet is also indicated by a comparison of *Hb M Saskatoon* and *Hb Zurich*. It is the β 63 His in both haemoglobins which is substituted by Tyr in the case of *Hb M Saskatoon*, and by arginine in the case of *Hb Zurich*. The functional disorder of *Hb M Saskatoon* has been already described. It is very suggestive that with *Hb Zurich* there is no functional disorder, but the stability of the molecule is markedly decreased.

A comparison of His and Arg molecules will show that these molecules are closely related (Fig. 1). Both molecules are basic, but more than that they contain the same number of atoms in the same order. The models in Fig. 2 demonstrate



Histidine Arginine

Fig. 1. Comparison of the structural formulae of histidine and arginine

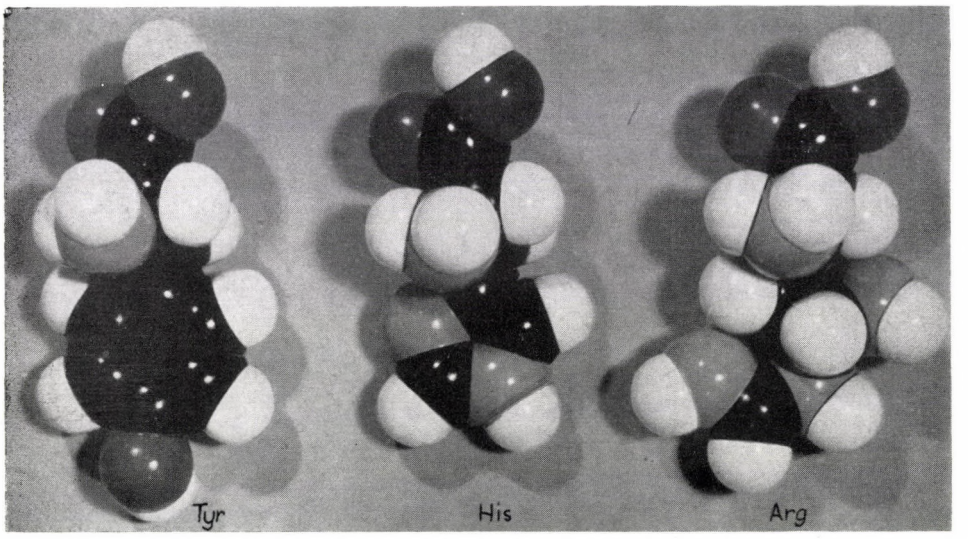


Fig. 2. Calotte models of the molecules of histidine, arginine and tyrosine

that an arginine side chain can assume a similar conformation as a histidine side chain, with respect to both steric requirements and orientation of polar groups.

In spite of all the above similarities between Arg and His, *Hb Zurich*, which is a result of a His to Arg substitution, is markedly different from *Hb A*. This is certainly a further support of our suggestion that the π-electron sextet of the histidines which are affected in the *Hb M* mutations do play a role in the maintenance of haemoglobin conformation. The decreased stability of *Hb Zurich* is indicated by the formation of Hb-precipitates, i.e. inclusion bodies in the red blood

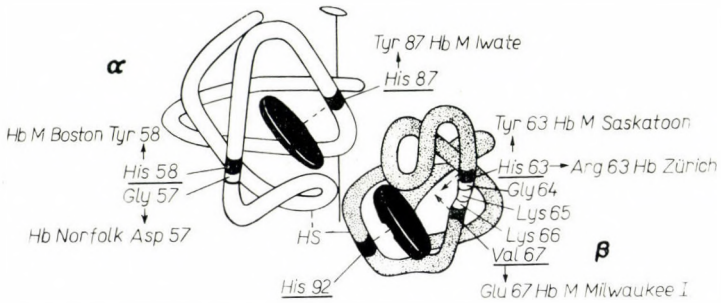


Fig. 3. The location in the sterical model of haemoglobin of the amino acid residues which are concerned in the *Hb M* mutations. In *Hb M* Hyde Park the His 92 is replaced by Tyr

cells of patients following sulphonamide treatment. Therefore the basic Arg might replace the His from the point of view of the function of haemoglobin. On the contrary the stability of the protein molecule is better maintained by a His to Tyr replacement.

On the other hand, the replacement of only some of the histidines of haemoglobin leads to a functional disorder of the *Hb M* type. All the "essential" histidines are in the vicinity of the heme, in the so-called basic centers (Fig. 3), and their side chains are oriented towards the heme. The functional significance of the polypeptide chain is indicated by the fact that the α 58 – 63, and β 63 – 68 sequences, respectively, of human α, β, γ and δ chains are identical in spite of evolutionary alterations in all other parts of these chains (Ingram, 1961). The functional role of His and Gly in these constant Hb-sequences is known. His contributes with a H-bond to the binding of molecular oxygen, and as we have suggested above, its aromatic side chain also takes part in the stabilization of protein conformation. The Gly in positions α59 and β64 interacts with another Gly in positions α25 and β24, respectively (Cullis et al., 1962).

Perutz et al. (1965) have compared the amino acid sequences of human haemoglobins to other products of haemoglobin evolution, such as the haemoglobins of various mammals, including whale myoglobin. According to their conclusion,

there are only a few amino acids which have not changed in the course of evolution. The number of these amino acids is apparently nine. Of the nine amino acids, four are involved in a direct contact with the heme. Two histidines which are involved in the *Hb M* mutations, namely E7 and F8 are among the four. The F8 histidine is substituted by Tyr in *Hb M Iwate (= Hb M Kankakee)* and in *Hb M Hyde Park*. In these haemoglobin variants the heme group is held in position by coordination to α58 His and β 63 His, respectively. The new Tyr residue forms a stable complex with the ferric-ion. This substitution again does not interfere with molecular stability.

The discussed correlations support our claim that the quality of the mutant plays an essential role in the maintenance of the molecular conformation.

References

Baglioni, C. (1962a) Biochim. Biophys. Acta *59* 437

Baglioni, C. (1962b) J. Biol. Chem. *237* 69

Baglioni, C., Ingram, V. M. (1961) Biochim. Biophys. Acta *48* 253

Baglioni, C., Lehmann, H. (1962) Nature *196* 229

Baglioni, C., Weatherhall, D. J. (1963) Biochim. Biophys. Acta *78* 637

Bayracki, C., Josephson, A., Singer, L., Heller, P., Coleman, R. D. (1964) appeared in Proc. Int. Soc. Hemat. *10* L6 1965

Beale, D., Lehmann, H. (1965) Nature *207* 259

Bowman, B. H., Oliver, C. P., Barnett, D. R., Cunningham, J. E., Schneider, R. G. (1964) Blood *23* 195

Cullis, A. F., Muirhead, H., Perutz, M. F., Rossmann, M. G. (1962) Proc. Roy. Soc. A. *265* 161

Gerald, P. S. (1958) Blood *13* 936

Gerald, P. S., Efron, M. L. (1961) Proc. Nat. Acad. Sci. *47* 1758

Gottlieb, A. J., Restrepo, A., Itano, H. A. (1964) Fed. Proc. *23* 172

Hanada, M., Rucknagel, D. L. (1963) Biochem. Biophys. Res. Commun. *11* 229

Heller, P., Coleman, R. D., Yakulis, V. J. (1966) 11th Congress of the International Society of Blood Transfusion, Sydney, p. 184

Hill, R. L., Swenson, R. T., Schwartz, H. T. (1960) J. Biol. Chem. *235* 3182

Hollán, S. R., Szelényi, J. G. (1962) 9th Congress of the International Society of Haematology, Mexico City

Hollán, S. R., Szelényi, J. G. (1963) Proc. 9th Congress of the European Society of Haematology, Lisboa, p. 538

Hollán, S. R., Szelényi, J. G., Lehmann, H., Beale, D. (1965) in press

Huehns, E. R., Shooter, E. M. (1965) J. Med. Gen. *2* 1

Hunt, J. A., Ingram, V. M. (1960) Biochim. Biophys. Acta *42* 409

Hunt, J. A., Ingram, V. M. (1961) Biochim. Biophys. Acta *49* 520

Ingram, V. M. (1959) Biochim. Biophys. Acta *36* 402

Ingram, V. M. (1961) Nature *189* 704

Jones, R. T., Koler, R. D., Lisker, R. (1963) Clin. Res. *11* 105

Lehmann, H., Beale, D., Boi Doku, F. S. (1964) Nature *203* 363

Liddel, J., Brown, D., Beale, D., Lehmann, H., Huntsman, R. G. (1964) Nature *204* 269

Muller, C. J., Kingma, S. (1961) Biochim. Biophys. Acta *50* 595

Murayama, M., Ingram, V. M. (1959) Nature *183* 1798

Perutz, M. F. (1965) J. Mol. Biol. *13* 646
Perutz, M. F., Kendrew, J. C., Watson, H. C. (1965) J. Mol. Biol. *13* 669
Pierce, L. E., Rath, C. E., McCoy, K. (1963) New Engl. J. Med. *268* 862
Shibata, S., Miyaji, T., Iyuchi, I., Ueda, S., Takeda, I. (1964) Clin. Chim. Acta *10* 101
Shibata, S., Tamura, A., Iyuchi, I., Takahashi, H. (1960) Acta Haemat. Japan *24* 486
Schneider, B. H., Barnett, D. R. (1964) Science *143* 697
Swenson, R. T., Hill, R. L., Lehmann, H., Jim, R. T. S. (1962) J. Biol. Chem. *237* 1517

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 219—225 (1966)

Effect of Temperature on Penicillinase Synthesis by B. cereus

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(Received January 27, 1966)

There are marked differences in the basal level of penicillinase of *B. cereus* cells grown at different temperatures. The rate of induced penicillinase synthesis is not significantly affected by temperatures in the range of 26 °C to 39 °C. A temperature effect can be observed in the lag period of induced penicillinase synthesis, which is reduced as the temperature increases. Experimental values of the apparent activation energy of both basal and induced penicillinase synthesis are reported.

Introduction

We have shown in a previous paper that the basal rate of penicillinase synthesis in *B. cereus* is markedly affected by cultural conditions (Csányi, 1966a). Temperature is one of the factors which have a consistently observable effect on both basal and induced penicillinase synthesis. A detailed report on our experimental findings is given in this paper.

Materials and Methods

Strain NRRL-B-569 of *B. cereus* was used throughout the experiments. Conditions of culture have already been described (Csányi, 1966a). Dry matter content of the cells was determined by spectrophotometry. Optical density was read in an Extinctiometer and the readings were converted to mg dry matter by using a calibration curve obtained by direct weight measurements.

The assay conditions for the determination of penicillinase activity have been published in a previous communication (Csányi, 1966a). All cultures used for activity determinations were grown in glass vessel coated with a 5 mm layer of paraffin wax to avoid losses due to the adsorption of the enzyme.

Results

Pre-cultures were started with spores of *B. cereus*. A glucose-free nutrient containing casein hydrolyzate was inoculated with the spores and these pre-cultures were grown for 15 h at 30 °C and 39 °C, respectively. The pre-cultures were subsequently divided into two equal parts, glucose was added to a final con-

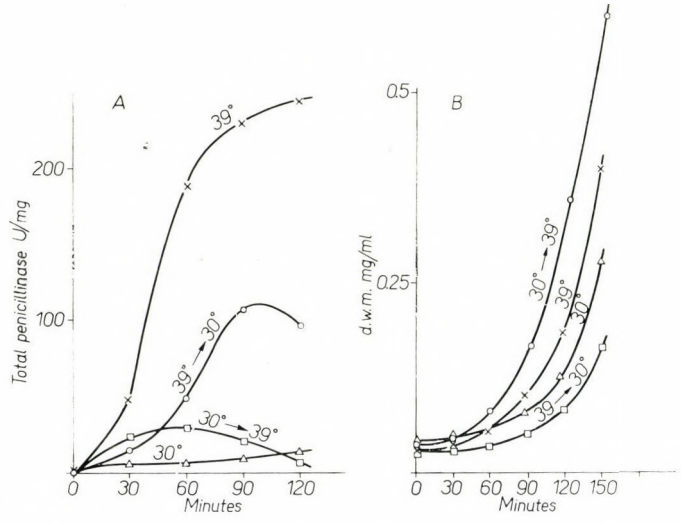

Fig. 1. Effect of temperature on the basal rate of penicillinase synthesis

Pre-cultures were grown for 15 h at 30 °C and 39 °C, respectively. These pre-cultures were subsequently divided into two parts, glucose was added to a final concentration of 0.4 per cent [w/v], and shaking was started. One part was always grown at the temperature of the corresponding control. A) Time course of the rate of penicillinase synthesis in differential units. B) Time course of dry weight accumulation by the cultures.

centration (w/v) of 0.4 per cent, and these cultures were grown under continuous shaking. One part of the halved cultures was grown at the temperature of the pre-culture, while the other part was grown at the temperature of the control. The results are shown in Fig. 1. The values on the ordinate are given in differential units, which refer to unit dry weight. The advantages of this way of expressing the results have already been discussed (Csányi, 1966a). Fig. 1 B shows the simultaneous increase in dry weight. It may be seen that the basal level of penicillinase is very low at 30 °C, and markedly higher at 39 °C. It is also quite clear that the temperature of the pre-culture has a decisive effect on the basal level of enzyme synthesis. Cultures which were grown at 30 °C from an inoculum which had been obtained at 39 °C grew at the slow rate characteristic of low-temperature cultures,

and still had a relatively high basal level of penicillinase synthesis. We have also found that it takes a few hours before the effect of temperature during pre-culture is no more observable in the basal rate of penicillinase synthesis. It is worth noting that the basal endopenicillinase content of *B. cereus* cells as well as the basal exopenicillinase in the nutrient were markedly higher at 39 °C than at 30 °C. The endopenicillinase was about 10 per cent of the total enzyme in these cultures.

Effect of temperature on penicillinase induction

In our subsequent experiments we tried to answer the following question: Is it the synthetic mechanism or the regulatory system, which is directly affected by temperature? The synthesis of penicillinase was induced in cultures grown at different temperatures. Pre-cultured inocula were obtained at both 30 °C and

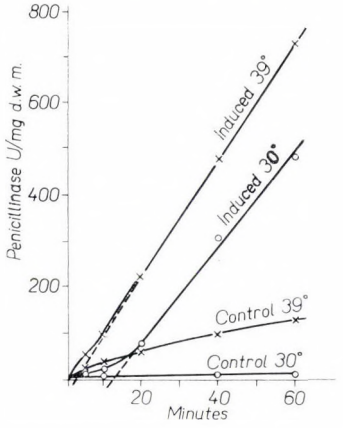

Fig. 2. Effect of temperature on the induced rate of penicillinase synthesis
Pre-cultures were grown for 15 h at 30 °C and 39 °C, respectively. Glucose was then added and shaking of the cultures was started. Cultures were grown until the beginning of the logarithmic growth phase. The cells were then centrifuged off and resuspended in a fresh medium. Induction was carried out by the addition of 10 units of penicillin G, potassium salt, per ml, at the same temperature as pre-culturing

39 °C, then 0.4 per cent glucose (w/v) was added and shaking was started. Subsequently enzyme synthesis was induced by the addition of penicillin. The results are shown in Fig. 2. It is remarkable that the lag period was significantly shorter at 39 °C, than at the lower temperature. The lag period of induction was not longer than 3–4 min at 39 °C, whereas it took about 15 min at 30 °C. Although the basal enzyme level proved to be higher in cells precultivated at 39 °C, the lack of the latency period in non-induced (control) cells cannot be explained by the high rate of basal enzyme synthesis found in these cells, as enzyme synthesis proceeded considerably more rapidly in the induced cells, even in the first minutes

of the experiments, as compared to the control. When cells were cultivated at 30 °C, a latency period of 12 minutes, characteristic for the inductive synthesis of penicillinase, was found. It should be noted that about 20 min after induction the rates of enzyme synthesis were roughly equal at 30 °C and 39 °C.

Apparent activation energy of enzyme synthesis at 30 °C and 39 °C

The rates of both basal and induced enzyme synthesis were determined at different temperatures. The Arrhenius-plot of the results is shown in Fig. 3. It is seen that on the curve for basal enzyme synthesis two linear phases can be distinguished, and that the slopes of these two straight lines are widely different.

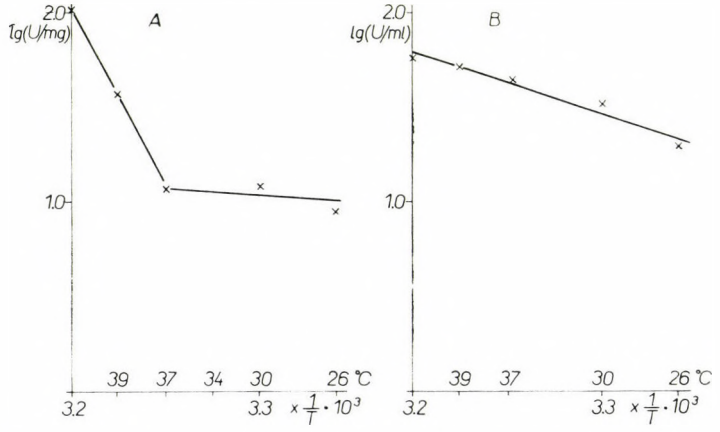

Fig. 3. Temperature dependence of the basal rate and induced rate of penicillinase synthesis

The cells were first grown without shaking at the indicated temperature, then glucose was added and the cultures were shaken until the beginning of the logarithmic growth phase. The cells were subsequently centrifuged off and resuspended in a fresh nutrient, in which they were further grown at the same temperature as during pre-culture. The results shown refer to the linear phase of both basal and induced enzyme synthesis. In the case of basal synthesis the ordinate shows the amount of enzyme synthesized in 20—40 min per unit dry weight. In the case of penicillin-induced synthesis the ordinate refers to the amount of enzyme synthesized in 20—40 min per unit volume of the culture, because dry matter production was not induced. A) Basal enzyme synthesis; B) induced enzyme synthesis

The apparent activation energy of basal enzyme synthesis was about 5000 cal/mole up to 34 °C. The corresponding figure for temperatures above 34 °C was about 92 000 cal/mole. On the contrary, there was no variation in the apparent activation energy of induced enzyme synthesis in the range of 26 °C to 39 °C. The calculated constant apparent activation energy of induced enzyme synthesis was 16 500 cal/mole.

Discussion

The results presented demonstrate that penicillinase synthesis is markedly affected by the temperature at which the cells are grown. Prolonged growth at elevated temperatures leads to an increased basal rate of enzyme synthesis. This must be the expression of a change which is preserved during a number of generations, since on changing the temperature from 39 °C to 30 °C there is a remarkable lag period before the basal level of enzyme synthesis will start to drop. Another effect of temperature was the marked shortening of the lag period on induction with penicillin.

Similar observations have already been described with other microbial enzymes. Temperature sensitivities of the inducible β-galactosidase and alkaline phosphatase of some mutant strains of *E. coli* have been described by Horiuchi (1961), and by Gallant and Stapleton (1964). When these mutants were grown at 30–32 °C, they produced β-galactosidase on induction only, and alkaline phosphatase under conditions of depression, respectively. The same mutants showed a constitutive character at 37–39 °C. These results have been interpreted by suggesting that the synthesis of the specific repressor substance was temperature sensitive.

By the use of mutants which contained a temperature sensitive repressor system, it was shown by Sadler and Novick (1965) that the repressor substance was a metabolically labile molecule, and that the molecular structure of the repressor would change by combining with the low molecular weight inducer substance.

It is a special feature of the results described in this paper, that the observed temperature sensitivity of penicillinase synthesis is the characteristic of wild type *B. cereus* cells.

It is suggested that the observed effects of temperature may be due to primary effects on either the protein synthesizing system or the regulatory mechanism. We further suggest that this question may be answered by the results presented in this paper. The fact that the rate of penicillinase synthesis (relative to the rate of dry matter production) was roughly equal at 30 °C and 39 °C when in the linear stage is regarded to be a conclusive proof against the idea that the enzyme synthesizing system is directly affected by temperature. This conclusion is further supported by the finding (Fig. 3) that the Arrhenius-plot of induced enzyme synthesis gives a straight line. On the other hand, the lag period of induction was markedly shortened, when the cells were both grown and induced at 39 °C. This is possibly a direct indication of a temperature effect on the regulatory system. The amount of the repressor substance might be lower, or the rate of its decomposition might be higher at the higher temperature. At any rate, it is a plausible interpretation of the shortened lag period, that the enzyme synthesizing system is relieved of the inhibition in a shorter period of time upon induction with penicillin. It has already been proposed (Csányi, 1966b) that the stable messenger

of penicillinase is synthesized by, and kept at a constant concentration in, dividing cells. According to this hypothesis, induction releases the inhibition of the functioning of the stable messenger. The present results are in a general agreement with this idea. Indeed, it is hard to postulate that the penicillinase messenger is synthesized during the lag period, because this would involve an unusually high increase in the rate of messenger synthesis due to temperature. In this connection we should like to refer to the figure in which the temperature dependence of the apparent activation energy of enzyme synthesis is given (Fig. 3).

The figure for the apparent activation energy of basal enzyme synthesis at lower temperatures as well as that of induced enzyme synthesis (when calculated for the linear phase of synthesis) falls within the range of the activation energy of enzyme reactions (Sizer, 1943). The apparent activation energy of basal enzyme synthesis at elevated temperatures is much higher (92 000 cal/mole), than the upper limit of the same range, and it is comparable to the activation energy of processes which involve protein denaturation. It is suggested that the common cause of a (a) higher basal level and a (b) shorter lag period upon induction at elevated temperature is protein denaturation, that is, the partial inactivation of the repressor protein.

Acknowledgements

My sincere thanks are due to Professor F. B. Straub and Dr. I. Horváth for their valuable advices during the course of this work and to Miss Gy. Prágai for excellent technical assistance.

References

Csányi, V. (1966a) Acta Biochim. Biophys. Acad. Sci. Hung. In the press
Csányi, V. (1966b) in preparation
Gallant, J., Stapleton, R. (1964) J. Mol. Biol. *8* 431
Horiuchi, T., Horiuchi, S., Novick, A. (1961) J. Mol. Biol. *3* 703
Sadler, J. R., Novick, A. (1965) J. Mol. Biol. *12* 305
Sizer, I. W. (1943) Adv. Enzymol. *3* 35
Sizer, I. W. (1944) J. Biol. Chem. *154* 461

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 225—232 (1966)

Effect of some Mucopolysaccharides on Beta-Glucuronidase Activity

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(Received March 22, 1966)

CSA* activated liver beta-glucuronidase in a concentration above 0.5 mg/ml, but had no effect on the activity of Helix beta-glucuronidase. Aortic MPS mixture had no effect on beta-glucuronidase activity. Beta-glucuronidases of liver, Helix and serum displayed similar enzyme activity patterns in starch gel electrophoresis, although they differed to some extent in protein fractions and esterase activity.

Beta-glucuronidase is activated by substances which contain at least two basic groups per molecule. Chitosan, protamine, serum albumin DNA (salmon milt, fish sperm, thymus), gelatin, chymotrypsin, spermine, lysine, ornithine, etc. in decreasing order of efficiency are such substances. The activation prevents the dissociation of beta-glucuronidase at high dilutions (Bernfeld et al., 1953, 1954). Another activating substance, which is independent of the enzyme concentration has been purified from rat liver. It does not contain carbohydrates, amino acids or nucleotides in any considerable amounts. The activator is effective only with liver beta-glucuronidase (Fialkow, Fishman, 1961).

Among polysaccharides acting as inhibitors heparin is known to inhibit beta-glucuronidase activity by 50 per cent at a concentration of 0.25 mg/ml. Using the same concentration of chondroitin sulphuric acid the enzyme activity was decreased to 25 per cent at pH 4.2 and to 5 per cent at pH 5.2 (Walker, Lewy, 1953). Hyaluronic acid and ascorbic acid in concentrations of 40 mg per cent and 20 mg per cent, also inhibited beta-glucuronidase activity by 50 per cent and 90 per cent, respectively (Becker, Friedenwald, 1949).

Beta-glucuronidase is a lysosomal enzyme (De Duve et al., 1953). Its activity is increased both in human and experimental atherosclerotic changes of the arterial wall (Dyrbye, Kirk, 1956; Branwood, Carr, 1960; Mrhova et al., 1963). Recently Miller et al. (1965) have found a correlation between the serum beta-glucuronidase activity and the susceptibility of the species to spontaneous or induced atherosclerosis. It seemed to be of interest whether the raised activity

* Abbreviations used: CSA = chondroitin sulphuric acid
DNA = deoxyribonucleic acid
MPS = mucopolysaccharide

of beta-glucuronidase in atherosclerosis could be explained by the activating effect of some MPS component of the wall of blood vessels. To this end the effect of CSA and that of an aortic MPS mixture on the beta-glucuronidase activity was investigated.

Materials and Methods

Beta-glucuronidase preparations purified from beef liver with minimum activities of 4.2 units/gr (Fluka A. G., Basel), and 2 million units/gr (Sintex, Kankakee), and from Helix pomatia (670 units/mg, Reanal, Budapest) were used. Phenolphthalein glucuronide (Sigma Chemical Co., St. Louis) was used as substrate. Heparin (Gedeon Richter, Budapest), different chondroitin sulphuric acid preparations (Light and Co. Ltd., Colnbrook) and a MPS mixture isolated from pooled sclerotic aortic intimas according to the method of Dyrbye and Kirk (1956) modified in our laboratory (Dévényi et al., 1963; Bihari-Varga et al., 1966), were used as activating or inhibiting substances. The chondroitin sulphuric acid and MPS preparations did not contain protein. The analytical data were as follows:

2.7 per cent N, 6.8 per cent S for chondroitin sulphuric acid, and 2.9 per cent N, 4.5 per cent S, 8 per cent Na, 23.5 per cent galactosamine for the Na salt of chondroitin sulphuric acid. The MPS mixture from aorta contained 27.2 per cent hexose, 6.4 per cent hexosamine, 14.4 per cent sialic acid, 5.3 per cent hexuronic acid, 5.3 per cent N, 5.1 per cent SO_4, 10.2 per cent ash, 5.0 per cent moisture; the main constituents of the acid MPS-fraction in the preparation were: 67 per cent CSA, 12 per cent heparitin-sulfate, 7 per cent hyaluronic acid.

The beta-glucuronidase activity was determined according to Talalay et al. (1946) at pH 5.0 and 37°C. The preparations used were preincubated for 10 min at 37°C with the enzyme and buffer before adding the substrate.

Vertical starch gel electrophoresis was performed according to Smithies (1959) at pH 8.0 in borate buffer in order to compare protein fractions and enzyme activities from different sources. 40 μl samples were placed into the slots. Electrophoresis was carried out at room temperature, 90 V and 15 mA for 17 hours. After completion of the run the gels were sliced horizontally into two parts, one part was stained for protein for 3 minutes with 1 per cent Amido Schwarz B in methanol–acetic acid–water (5 : 2 : 5). The remaining stain was removed by washing several times with the same solvent.

Beta-glucuronidase activity in the gel was stained by the same procedure as employed for the quantitative determination of enzyme activity (Talalay et al., 1946).

Esterase activity was detected by the use of alpha-naphthylacetate (Nachlas, Seligman, 1949) using Fast Blue B (Sigma Chemical Co., St. Louis) as the coupling agent. Incubation was carried out for one hour at room temperature.

Results

In Table 1 the effect of various MPS preparations on the beta-glucuronidase activity of liver and Helix pomatia is compared.

Table 1

The effect of different MPS preparations on the beta-glucuronidase activity

	Liver		Helix pomatia
	beta-glucuronidase activity μg phenolphtalein/mg protein/hr		
	Fluka enzyme	Sintex enzyme	
Control	14	124 000	576
Chondroitin sulphuric acid 1.5 mg/ml	30	256 000	500
Chondroitin sulphuric acid 1.5 mg/ml hydrolysed in 0.5 n H_2SO_4	14	115 000	—
Chondroitin sulphuric acid Na salt No. J. 60080, 1.5 mg/ml	20	182 000	564
Chondroitin sulphuric acid C No. 6026 Y 13, 1.5 mg/ml	20	187 000	624
Heparin 1.5 mg/ml	8	75 300	455
MPS aorta 610713, 6 mg/ml	16	125 000	—

The data in Table 1 show that chondroitin sulphuric acid had a strong activating effect on the liver beta-glucuronidase. Whereas heparin inhibited the

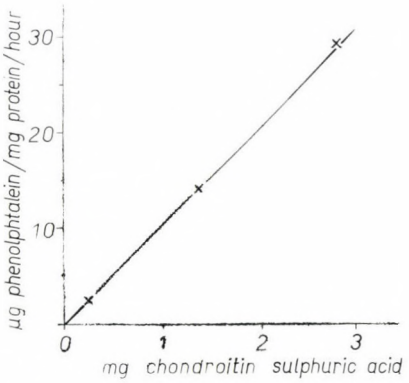

Fig. 1. Activating effect of different concentrations of chondroitin sulphuric acid on beta-glucuronidase activity (Fluka)

enzyme activity in the same concentration, MPS preparations of the aorta had no effect. There was no significant change in the activity of beta-glucuronidase prepared from Helix pomatia, except when using heparin. In order to prove that the activating effect on beta-glucuronidase was due to CSA it was destroyed by

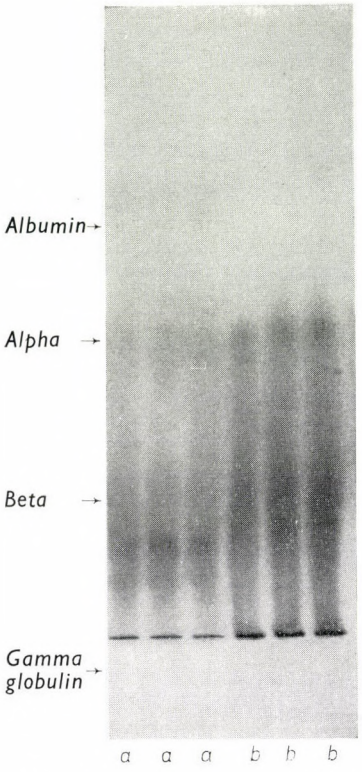

Fig. 2. Protein fractions of beta-glucuronidase from snail and liver in starch gel electrophoresis
a = beta-glucuronidase from Helix pomatia (50 mg/ml)
b = beta-glucuronidase from beef liver (50 mg/ml)

Fig. 3. Beta-glucuronidase activity as demonstrated by starch gel electrophoresis
a = normal human serum
b = beta-glucuronidase from Helix pomatia (50 mg/ml)
c = beta-glucuronidase from beef liver (Fluka) (100 mg/ml)

hydrolyzing it for 4 hours in 0.5 n H_2SO_4 at 100°C. The decomposed molecule was deprived of any activating effect as seen from Table 1.

The activating and inhibiting effects of these substances did not change to any considerable extent after dialyzing both enzyme preparations for 24 hours at 5°C against 0.01 M acetate buffer of pH 5.

Using different concentrations of chondroitin sulfate a direct relationship between the effective concentrations of the activator and the increase in beta-glucuronidase activity was established. This is shown in Fig. 1. Changes in the

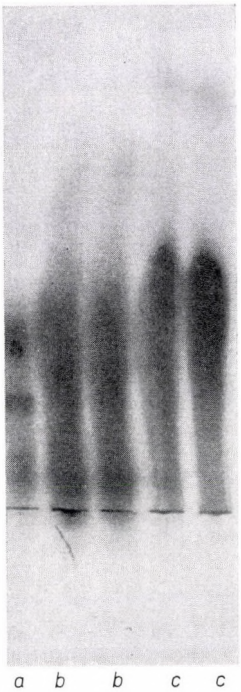

Fig. 4. Esterase activity in starch gel electrophoresis
a = normal human serum
b = helix pomatia (50 mg/ml)
c = beef liver (Fluka) (100 mg/ml)

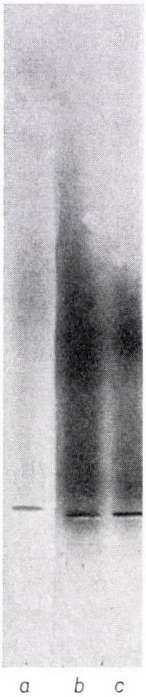

Fig. 5. Inhibition of esterase activity by neostigmine in starch gel electrophoresis
a = normal human serum
b = beta-glucuronidase from Helix pomatia (50 mg/ml)
c = beta-glucuronidase from beef liver (100 mg/ml) (Fluka) preincubated for 1 hour with 0.5 mg Neostigmine (100 ml pH 7 0.1 M phosphate buffer)

concentration of the enzyme did not influence the activating effect of chondroitin sulphuric acid.

In order to explain the differences found between liver and snail beta-glucuronidases, starch gel electrophoresis was performed with both enzymes. From the protein pattern (Fig. 2) it is evident, that whereas the snail enzyme contains more albumin and less alpha globulin, the liver enzyme is poor in albu-

min, but more abundant in alpha globulin than the helix enzyme. Comparing the beta-glucuronidase activities on starch gel of both enzymes and human serum, activity appeared in one broad band in the beta-globulin region (Fig. 3) which

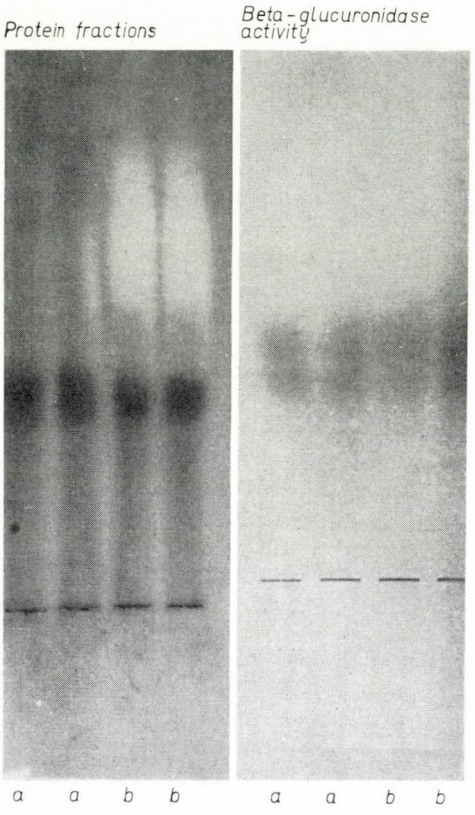

Fig. 6. Protein fractions of beta-glucuronidase from beef liver and beta-glucuronidase activity activated by chondroitin sulphuric acid as demonstrated by starch gel electrophoresis. a = beta-glucuronidase from beef liver (Sintex) 10 mg/ml. b = beta-glucuronidase from beef liver 10 mg/ml + 10 mg CSA

was activated by chondroitin sulphuric acid in the case of the liver beta-glucuronidase (Fig. 6).

Staining the gel with alpha-naphthyl acetate we obtained strong esterase activities with both the liver and snail enzymes located in all globulin fractions (alpha-beta-gamma) in decreasing order (Fig. 4). The activity was not due to cholinesterase because neostigmine abolished only the serum-cholinesterase activity (Fig. 5).

Discussion

There are different, to some extent contradictory data in the literature on activators and inhibitors of beta-glucuronidase (Fishman, 1955). Therefore it seems worthwile to discuss the activating effect of chondroitin sulphuric acid on beta-glucuronidase activity. The data of Walker and Lewy (1953) on its inhibiting effect (5 per cent inhibition at pH 5.2) are not convincing enough and the concentrations used by them are below the effective activator concentrations. On the other hand, chondroitin sulphuric acid has not two basic groups per molecule, even if we suppose that acetic acid or sulphuric acid might be hydrolyzed by some other enzymatic contaminations in the liver enzyme. Its activating effect does not change when using different concentrations of enzyme solutions. In this respect and because it activates only liver beta-glucuronidase it seems to be similar to the rat liver activator of Fialkow and Fishman (1961). On the other hand chondroitin sulphuric acid structurally somewhat resembles chitosan, which is the strongest known activator of beta-glucuronidase. (It is already effective in 10 μg/ml concentration.)

Another interesting problem is whether chondroitin sulphuric acid A or C or its building element chondrosin, having a beta-d-glucopyruronoside structure, might serve as substrate for beta-glucuronidase.

The chondroitin sulphuric acid preparation with the strongest activating effect showed a slight opalescence when added to the liver enzyme. The pH of the incubation mixture did not change noticeably. It is known that CSA forms salt-like compounds with globulins, albumin, gelatin, edestin (Meyer et al., 1937), and serum beta-lipoprotein (Amenta, Waters, 1960; Bihari-Varga, Gerő, 1966). (See also Fig. 6.) It is possible that this property plays a role in the activating effect of CSA on beta-glucuronidase of liver.

The lack of the activating effect of the aortic MPS mixture used by us could be explained by its heparitin sulphate and hyaluronic acid contents beside CSA. Heparin and hyaluronic acid are known to inhibit beta-glucuronidase activity (Becker, Friedenwald, 1949).

It has been discussed whether one or more beta-glucuronidases are present in the liver (Fishman, 1955). In our experiments performed with starch gel electrophoresis liver, helix and serum beta-glucuronidases gave one strong band in the region of beta-globulin, whereas their protein fractions and esterase bands were different. These results suggest that beta-glucuronidase activity was bound in all cases to the same fractions of beta-globulin.

Our results point to the possibility that the increased activity of beta-glucuronidase in atherosclerosis might derive from the activating effect of CSA. CSA as an acidic substance may have also an activating effect on other lysosome bound enzymes (acid phosphatase, chymotrypsin, ribonuclease). On the other hand, it is known that CSA has an inhibitory effect on the lipolytic activity of the aorta (Gerő et al., 1962). These findings give further support to the view that changes

in the MPS content of the wall of blood vessels or serum may play a role in atheroma formation.

References

Amenta, J. S., Waters, L. L. (1960) Yale J. Biol. Med. *33* 112
Becker, B., Friedenwald, J. S. (1949) Arch. Biochem. Biophys. *22* 101
Bernfeld, P., Bernfeld, H. C., Fishman, W. H. (1953) Fed. Proc. *12* 177
Bernfeld, P., Bernfeld, H. C., Eisselbaum, J. S., Fishman, W. H. (1954) J. Am. Chem. Soc. *76* 4872
Bihari-Varga, M., Gerő, S. (1966) Acta Physiol. Acad. Sci. Hung. In press
Branwood, A. W., Carr, A. J. (1960) Lancet *2* 1254
De Duve, C., Gianetto, R., Appelmans, F., Wattiaux, R. (1953) Nature *172* 1143
Dévényi, T., Bihari-Varga, M., Jakab, L., Gergely, J. (1963) Kísérl. Orvostud. *15* 326
Dyrbye, M., Kirk, J. E. (1956) J. Geront. *2* 33
Fialkow, P. J., Fishman, W. H. (1961) J. Biol. Chem. *236* 2169
Fishman, W. H. (1955) Adv. in Enzymol. *16* 361
Gerő, S., Gergely, J., Dévényi, T., Virág, S., Székely, J., Jakab, L. (1962) Nature *194* 1181
Gerő, S., Gergely, J., Dévényi, T., Jakab, L., Székely, J., Virág, S. J. (1961) Atheroscler. Res. *1* 67
Meyer, K., Linke, A., Rapaport, M. M. (1951) J. Biol. Chem. *192* 275
Meyer, K., Palmer, J. W., Smyth, E. M. (1937) J. Biol. Chem. *119* 501
Miller, B. F., Keyes, F. P., Curreri, P. M. (1965) Proc. Soc. Exp. Biol. Med. *119* 1129
Mrhova, O., Zemplényi, T., Lojda, Z. (1963) J. Atheroscler. Res. *3* 44
Nachlas, M. M., Seligman, A. M. (1949) Anat. Rec. *105* 677
Smithies, O. (1959) Biochem. J. *71* 585
Talalay, P., Fishman, W. H., Huggins, C. (1946) J. Biol. Chem. *166* 757
Walker, P. G., Lewy, G. A. (1953) Biochem. J. *54* 56

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 233—244 (1966)

Some Properties of the Sarcoplasmatic Reticular Fraction of Fish (Amiurus nebulosus) Muscle

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(Received April 12, 1966)

Adenosine triphosphatase and cholinesterase activities and calcium uptake in fractions obtained from fish muscle homogenizates by differential centrifugations at 1000 × g, 8000 × g, and 25,000 × g, respectively, were studied. These enzyme activities and calcium uptake are characteristic for the 1000 × g to 8000 × g (Fr. II) and for the 8000 × g to 25,000 × g (Fr. IV or SRF) fractions. These fractions behave similarly with respect to Ca^{++} uptake and adenosine triphosphatase activity in the presence of sodiumazide and dinitrophenol. During ageing the Ca^{++} uptake of Fr. IV or SRF decreases with a simultaneous increase in adenosine triphosphatase and cholinesterase activities. The cholinesterase activity is high (60—90 μM acetylcholine per mg of protein per hour) and from the histochemical reaction on the cellulose column it seems probable that this is not due to adsorption. The adenosine triphosphatase activity of Fr. IV increases until a certain phase of ageing (48 to 60 hours) both with and without oxalate in the presence of 0.12 mM Ca^{++} in the medium. Under the same experimental conditions the enzyme activity is inhibited from the third to fourth day onward. A certain difference was found in the Ca^{++} uptake of Fr. IV between rabbit and fish muscles, especially with respect to the effect of oxalate and Ca^{++} concentration. These findings suggest that the structure of the sarcoplasmatic reticulum of the two species is different.

Introduction

The intracellular structure of the sarcoplasmatic reticulum of fish muscle is well-known (Fawcett, Revel, 1961; Franzini-Armstrong, Porter, 1964), but only few data are available on its ability to accumulate Ca^{++} and on its other biochemical characteristics. The study of the sarcoplasmatic reticulum of fish muscle was undertaken because as shown by earlier experiments (Connell, 1958, 1961; Hamoir et al., 1960; Stainier-Lambrecht, 1962; Kövér et al., 1963; Szabolcs et al., 1963) contrary to the contractile proteins prepared from rabbit those of fish muscle display a high lability and their physicochemical and enzymatic properties are also different. These data suggest that the remarkable differences in the contractile proteins are reflected in the structure and function of the sarcoplasmatic reticulum (SR).

In the present paper some experimental data concerning the properties of some subcellular fractions, mostly those of the sarcoplasmatic reticular fraction (SRF) of fish muscle are reported.

Materials and Methods

Preparation of the subcellular fractions from the white skeletal muscle of fish. – All operations were performed at temperatures below 4°C. The muscle was minced, mixed with four volumes of a solution containing 0.1 M potassium chloride, 0.005 M of potassium oxalate and 0.01 M phosphate buffer (pH 7.0) and homogenized four times for 30 sec in a Waring Blendor. The temperature was checked at each step. After homogenization the suspension was centrifuged for 20 minutes at $1000 \times g$. The supernatant (Fr. I) was again centrifuged at $8000 \times g$ for 20 minutes. The precipitate (Fr. II) was suspended in a solution containing 0.055 M potassium chloride, 0.005 M magnesium chloride, 0.005 M potassium oxalate and 0.01 M phosphate buffer (pH 7.0) (suspending solution). The supernatant was again centrifuged at $25{,}000 \times g$ for 60 minutes to separate the microsomes and the soluble proteins. The supernatant was used as Fr. III. and the precipitate was used as Fr. IV or SRF after suspension in the suspending solution. Essentially this method resembles the one applied by Martonosi and Feretos (1964a). To compare the characteristics of the SRF of fish and rabbit muscle SRF prepared by the method of Nagai et al. (1960) were used. Freshly prepared fractions were used unless stated otherwise. The protein content of the fractions was determined by the micro-Kjeldahl method.

Estimation of Ca^{++} uptake. – Ca^{++} uptake was measured by the method described in an earlier communication (Szabolcs, Kövér, 1966) with isotope technique on a cellulose column. The extent of Ca^{++} uptake was expressed in μM Ca^{++} per mg protein.

Determination of ATP-ase activity. – Two different incubation mixtures were used. The first (I) contained 0.05 M potassium chloride, 0.002 M magnesium chloride, 0.002 M potassium oxalate, 0.002 M adenosine triphosphate (ATP) and 0.01 M TRIS-maleate buffer (pH 7.0), the second (II) consisted of 0.05 M potassium chloride, 0.005 M magnesium chloride, 0.005 M potassium oxalate, 0.005 M ATP and 0.01 M TRIS-maleate buffer (pH 7.0). The incubation temperature was 23°C. In some cases the incubation mixture contained no oxalate. The protein content of each fraction in the incubation mixture was different. During incubation one ml samples were taken at given intervals, activity was stopped by the addition of trichloroacetic acid and the inorganic phosphorus (P) content was determined by the method of Taussky and Shorr (1953).

Measurement of cholinesterase activity. – Incubation was carried out at 37°C for 30 minutes. The composition of the incubation mixture was the following: 0.05 M potassium chloride, 0.002 M magnesium chloride, 0.002 M potassium oxalate, 0.001 M acetylcholine chloride, 0.01 M TRIS-maleate buffer (pH 7.0). The acetylcholine content of an aliquot of the incubation mixture was determined at 0 and 30 minutes by Hestrin's (1949) method.

Localization of cholinesterase activity on the cellulose column. – The solutions were prepared in the way described by Karnovsky (1964) with the differ-

ence that the concentration of acetylthiocholine iodide was raised to 4 mM. Cholinesterase activity was localized in the following way: 0.5 ml of the SRF with a protein concentration of 2 mg per ml was applied to a cellulose column (50 mm by 8 mm). The sample was washed in with 5–10 ml of a 0.1 M potassium chloride solution. 25 ml of Karnovsky's incubation solution was passed through the column in 25–30 min, and the column was then rinsed three times with 5 ml of 0.1 M potassium chloride solution. Cholinesterase activity appeared in the form of a brownish red ring at both pH 6.0 (sodium hydrogen maleate buffer) and pH 7.0 (TRIS-maleate buffer). The inhibition of cholinesterase activity was examined in the presence of 10^{-4} M physostigmine sulphate. In such cases both the 0.1 M potassium chloride washing solution and the incubation solution contained physostigmine. The same procedure was used in the experiments with butyrylthiocholine iodide, but instead of acetylthiocholine iodide butyrylthiocholine iodide was applied.

All reagents were of analytical grade. ATP (Reanal, Budapest) was purified on a Dowex 50 ion exchanger by the method of Seidel and Gergely (1963). $^{45}CaCl_2$ was obtained from the Isotope Institute of the National Nuclear Energy Commission.

Results

I

Distribution of protein content, cholinesterase and ATP-ase activities and of calcium uptake among the different cellular fractions. – Table 1 shows that proteins extracted under the given conditions were not distributed uniformly among the different cellular fractions. The bulk of the proteins of Fr. I was found in Fr. III which consisted mainly of soluble proteins, but there was a relatively significant amount of protein also in Fractions II and IV. It is remarkable that fractions with relatively high ATP-ase activities displayed also significant cholinesterase activities. Ca^{++} uptake by the fractions was studied with and without oxalate. Maximum Ca^{++} uptake was found in the fraction which sedimented between 8000 × g and 25,000 × g (Fr. IV or SRF); but the fraction sedimenting between 1000 × g and 8000 × g (Fr. II) also had a significant calcium binding activity. In a medium without oxalate Ca^{++} uptake was about 70–75 per cent lower than in the oxalate containing medium with both Fr. II and Fr. IV.

Effect of sodium azide and dinitrophenol on the Ca^{++} uptake and ATP-ase activity of the cellular fractions of fish muscle. – As shown in Table 1 the ATP-ase and cholinesterase activities of Fr. II and Fr. IV were almost identical, but there was a difference in Ca^{++} uptake. To determine whether or not the Ca^{++} uptake by these fractions was of mitochondrial origin the effect of sodium azide or dinitrophenol was investigated, as these reagents are known to inhibit the Ca^{++} uptake of mitochondria (Brierley et al., 1964). As shown in Table 2 neither azide nor

Table 1

Distribution of protein, cholinesterase activity, ATP-ase activity and Ca++ uptake among the subcellular fractions of fish muscle

Incubation mixture I (see methods) was used for the determination of ATP-ase activity, the fractions were 24 hours old. For the measurement of Ca++ uptake the same incubation mixture I was used. Concentration of $^{45}CaCl_2$ was 0.12 mM and 0.06 mM, respectively (the latter in the experiment without oxalate). Cholinesterase activity was measured by the method described

Fraction	g protein / 100 g muscle	Cholinesterase activity [μM acetylcholine/mg protein/hour]	ATP-ase activity [μM P/mg protein/5 min]	Ca++ uptake [μM Ca++/mg protein]	
				with oxalate	without oxalate
I.	2.032 ±0.203	24.81±6	0.323±0.127	0.0798±0.0032	—
II.	0.1718±0.033	60.85±6.25	2.10 ±0.51	0.329 ±0.055	0.063
III.	1.530 ±0.145	8.05±1.9	0.126±0.07	0.009	—
IV.	0.226 ±0.071	67.7 ±5.08	1.93 ±0.72	0.512 ±0.058	0.15±0.03

dinitrophenol inhibited the Ca++ uptake by either Fr. II or Fr. IV. ATP-ase activity did not change appreciably in the presence of 0.2 mM dinitrophenol, but was significantly lower in both fractions to which 2 mM sodium azide had been added. Table 2 shows further that the ATP-ase activity of Fr. II and IV was

Table 2

Effect of sodium azide and of dinitrophenol on the Ca++ uptake and ATP-ase activity of the subcellular fractions of fish muscle

ATP-ase activity was measured in the incubation mixture II at inhibitor concentrations shown in the Table. Ca++ uptake was measured with incubation mixture II in the presence of 0.12 mM $^{45}CaCl_2$

Fraction	Dinitrophenol (mM)	Sodium azide (mM)	Ca++ uptake μM/mg protein	ATP-ase* activity per cent	ATP-ase** activity per cent
II	—	—	0.501	100.0	110.0
	0.2	—	0.496	105.0	119.0
	—	2.0	0.497	54.0	74.0
IV	—	—	0.770	100.0	120.0
	0.2	—	0.745	110.0	118.0
	—	2.0	0.790	45.0	59.0

* without Ca++
** with 0.12 mM Ca++

higher in the presence of 0.12 mM Ca^{++}, and this increase did not change when dinitrophenol or sodium azide was added. This agrees with the finding that Ca^{++} uptake is not inhibited by the presence of these two reagents. From the data of Table 2 the mitochondrial origin of Fr. II seems improbable, as ATP-ase activity was not enhanced neither was Ca^{++} uptake inhibited by dinitrophenol.

Localization of cholinesterase activity on the cellulose column. – It appears from the data in Table 1 that both Fr. II and Fr. IV have a significant cholin-

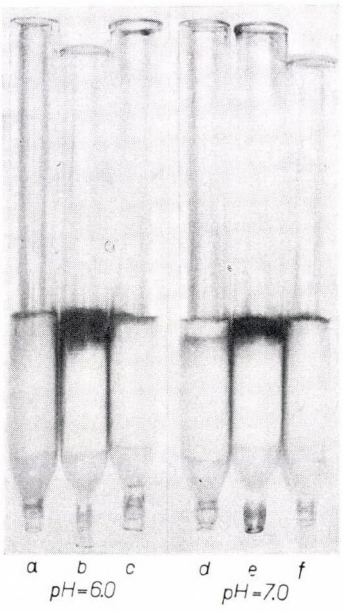

Fig. 1. Localization of cholinesterase activity on the cellulose column

Columns a and d: Cholinesterase reaction with butyrylthiocholine iodide. Columns b and e: Cholinesterase reaction with acetylthiocholine iodide. Columns c and f: Cholinesterase reaction with acetylthiocholine iodide in the presence of physostigmine. Columns a, b and c: at pH 6.0. Columns d, e and f: at pH 7.0. For further details see the text

esterase activity. It has been reported in an earlier paper (Szabolcs, Kövér, 1966) that Fr. IV remains in the top layer of a cellulose column (50 × 8 mm) while the dissolved and soluble proteins pass the column. It was therefore assumed that by using suitable histochemical reactions on the cellulose column an answer may be found to the question whether cholinesterase activity of Fr. IV is due to an adsorption on the structure during extraction or an intrinsic property of Fr. IV.

Fig. 1 shows that Karnovsky's histochemical reaction developed in spite of a prolonged preliminary washing of Fr. IV (Figs 1 b and e) at both pH 6.0 and

7.0. The cholinesterase activity was inhibited by physostigmine (Figs 1 c and f). The specificity of cholinesterase was demonstrated by the fact that the enzyme gave a minimum histochemical reaction with butyrylthiocholine iodide (Figs 1 a and d). Thus the cholinesterase activity of Fr. IV prepared from fish muscle is not of a pseudocholinesterase nature.

Changes in the properties of Fr. IV (SRF) during ageing. – Preliminary experiments have shown that the Ca^{++} uptake by Fr. IV of fish muscle rapidly

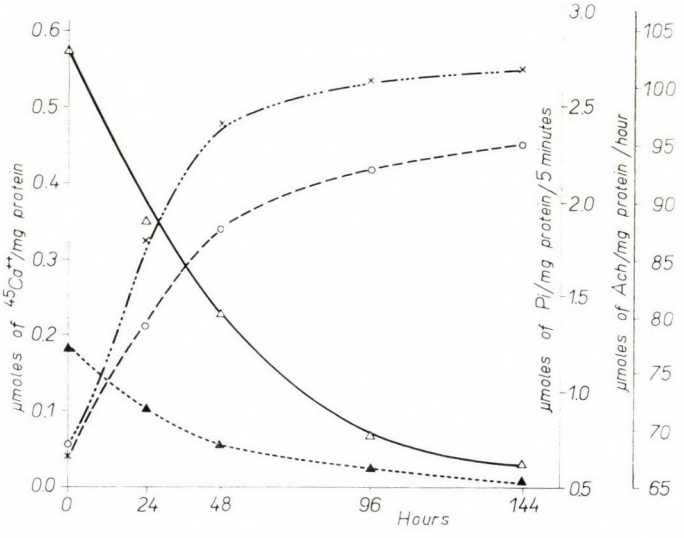

Fig. 2. Changes in the characteristics of Fr. IV (SRF) during ageing

For experimental conditions see Table 1. Ordinates: Ca^{++} uptake μM Ca^{++} per mg protein; μM P_i per mg protein and μM Ach per mg protein per hour. Abscissa: ageing time in hours. △———△ Ca^{++} uptake in the presence of 0.002 M potassium oxalate. ▲ ..... ▲ Ca^{++} uptake without oxalate. ○- - -○ ATP-ase activity. × ···—···× cholinesterase activity

decreases during ageing indicating a labile structure of the sarcoplasmatic reticulum. It was therefore of interest to examine how far this lability of the structure is reflected in the ATP-ase and cholinesterase activities of Fr. IV. Fig. 2 shows that the decrease of Ca^{++} uptake with and without oxalate was accompanied by an increase in the ATP-ase and cholinesterase activities. It is highly remarkable that ATP-ase and cholinesterase activities change in exactly the same way and that this change shows a trend opposite to that of Ca^{++} uptake.

Effect of Ca^{++} on the ATP-ase activity of Fr. IV during ageing. – The ATP-ase activity of Fr. IV was studied in an oxalate containing medium without the

addition of Ca^{++} (Fig. 2). Martonosi and Feretos (1964b) have found that the ATP-ase activity of Fr. IV or SRF is controlled by the Ca^{++} concentration of the solution. The aim of our experiments was to study the effect of Ca^{++} on the ATP-ase activity of Fractions IV of different ages.

As shown in Fig. 3 using a fresh preparation of Fr. IV 0.12 mM Ca^{++} raises ATP-ase activity in media both with and without oxalate. In the presence of 2 mM oxalate, however, the increase in ATP-ase activity is more pronounced.

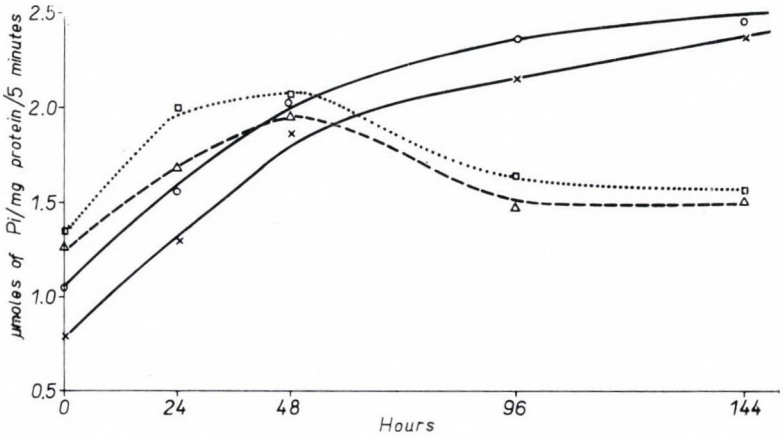

Fig. 3. Effect of Ca^{++} on the ATP-ase activity of Fr. IV (SRF) during ageing
Incubation mixture I (see methods). ○——○ ATP-ase activity without Ca^{++} and oxalate; △- - -△ ATP-ase activity in the presence of 0.12 mM Ca^{++} without oxalate; ×——× ATP-ase activity in the presence of 0.002 M oxalate without Ca^{++}; □····□ ATP-ase activity in the presence of both 0.12 mM Ca^{++} and 0.002 M oxalate

Activation of the ATP-ase activity by Ca^{++} gradually decreases during ageing. ATP-ase activity of Fr. IV stored at 0°C for 48 hours is no longer activated by Ca^{++} if enzymatic activity is measured in an oxalate free medium. An activation by Ca^{++} was observed, however, when the enzyme activity of the same preparation was determined in a medium containing 2 mM oxalate. When the ATP-ase activity of Fr. IV was measured after a 4–6 day storage at 0°C a decrease in the activity was found both with and without oxalate.

II

In the second part of the experiments certain characteristics of Fr. IV (SRF) prepared from rabbit and from fish muscle, respectively, were compared.

Effect of ATP on Ca^{++} uptake. – As may be seen from Fig. 4 with ATP concentrations over 3 mM the Ca^{++} uptake by neither rabbit nor fish muscle

SRF displayed a significant increase in oxalate containing medium. At an ATP concentration of 1 mM the Ca^{++} uptake by the two types of SRFs was practically identical, but there was a decisive change at an ATP concentration of 2 mM. In this latter case rabbit SRF took up twice as much Ca^{++} as fish SRF. This

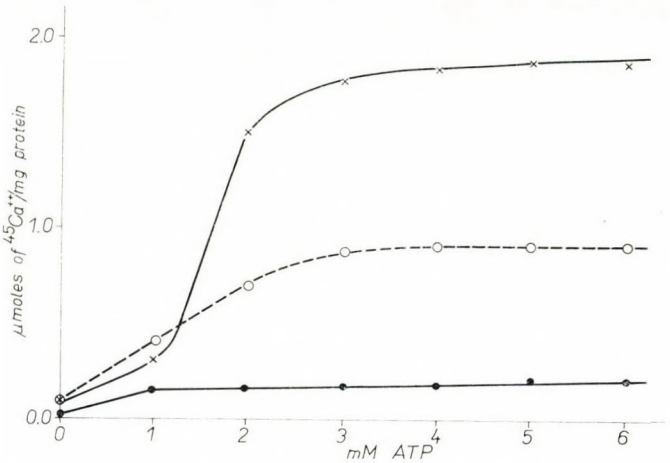

Fig. 4. Effect of ATP on the Ca^{++} uptake by Fr. IV (SRF)

Ca^{++} uptake was measured in the presence of 50 mM potassium chloride, 5 mM magnesium chloride, 5 mM potassium oxalate, 10 mM TRIS-maleate buffer (pH 6.8) and 0.12 mM or 0.06 mM $^{45}CaCl_2$ (the latter without oxalate) at ATP concentrations shown on the abscissa. ×——× Amount of protein on the cellulose column with rabbit SRF, 0.26 mg. ○- - -○ Amount of protein on the cellulose column with fish SRF in oxalate medium, 0.64 mg. ●——● Amount of protein on the cellulose column with fish SRF in oxalate free medium, 1.3 mg

difference was maintained at higher concentrations. In oxalate free medium fish muscle SRF had a significant ability to take up Ca^{++}. This also depended on the ATP concentration.

Effect of oxalate on Ca^{++} uptake. – It appears from Fig. 5 that the Ca^{++} uptake by rabbit SRF increases quite abruptly up to an oxalate concentration of 4 mM, while the Ca^{++} uptake by fish SRF shows a slight increase up to the highest oxalate concentrations tested (6 mM). It can be seen from the same figure that in oxalate free medium fish muscle SRF takes up about three times as much Ca^{++} as rabbit muscle SRF.

Effect of Ca^{++} concentration on Ca^{++} uptake. – The Ca^{++} uptake by rabbit muscle SRF increased first linearly with increasing Ca^{++} concentrations, followed by a decrease in the rate of increase of Ca^{++} uptake between 0.24 and 0.36 mM. Ca^{++} uptake no longer increased over a Ca^{++} concentration of 0.36

mM. The Ca^{++} uptake by fish muscle SRF showed a slight linear increase in oxalate medium with increasing Ca^{++} concentration. The same was true for experiments in oxalate free medium.

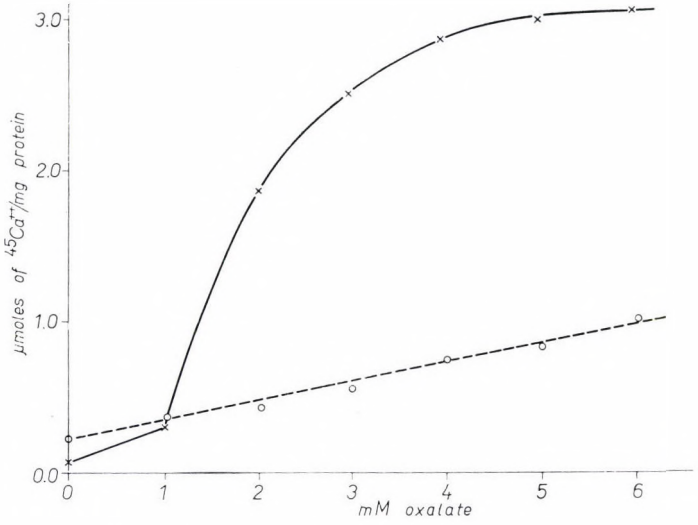

Fig. 5. Effect of oxalate on the Ca^{++} uptake by Fr. IV (SRF)

Ca^{++} uptake was measured in the presence of 50 mM potassium chloride, 5 mM magnesium chloride, 5 mM ATP, 10 mM TRIS-maleate buffer (pH 6.8), 0.12 mM $^{45}CaCl_2$ and at oxalate concentrations shown on the abscissa. ×——× Amount of protein on the cellulose column with rabbit SRF, 0.16 mg. ○——○ Amount of protein on the cellulose column with fish SRF, 0.42 mg

Effect of Mg^{++} on Ca^{++} uptake. – In the case of rabbit muscle SRF up to 5 mM Ca^{++} uptake increased linearly with increasing Mg^{++} concentrations. Between 5 and 10 mM there was still a slight increase, but at Mg^{++} concentrations over 10 mM the rate of Ca^{++} uptake gradually decreased. With fish muscle SRF the optimum Ca^{++} uptake was observed with 5 mM Mg^{++}, at higher Mg^{++} concentrations the Ca^{++} uptake by fish muscle SRF decreased.

Discussion

A few years ago a number of data pointed to the great differences in the structure of contractile proteins depending on the species of animal studied and on the function and innervation of the muscle (Varga et al., 1957a, b; Kövér, Kovács, 1961; Varga et al., 1962a, b). Recently other authors (Seidel et al., 1964;

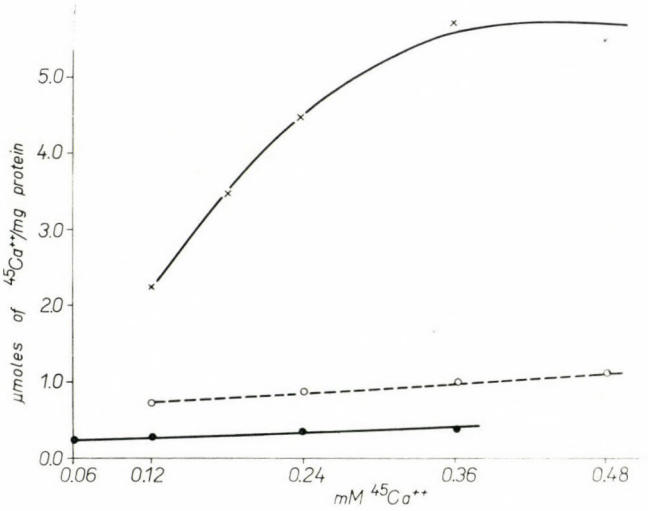

Fig. 6. Effect of $^{45}Ca^{++}$ concentration on the Ca^{++} uptake by Fr. IV (SRF)

Ca^{++} uptake was measured in the presence of 50 mM potassium chloride, 5 mM magnesium chloride, 5 mM ATP, 5 mM potassium oxalate, 10 mM TRIS-maleate buffer (pH 6.8) and at $^{45}CaCl_2$ concentrations shown on the abscissa. ×——× Amount of protein on the cellulose column with rabbit SRF, 0.25 mg. ○--○ Amount of protein on the cellulose column with fish SRF in 5 mM oxalate medium, 0.43 mg. ●——● Amount of protein on the cellulose column with fish SRF without oxalate, 1.04 mg

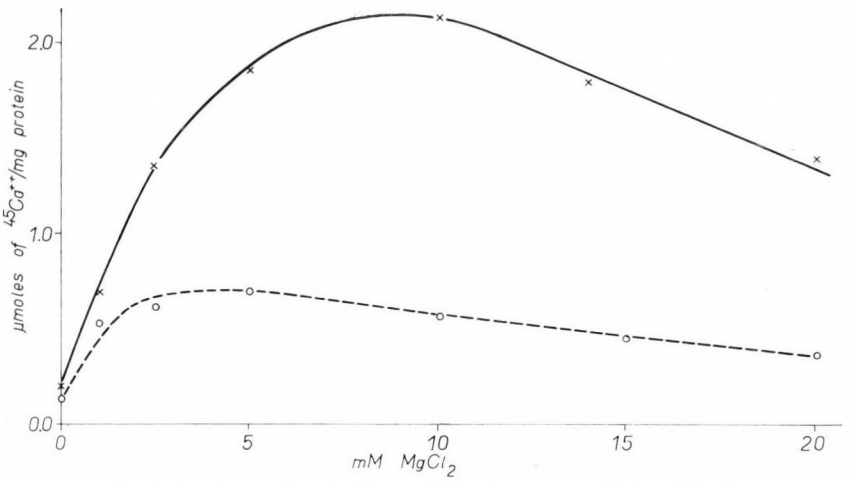

Fig. 7. Effect of Mg^{++} on the Ca^{++} uptake by Fr. IV (SRF)

Ca^{++} uptake was measured in the presence of 50 mM potassium chloride, 5 mM ATP, 5 mM potassium oxalate, 10 mM TRIS-maleate buffer (pH 6.8) and at magnesium chloride concentrations shown on the abscissa. ×——× Amount of protein on the cellulose column with rabbit SRF 0.23 mg. ○--○ Amount of protein on the cellulose column with fish SRF, 0.51 mg

Bárány et al., 1965) have pointed out the differences in the enzymatic activities and other properties of the myosins and actomyosins of the red (slow) and white (fast) muscles. Sréter and Gergely (1964) and Samaha and Gergely (1965) have found that the ATP-ase activity, Ca^{++} uptake and the changes during ageing of SRF prepared from red muscle differ from those of white muscle SRF.

Our studies on the properties of subcellular fractions of the fish white muscle SRF exhibits a pronounced cholinesterase activity in addition to ATP-ase activity. According to electron microscopic histochemical investigations the longitudinal elements of SR possess a cholinesterase activity, while the transversal elements (Karnovsky, 1964) do not. We have demonstrated that cholinesterase activity is intrinsic property of fish muscle SRF (Fig. 1). The fact that ATP-ase and cholinesterase activities show a practically identical increase during the ageing of SRF (Fig. 2) renders probable that the proteins responsible for cholinesterase activity are incorporated into the structure of SR.

Ca^{++} uptakes by Fr. II and Fr. IV (or SRF) remain unchanged in the presence of sodium azide (Table 2) in spite of the significant decrease in the ATP-ase activity of both fractions under the same conditions. Dinitrophenol has no effect either on the Ca^{++} uptake or the ATP-ase activity of the two fractions. The Ca^{++} uptake and ATP-ase activity of the $1000-8000 \times g$ fraction (corresponding to our Fr. II) of heart muscle change significantly in the presence of sodium azide and of dinitrophenol (Fanburg, Gergely, 1965). Similar results were obtained for the $1000-8000 \times g$ fractions prepared from human skeletal muscle (Samaha, Gergely, 1965). At the same time the Ca^{++} uptake and ATP-ase activity of the $8000-30{,}000 \times g$ fraction (corresponding to our Fr. IV or SRF) prepared from either the heart muscle or the human skeletal one show no significant change if treated with these inhibitors. The Ca^{++} uptake by Fr. II is 0.33 μM Ca^{++} per mg of protein in the presence of 2 mM oxalate, 2 mM ATP, 2 mM magnesium chloride and 10 mM TRIS buffer (Table 1) and 0.50 μM Ca^{++} per mg of protein in the presence of 5 mM oxalate, 5 mM ATP, 5 mM $MgCl_2$ and 10 mM TRIS buffer (Table 2), indicating that oxalate promotes Ca^{++} uptake, as with Fr. IV (Fig. 5). The increase in Ca^{++} accumulation with increasing oxalate concentration (Hasselbach, Makinose, 1963; Martonosi, Feretos, 1964a) and the insensitivity of Ca^{++} uptake to the presence of sodium azide and dinitrophenol, as well as the unchanged ATP-ase activity in the presence of dinitrophenol point to the fact that the $1000-8000 \times g$ fraction (Fr. II) of fish muscle is of sarcoplasmatic reticular origin rather than of mitochondrial origin.

There is a difference in the behaviour of ATP-ase activity of SRF during ageing in the presence or absence of Ca^{++} (Fig. 3). This is presumably in correlation with a reduced ability to accumulate Ca^{++} (Fig. 2), as due to lower Ca^{++} accumulation during ageing the Ca^{++} concentration of the medium stays at a higher level during the measurement of ATP-ase activity. According to Martonosi and Feretos (1964a) the ATP-ase activity of SRF is greatly inhibited by Ca^{++} concentrations lower than 1 μM or higher than 0.1 mM. It may be as-

sumed that from the fourth day of ageing the latter conditions prevail in fish muscle SRF because of greatly reduced Ca^{++} accumulation.

The characteristics of the Ca^{++} uptake by fish muscle SRF are significantly different from those of rabbit muscle SRF especially with respect to the effect of oxalate (Fig. 5) and of Ca^{++} concentration (Fig. 6). It was found that in the presence of oxalate fish muscle SRF accumulates half or one third as much Ca^{++} as rabbit muscle SRF does, suggesting a difference in the structure of the sarcoplasmatic reticulum of the two species.

Acknowledgement

The authors wish to express their thanks to Prof. Emil Varga for his interest in this work and for his useful advices, and to Mrs. Sándor Csorba and István Moldvai for their valuable technical assistance.

References

Bárány, M., Bárány, K., Reckard, T., Volpe, A. (1965) Arch. Biochem. Biophys. *109* 185
Brierley, G. P., Murer, E., Bachmann, E. (1964) Arch. Biochem. Biophys. *105* 89
Connell, J. J. (1961) Arch. Biochem. Biophys. *94* 128
Connell, J. J. (1958) Biochem. J. *69* 5
Fanburg, B., Gergely, J. (1965) J. Biol. Chem. *240* 2721
Fawcett, D. W., Revel, J. P. (1961) J. Biophys. Biochem. Cytol. *10* No. 4. Suppl. 89
Franzini-Armstrong, C., Porter, K. R. (1964) J. Cell Biol. *22* 675
Hamoir, G., McKenzie, H. A., Smith, M. B. (1960) Biochim. Biophys. Acta *40* 141
Hasselbach, W., Makinose, M. (1963) Biochem. Z. *339* 94
Hestrin, S. (1949) J. Biol. Chem. *180* 249
Karnovsky, Morris, J. (1964) J. Cell Biol. *23* 217
Kovács, T., Kövér, A., Balogh, G. (1961) J. Cell Comp. Physiol. *57* 63
Kövér, A., Kovács, T. (1961) J. Cell Comp. Physiol. *57* 73
Kövér, A., Szabolcs, M., Benkő, K. (1963) Acta Physiol. Acad. Sci. Hung. *23* 229
Martonosi, A., Feretos, R. (1964a) J. Biol. Chem. *239* 648
Martonosi, A., Feretos, R. (1964b) J. Biol. Chem. *239* 659
Nagai, T., Makinose, M., Hasselbach, W. (1960) Biochim. Biophys. Acta *43* 223
Samaha, F. J., Gergely, J. (1965) J. Clin. Invest. *44* 1425
Seidel, J. C., Gergely, J. (1963) J. Biol. Chem. *238* 3648
Seidel, J. C., Sréter, F. A., Thompson, M. M., Gergely, J. (1964) Biochem. Biophys. Res. Comm. *17* 662
Sréter, F. A., Gergely, J. (1964) Biochem. Biophys. Res. Comm. *16* 438
Stainier-Lambrecht, A. (1962) Arch. Intern. Physiol. Biochim. *70* 5
Szabolcs, M., Kövér, A. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 159
Szabolcs, M., Kövér, A., Benkő, K. (1963) Acta Physiol. Acad. Sci. Hung. *23* 239
Taussky, H. H., Shorr, E. (1953) J. Biol. Chem. *202* 675
Varga, E., Kövér, A., Kovács, T., Heténvi, E. (1957a) Acta Physiol. Acad. Sci. Hung. *11* 235
Varga, E., Kövér, A., Kovács, T., Heténvi, E. (1957b) Acta Physiol. Acad. Sci. Hung. *11* 243
Varga, E., Kövér, A., Kovács, T., Jókay, I., Szilágyi, T. (1962a) Acta Physiol. Acad. Sci. Hung. *22* 21
Varga, E., Kövér, A., Kovács, T., Szabolcs, M., Jókay, I. (1962b) Acta Physiol. Acad. Sci. Hung. *22* 119

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 245—252 (1966)

Penetration of Benzoic Acid into Cells of Mycobacterium Friburgensis in the Presence of Streptomycin

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(Received April 25, 1966)

The effect of streptomycin on the uptake of ^{14}C-benzoic acid by M. Friburgensis cells was studied. Certain conclusions were drawn from the observed inhibition of the induction of the benzoic acid oxidase system in the presence of streptomycin.

The penetration of ^{14}C-benzoic acid into cells incubated without streptomycin was observed from the 20th minute onwards; streptomycin had an inhibiting effect on penetration. Up to the 9th minute streptomycin had no effect on the benzoic acid uptake by cells which had been pretreated with ^{14}C-benzoic acid. Upon pretreatment with benzoic acid streptomycin resistance developed earlier in the systems which regulate benzoic acid transport than in those which synthetize benzoic acid oxidase.

Introduction

There are many avirulent M. tuberculosis strains which are capable of oxidizing benzoic acid. Fitzgerald and Bernheim (1947) were the first to observe the inhibiting action of streptomycin (SM) on this oxidation process. They tried to explain this phenomenon by assuming that the presence of SM inhibits the induced synthesis of benzoic acid oxidase which is necessary for the oxidative breakdown of benzoic acid (Fitzgerald et al., 1948). Erdős and Tomcsányi (1957) found later that as far as the effect of SM is concerned the latency period of benzoic acid oxidase can be divided into two stages, a SM sensitive and a SM resistant stage. The first i.e. the SM sensitive stage lasts for about 15 minutes after the addition of the inducer. In this stage of the latency period enzyme induction is practically fully inhibited by the presence of SM, while in the second stage SM is no longer able to exert its inhibitory effect.

The aim of the present work is to offer an explanation for the mechanism of this phenomenon. It was found that SM inhibits the penetration of the inducer into the cells. Inhibition was observed in the early stage of induction only. It was demonstrated that after induction the systems which control the penetration of the inducer become resistant to SM earlier than synthetizing systems.

Materials and Methods

The bacterium used in the experiments was Mycobacterium Friburgensis after 44 hours surface cultivation at 37 °C using sauton nutrient medium.

Preparation of the bacterium suspension: the bacteria were washed twice in M/15 phosphate buffer (pH 7.2), and suspended in the same phosphate buffer. The concentration of the suspensions was always adjusted to 25 mg fresh weight per ml.

Experiments with ^{14}C-benzoic acid: two types of benzoic acids were used in the experiments: 1. benzoic acid labelled in the ring with ^{14}C and 2. benzoic acid labelled in the carboxyl group with ^{14}C. 17 ml of the bacterium suspension were poured into Erlenmeyer flasks and the flasks incubated in water bath of 37 °C until there was no more difference between the outside and inside temperatures. Radioactive benzoic acid was then added and samples (usually 1 ml) were taken at given time intervals. The samples were poured into 1 ml of M/15 phosphate buffer (pH 7.2) which had been cooled to ice pulp consistency and which contained 2 mg of "cold" sodium benzoate per ml. The mixture was centrifuged at 0 °C. The precipitate was washed five times with the phosphate buffer and twice with distilled water at 0 °C. A suspension was prepared from the washed bacteria with distilled water and aliquots of the suspension were placed on planchets, dried and their radioactivity measured in a gas flow counter.

In the experiments in which the cells were pretreated with other substances (e.g. pretreatment with SM or inducer) the bacterium cells were centrifuged off after suitable pretreatment, washed twice with M/15 phosphate buffer (pH 7.2) and resuspended in phosphate buffer to the initial volume.

Results

As mentioned in the introduction, 15 minutes after induction the benzoic acid oxidase synthetizing system of M. Friburgensis cells became SM resistant. This statement is now supplemented by the finding (Fig. 1) that cells pretreated before induction with SM lose their ability to synthetize benzoic acid oxidase irreversibly.

To find the cause of this phenomenon the binding of the inducer to differently treated bacteria was investigated. The bacterium cells were pretreated with "cold" sodium benzoate or SM. After preincubation for 20 minutes with these substances the cells were centrifuged off, washed twice with phosphate buffer, suspended in fresh phosphate buffer and used to measure their ability to radioactive benzoic acid (inducer).

As shown in Fig. 2 if "cold" sodium benzoate was used for preincubation the uptake of radioactive benzoic acid increased in comparison to the uptake

of the control which had been preincubated in buffer solution only, while the benzoic acid uptake of cells pretreated with SM was inhibited.

Next to effect on benzoic acid uptake of SM added to the system at different time intervals was investigated. As shown in Fig. 3 SM inhibits the uptake

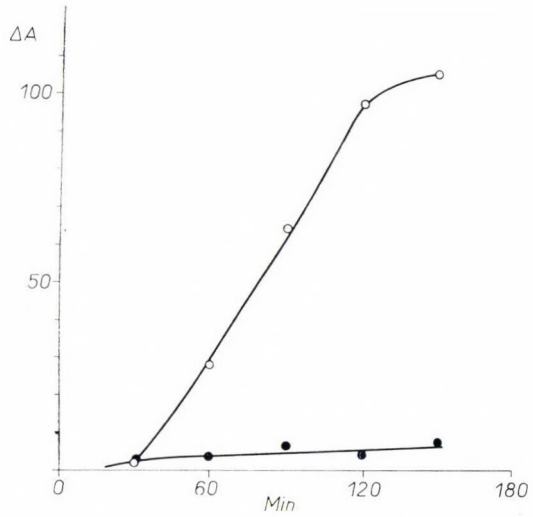

Fig. 1. Effect of preincubation with streptomycin on the synthesis of benzoic acid oxidase by M. Friburgensis

Pretreatment of bacteria: 17 ml of a bacterium suspension in M/15 phosphate buffer (pH 7.2) were measured into two Erlenmeyer flasks. The suspensions contained bacteria in an amount of 25 mg fresh weight per ml. Streptomycin was added to the second flask to a final concentration of 15 μg per ml. Both flasks were incubated at 37 °C for 20 minutes, the contents were centrifuged, the sediment washed twice with phosphate buffer and resuspended in 17 ml of phosphate buffer. Oxygen consumption was determined after the addition of an adequate amount of sodium benzoate. ○—○ Control experiments. The Warburg vessels contained 50 mg of bacteria (fresh weight) in M/15 phosphate buffer (pH 7.2) and 1 mg sodium benzoate. The final volume was 2.5 ml, temperature 37 °C. Sodium benzoate was added from the side vessel to the bacterium suspension at zero time. ●—● Bacterium suspension preincubated in the presence of 15 μg streptomycin per ml. Other experimental conditions were the same as those of the control. Ordinate represents the amount of oxygen consumed for the oxidation of benzoic acid in 30 minutes, expressed in μl. The data refer to 50 mg of bacteria (fresh weight)

of benzoic acid by the cells only if added to the suspension simultaneously with benzoic acid. No inhibition was, however, observed if SM was added to the cells 9 or 16 minutes after the addition of radioactive benzoic acid.

At the same time the benzoic acid oxidase synthesis of similarly treated cells was measured in the Warburg apparatus (Fig. 4).

It was found that SM added simultaneously with the inducer (benzoic acid) completely stopped the synthesis of the enzyme, while when SM was added 16 minutes after induction no inhibition was observed. This finding confirms our

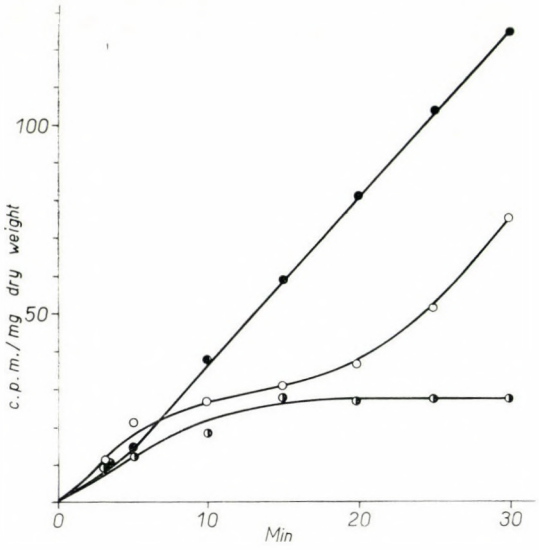

Fig. 2. Uptake of 7-^{14}C benzoic acid by M. Friburgensis cells pretreated with sodium benzoate and streptomycin

Pretreatment of bacteria: 17 ml of a bacterium suspension containing 25 mg bacteria (fresh weight) per ml were measured into three Erlenmeyer flasks. Flask 1: control experiment, incubated at 37 °C for 20 minutes. Flask 2: the same as the control + 3 mg of "cold" sodium benzoate. Flask 3: the same as the control + streptomycin to a final concentration of 15 μg per ml. After 20 minutes of incubation the contents of the flasks were centrifuged, the sediments washed twice at 0 °C with M/15 phosphate buffer (pH 7.2). After washing the bacteria were resuspended in 17 ml phosphate buffer. ○—○ Control. Bacterium cells preincubated in phosphate buffer. 7-^{14}C-benzoic acid was added to the system to obtain an activity of 1.47 μCi per ml. This corresponded to a final concentration of 0.125 mg per ml of benzoic acid. ●—● Cells preincubated with "cold" sodium benzoate. Other experimental conditions were the same as those of the control. ◑—◑ Bacterium cells preincubated in the presence of 15 μg streptomycin per ml. Other experimental conditions were the same as those of the control

earlier experimental results (Erdős, Tomcsányi, 1957) as well as the results of Bernheim et al. (Fitzgerald et al., 1948) which were mentioned in the introduction. The observation, however, is quite new that if SM is added 9 minutes after induction it still greatly inhibits the induced synthesis of benzoic acid oxidase, in spite of the fact that after this period of time it has no effect on the uptake of radioactive benzoic acid by the cells.

Discussion

The fact that SM inhibits the induction of inducible enzymes has been observed by several authors (Fitzgerald et al., 1948; Erdős, Tomcsányi, 1957; Creaser, 1955). Bernheim et al. (Fitzgerald et al., 1948) were the first to report such

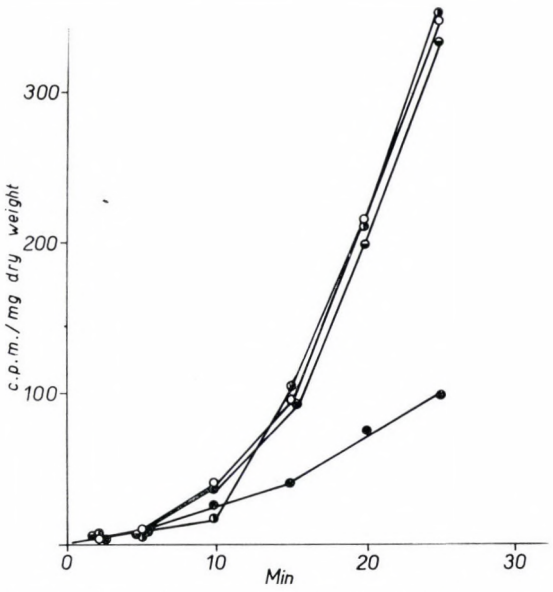

Fig. 3. Effect of streptomycin on the uptake of ^{14}C (ring labelled) benzoic acid by M. Friburgensis cells

○—○ Control: to 17 ml of a bacterium suspension containing 25 mg of bacteria (fresh weight) per ml to obtain ^{14}C (ring labelled) benzoic acid was added to a final activity of 1.18 μCi per ml. This corresponded to a benzoic acid concentration of 0.159 mg per ml. ●—● The same as the control except that streptomycin was added simultaneously with ^{14}C (ring labelled) benzoic acid. Final concentration of streptomycin: 15 μg per ml. ◑—◑ The same as the control except that streptomycin was added 9 minutes after the addition of ^{14}C (ring labelled) benzoic acid. Final concentration of streptomycin 15 μg per ml. ◒—◒ The same as the control except that streptomycin was added 16 minutes after the addition of ^{14}C benzoic acid. Final concentration of streptomycin 15 μg per ml

an observation in connection with benzoic acid oxidase. Later Creaser (1955) made the same observation with the induction of beta-galactosidase in a strain of Staph. aureus. The mechanism of this phenomenon is not quite clear yet. Most authors refer to Bernheim's observation as to the first evidence of the inhibition of protein biosynthesis by SM (Gale, 1963).

The fact that the benzoic acid oxidase synthetizing system loses its SM sensitivity if the cells are pretreated with the inducer for a sufficiently long time (15–20 min) has been observed earlier (Erdős, Tomcsányi, 1957) and confirmed

in our present experiments. No explanation was at first found for this phenomenon. At the present, however, with the literary data available it is no longer difficult to explain these findings. According to our present concepts induction involves the synthesis of a specific m-RNA which is bound to the ribosomes. This is

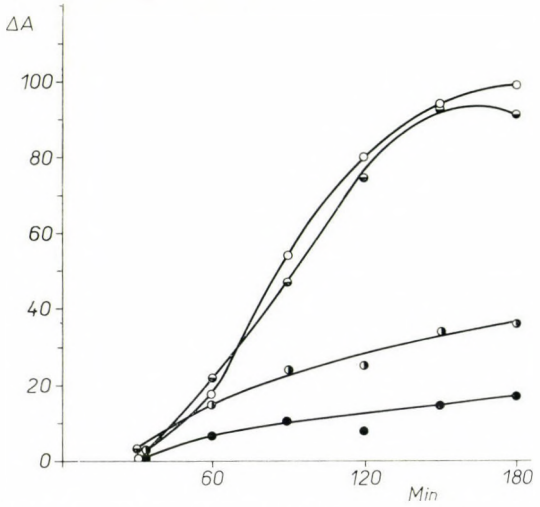

Fig. 4. Effect of streptomycin on the synthesis of benzoic acid oxidase by M. Friburgensis cells

○—○ Each Warburg vessel contained 50 mg of bacterium cells suspended in M/15 phosphate buffer (pH 7.2) and 1 mg of sodium benzoate. Final volume of the suspension was 2.5 ml, the experiments were carried out at 37 °C. ●—● The same as the control except that streptomycin was added at zero time to obtain a final concentration of 15 μg per ml. ◑—◑ The same as the control except that streptomycin was added at the 9th minute to obtain a final concentration of 15 μg per ml. ◒—◒ The same as the control except that streptomycin was added at the 16th minute to obtain a final concentration of 15 μg per ml. Ordinate represents the amount of oxygen consumed for the oxidation of benzoic acid in 30 minutes, expressed in μl. The data refer to 50 mg (fresh weight) of bacteria

followed by the *de novo* synthesis of the specific protein. Cox et al. (1964) have shown that SM can be attached to the ribosomes. These ribosomes – though they are still capable of binding m-RNA – do not function normally (Cox et al., 1964; Davies et al., 1964). It may therefore seem possible that the benzoic acid oxidase synthetizing system becomes SM resistant when the binding of the m-RNS by the ribosomes has already taken place. This explanation is confirmed by the results obtained in cell-free systems. In connection with the synthesis of polyphenyl alanine Flaks et al. (1962) and Rosano et al. (1966) have found that SM has a lesser inhibitory effect if the synthetic messenger (Poly-U) is first added to the system.

Our first experiment provides further evidence of the decisive role in the induction of benzoic acid oxidase of the order in which are added to the cells benzoic acid and SM, respectively. Cells pretreated for 20 minutes with SM could not be induced even after washing. Hurwitz and Rosano (1965) have shown that when SM is used in the pretreatment it penetrates the cells by the mediation of inducible streptomycin permeases. Gundersen (1965) has observed the resistance of intracellularly located SM to washing with inorganic ions. It may thus be assumed that in our experiments the formation of benzoic acid oxidase was inhibited by SM accumulated in the cells and irreversibly bound to the ribosomes.

Tests with radioactive benzoic acid have clearly shown that the inhibition of the system which synthetizes benzoic acid can only be partly explained by the direct action of SM on enzyme synthesis. Cohen and Monod (1957) were the first to point out the role of the specific protein components of the cell membrane in the active transport of low molecular weight substances. These proteins were called by them permeases. Similarly, in the penetration of benzoic acid specific permeases are involved. Our experiments have shown that when the bacteria were incubated with radioactive benzoic acid the uptake of the acid by the cells was first rather slow but later proceeded at an increasing rate. The maximum rate of uptake began after about 20 min. It was also observed that cells which had been pretreated with "cold" sodium benzoate for 20 minutes took up the benzoic acid immediately at the maximum rate.

The penetration of benzoic acid into the cells reaches obviously its maximum rate when the quantity of permeases the synthesis of which has been induced by benzoic acid also reaches its maximum level. When benzoic acid and SM were added simultaneously to the bacterium suspension a minimum benzoic acid uptake, similar to that observed in the control, occurred from the 20th minute onwards without an increase in the rate of uptake. This seems to indicate the inhibition by SM of the synthesis of permeases specific for benzoic acid. This will certainly affect the benzoic acid oxidase synthetizing system of the cell and will obviously lead to the inability of the bacteria to accumulate the amount of benzoic acid which is necessary for induction. Thus the permease system may be responsible indirectly for the inhibition of induction.

Further experiments have shown that like the benzoic acid oxidase synthetizing system the permease system, too, may become SM resistant if before the addition of SM the cells have been in contact with benzoic acid for a sufficiently long period of time. However, pretreatment with benzoic acid does not affect the SM sensitivity of the benzoic acid oxidase synthetizing system and of the permease system in the same way. Cells pretreated for 9 minutes with ^{14}C-benzoic acid showed under the effect of SM inhibited benzoic acid oxidase induction without any effect on the ^{14}C-benzoic acid uptake.

An explanation has to be found for the different trends of the curves in the control experiments without SM (Figs 2 and 3).

Erdős and Tomcsányi (1957) have shown that the induction of benzoic acid oxidase in the latency period – thus prior to the synthesis of the enzyme – is accompanied by the formation of detectable quantities of pyrocatechin. This indicates the presence of a minimum quantity of enzyme in the cells already before induction. In the oxidative breakdown of benzoic acid by benzoic acid oxidase in the first step pyrocatechin is formed by decarboxylation followed by the formation of beta-keto-adipic acid through cis-cis-muconic acid. If benzoic acid is added to the bacterium cells the enzyme immediately induces the oxidative breakdown of the benzoic acid which penetrates the cells. Thus in the experiments with benzoic acid labelled in the carboxyl group (Experiment No. 2) the radioactivity measured reflects the quantity of benzoic acid taken up by the cells and not yet degraded by benzoic acid oxidase. On the other hand when benzoic acid is labelled in the ring (Experiment No. 3) the radioactivity measured corresponds to the quantity of all the possible intermediary products.

These findings may explain the considerable difference between the trends of the control curves with two differently labelled benzoic acids and between the specific activities measured at different points of the curve.

Further experiments are, however, needed to decide whether the observed effect of SM affects merely cell permeability to benzoic acid or whether this effect is a generally valid phenomenon bearing also on the uptake of other substances of vital importance for the functioning of the cell.

Acknowledgements

The author expresses his thanks to Mrs. L. Gonda for her skillful technical assistance.

References

Cohen, G. N., Monod, J. (1957) Bacteriol. Rev. *21* 169
Cox, C., White, J. R., Flaks, J. G. (1964) Proc. Nat. Acad. Sci. *51* 703
Creaser, E. H. (1955) J. Gen. Microbiol. *12* 288
Davies, J. E. (1964) Proc. Nat. Acad. Sci. *51* 659
Erdős, T., Tomcsányi, A. (1957) Acta Physiol. Acad. Sci. Hung. *12* 311
Fitzgerald, R. J., Bernheim, F. (1947) J. Bact. *54* 671
Fitzgerald, R. J., Bernheim, F., Fitzgerald, D. B. (1948) J. Biol. Chem. *175* 195
Flaks, J. G., Cox, E. C., White, J. R. (1962) Biochem. Biophys. Res. Comm. *7* 385
Gale, E. F. (1963) Pharmacol. Rev. *15* 481
Gunderson, W. B. (1965) Acta Path. Microbiol. Scand. *65* 636
Hurwitz, C., Rosano, C. L. (1965) J. Bact. *90* 1233
Rosano, C. L., Hurwitz, C., Peabody, R. A. (1966) Biochim. Biophys. Acta *114* 188

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 253—266 (1966)

Structure and Function of Erythrocytes

I. Relation between the Energy Metabolism and the Maintenance of Biconcave Shape of Human Erythrocytes

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(Received May 2, 1966)

The disk-sphere transformation of erythrocytes follows only very slowly, and with some delay the rapid breakdown of ATP,* induced by IA or NaF. In contrast to this, the restoration of the biconcave shape after the fermentative resynthesis of ATP takes place at a much higher rate, i.e. the kinetics of the morphological degeneration and regeneration are different. While IA evokes the disk-sphere transformation of erythrocytes exclusively through the changes in ATP-levels NaF can exert also an additional direct inhibitory effect on the processes which ensure the maintenance of the biconcave shape (probably on an ATPase system). This inhibition is detectable at a NaF concentration as low as 8 mM and becomes pronounced above 10 mM NaF. NaF influences the disk-sphere transformation also by stopping ATP synthesis via the breakdown of 2,3 DPG but the latter compound plays a role in the control of shape transformation only inasmuch as it generates ATP.

Introduction

From the point of view of gas and liquid uptake, as well as flow and osmotic relations of erythrocytes, the biconcave disk shape is very advantageous. The maintenance of the biconcave shape was first explained on a physicochemical basis (Norris, 1882; Gough, 1924; Teitel-Bernard, 1934). A more dynamic aspect became necessary by the recognition that erythrocytes have not only one but two possible forms: the biconcave disk and the smooth sphere, and between these there exist several metastable forms (Hamburger, 1895). Ponder (1948) explained the maintenance of biconcave shape by the presence of an albumin-like material, the so-called antisphering factor. Prankerd and Altman (1954) found a correlation between the flatness of erythrocytes of various animal species (diameter to thickness ratio) and their 2,3-DPG content and called the attention to the possible connection between 2,3-DPG and ATP contents and biconcave shape. Nakao and his group (Nakao et al., 1961) brought about the decomposition of ATP in washed erythrocytes by 20 mM NaF. After several washings to remove NaF the resyn-

* *Abbreviations:* ATP = adenosinetriphosphate; 2,3-DPG = 2,3-diphosphoglycerate; IA = iodoacetate (sodium salt); I_m = morphological index.

thesis of ATP was induced by the addition of adenine, inosine and glucose. They examined along with the changes in ATP content the changes in the morphology of erythrocytes. In their opinion the two processes run parallel in both the degeneration and regeneration phases, and the shape of erythrocytes is determined only by the actual ATP level of the cells. According to their hypothesis in the erythrocyte membrane there is a contractile actomyosin-like protein, the structural change of which directly follows the changes in ATP content.

The effect of NaF on erythrocytes is of a complex nature. It inhibits the decomposition of 2,3-DPG even in a low concentration (2 mM) (Lennerstrand, 1940; Gárdos, 1966). In a concentration of 5 mM it induces the rapid decomposition of ATP in addition to the partial inhibition of glycolysis (Mányai, Székely, 1954). Above a concentration of 5 mM it causes suddenly dramatic changes in the permeability of the membrane. Lindemann and Passow (1960) refer to this effect as the "direct membrane effect" of NaF and in their opinion it is caused by the structural change of the membrane due to the formation of a complex between an erythrocyte membrane component, NaF and alkali earth metal. Considering the aforementioned we thought it necessary to examine whether the effects of NaF other than the inhibition of glycolysis, thus the influencing of the utilization of 2,3-DPG and first of all the so-called direct membrane effect, do affect the disk-sphere transformation phenomenon independent of the ATP content.

Experimental

In most experiments fresh defibrinated human blood was used. The experimental mixture contained 75 per cent normal blood, while the effective agents were contained in the remaining 25 per cent by volume. Thus we worked at a 30 per cent average hematocrit value. Part of the experiments was carried out with washed erythrocytes; in this case the cells were washed with three volumes of 166 mM sodium chloride or with 163 mM sodium chloride + 6 mM glucose. Centrifugations were performed at room temperature for 10 minutes at 3500 r.p.m. The original hematocrit values were attained by supplementing the erythrocyte suspensions with Tyrode-solution or fresh normal serum. These erythrocyte suspensions were applied, similarly to the defibrinated blood, in 75 per cent by volume of the experimental mixture. The mixtures were incubated at 37 °C.

The ATP resynthesis, following NaF treatment, was performed either in a fermentative or in an oxidative way (Dische, 1934; Mányai, Székely, 1954; Gárdos, Straub, 1957). In the former case erythrocytes were washed once with 10 volumes of cold 166 mM sodium chloride, thus reducing the NaF concentration below a critical level, then by the addition of glucose and incubation at 37 °C the regeneration of ATP was ensured. In the oxidative resynthesis of ATP sodium pyruvate, in a final concentration of 15 mM, was added to the experimental mixture containing NaF.

The morphology of erythrocytes was examined between slide and coverslip, in native preparations at an 800 × magnification, in a phase-contrast microscope. Two kinds of solution were used for the dilutions:

A) fresh normal compatible human serum and 166 mM sodium chloride in a proportion of 3 : 7;

B) 3 per cent trisodium citrate solution containing 1 per cent formalin (Dacie, 1956).

The blood sample (0.05 ml) was mixed with 3 ml of the diluting solution and the morphology of erythrocytes was evaluated immediately. Comparative examinations were carried out also with preparations suspended in 166 mM sodium chloride, between silicon- or paraffin-treated slides and coverslips. All methods applied gave identical results. The majority of the investigations was made in

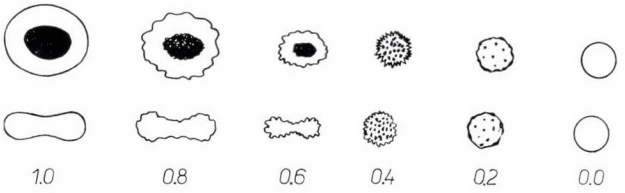

Fig. 1. The stages of disk sphere transformation of erythrocytes. 1.0: smooth disk, 0.8: crenated disk, 0.6: crenated discoid, 0.4: crenated spheroid, 0.2: crenated sphere, 0.0: smooth sphere

diluting solution *A*. In this medium, with the exception of mixtures containing IA, the morphology of erythrocytes did not change at all in any of our experimental systems within 30 minutes, while it changed only to a practically negligible extent within 60 minutes. In systems containing IA a continuous decay took place. In diluting solution *B* the picture at a given moment could be stabilized, the evaluation was, however, rendered difficult by the secondary shrinking of the cells.

Six cellular forms were distinguished: smooth disk, crenated disk, crenated discoid, crenated spheroid, crenated sphere, and smooth sphere forms (Fig. 1). For the quantitative evaluation the various forms were given, in the above order of enumeration, the following numerical values: 1.0, 0.8, 0.6, 0.4, 0.2, 0.0. After the examination of 200 cells, the values of percentage distributions of the various cellular forms were multiplied by the above factors, and the respective products were added up. Thus the morphology could be characterized by using a scale ranging from 100 to 0. This numerical value was referred to as morphological index (I_m). When diluting solution *A* was used, the scattering of parallel determined I_m values, between $I_m = 100$ and 40, was not appreciable ($s = \pm 0.4$), while below $I_m = 40$ it was more pronounced ($s = \pm 0.4 - 1.0$). In the latter case the crenated spheroid and crenated sphere forms were encountered in a high number, and these two forms could not be distinguished from each other so clearly as the other cellular forms. Rather significant individual differences were observed in the rates

of degeneration of the blood samples. (In Fig. 2 e.g. a slowly degenerating blood sample, while in the other figures blood samples degenerating at a medium rate are shown). The relative differences due to the effective agents were, however, well reproducible with all blood samples.

ATP determinations were carried out in two ways:

1. by the spectrophotometric analysis at 260 mμ of the fractions obtained from a Dowex-1 ion exchange column (Deutsch, Nilsson, 1953);

2. enzymically, with the aid of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase, according to the Boehringer-test (Bücher, 1947). The measured values were expressed as percentages of the original values.

The determination of 2,3-DPG was performed by separation on ion exchange column followed by the chromotropic acid color reaction according to the method of Bartlett (1959).

Results

Effect of glycolytic inhibitors on morphology

The effect of various concentrations of the generally applied glycolytic inhibitors, IA and NaF, on the ATP-content and morphological index of erythrocytes was examined in detail. With washed erythrocytes, the rate of decrease in ATP-level due to glycolytic inhibitors strongly depended on the number of washings and on the composition of the washing medium. Increasing the number of washings (to 3 or more) and omitting glucose from the washing medium resulted in a gradual slowing down of the fall of ATP-level caused by glycolytic inhibitors. This phenomenon could be observed even if the washed erythrocytes were resuspended in fresh normal serum. The significant differences in the rate of ATP-breakdown due to the washing of erythrocytes, and the fact that in experiments on the inhibition of glycolysis the presence of serum proteins did not influence appreciably the morphology urged us to perform the experiments under the most physiological conditions, by the omittance of washings, with defibrinated blood.

The effect of glycolytic inhibitors on the ATP-content and morphology of erythrocytes is shown in Fig. 2. It was found that with NaF concentrations lower than 4 mM the ATP content decreased, but slowly. In the presence of 5 mM NaF there was already a rapid ATP-breakdown, which slightly increased with increasing NaF concentrations. In case of IA the concentration limit was 0.5 mM, above this the rate of ATP-breakdown did not change appreciably within a wide concentration range (up to 10 mM). As to the morphology of the erythrocytes the following was observed: 1. The rate of decrease of the morphological index was considerably slower than that of ATP-breakdown; ATP-breakdown was followed by the morphological change with some delay. 2. The relatively small difference in the ATP-contents of the two systems was accompanied by a much more pronounced difference in the I_m values. Because of this, with IA used in concentrations higher than 0.5 mM the morphology did not change further, while the

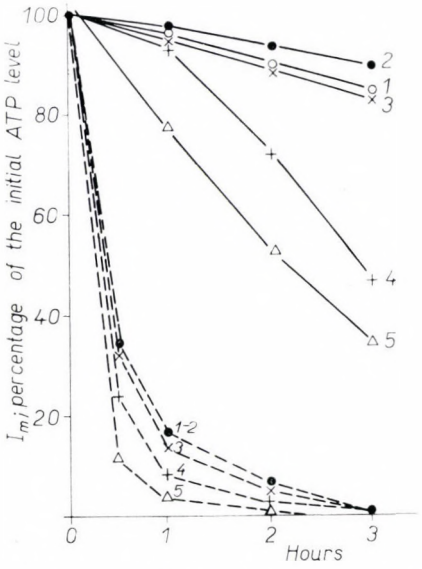

Fig. 2. Effect of IA and NaF on the ATP content and morphological index (I_m) of human erythrocytes. The broken lines indicate the ATP content of the cells, the solid lines mark the morphological index. 1: 0.5, 1 and 5 mM IA; 2: 5 mM NaF; 3: 8 mM NaF; 4: 16 mM NaF; 5: 110 mM NaF

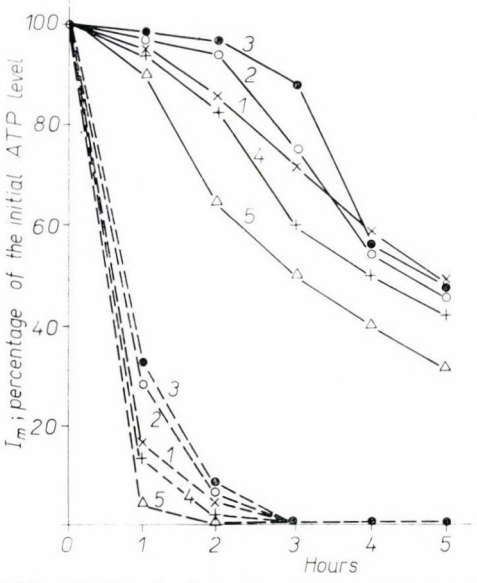

Fig. 3. Effect of $NaHSO_3$, NaF and adenosine on the ATP content and morphological index (I_m) of human erythrocytes in the presence of IA. 1: 1 mM IA; 2: 1 mM IA + 15 mM $NaHSO_3$; 3: 1 mM IA + 30 mM $NaHSO_3$; 4: 1 mM IA + 2 mM NaF; 5: 1 mM IA + + 10 mM adenosine. For symbols cf. Fig. 2

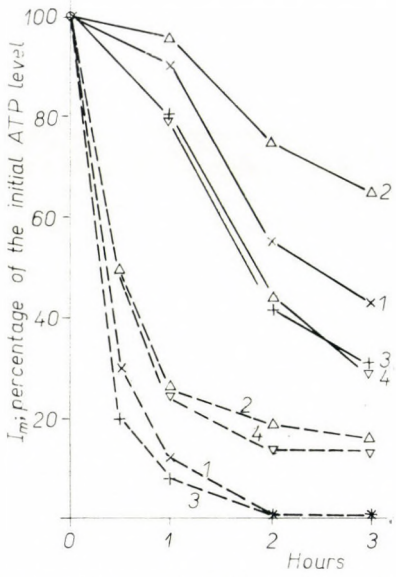

Fig. 4. Effect of NaF and adenosine on the ATP content and morphological index (I_m) of human erythrocytes. 1: 8 mM NaF; 2: 8 mM NaF + 10 mM adenosine; 3: 16 mM NaF; 4: 16 mM NaF + 10 mM adenosine. For symbols cf. Fig. 2

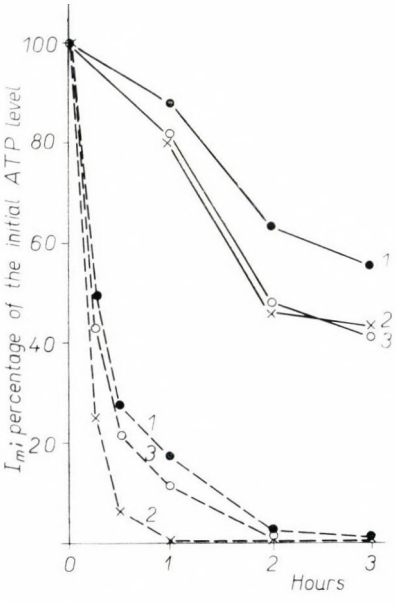

Fig. 5. Effect of NaF and Na_2HAsO_4 on the ATP content and morphological index (I_m) of human erythrocytes. 1: 8 mM NaF; 2: 8 mM NaF + 2.5 mM Na_2HAsO_4; 3: 16 mM NaF. For symbols cf. Fig. 2

increase in NaF concentration considerably enhanced the rate of fall of the morphological index.

The ATP-breakdown could be accelerated by the application, in addition to IA, of adenosine (10 mM) and of small concentrations of NaF (2 mM) or it could be slowed down with $NaHSO_3$ (15 mM). Parallel shifts were observed in the decrease of the morphological index, but here there was also some delay as related to the changes in ATP-levels (Fig. 3).

If some other compounds affecting ATP-breakdown, such as adenosine (10 mM) and Na_2HAsO_4 (2.5 mM) are applied together with NaF, quite different results will be obtained. Adenosine applied with 8 mM NaF will slow down the breakdown of ATP and consequently the morphological degeneration, too. Adenosine applied with 16 mM NaF, although it markedly slows down ATP-breakdown, so that the ATP-level is higher than in the system containing 8 mM NaF, does not influence morphological degeneration at all: results obtained with systems containing adenosine are completely identical with those obtained with systems which contain no adenosine (Fig. 4). Similar paradoxical effect can be observed with the $NaF - Na_2HAsO_4$ combination, too. Na_2HAsO_4 applied with 8 mM NaF considerably increases the rate of ATP-breakdown resulting in an ATP-level much lower than that found in the system which contains only 16 mM NaF. In spite of this, the extent of morphological degeneration in the system containing 8 mM NaF + Na_2HAsO_4 and in the AsO_4^{3-}-free system containing only 16 mM NaF, is almost identical (Fig. 5).

Effect of ATP resynthesis on morphology

In addition to the disk-sphere transformation, the reverse process, the sphere-disk restoration was also investigated. After treatment with 8 mM NaF, the fermentative resynthesis of ATP was accomplished. It was found that in the regeneration phase the improvement of the morphological picture followed ATP resynthesis to a much higher rate than the morphological changes did ATP-breakdown in the degeneration phase. Consequently, in the degeneration and regeneration phases very different I_m values belong to the same ATP values (Fig. 6).

When the partial, so-called oxidative resynthesis of ATP was performed with sodium pyruvate, different reactions were obtained depending on the concentration of NaF. Using 8 mM NaF we observed that while the resynthesis of ATP started immediately after the addition of pyruvate and attained a constant level in about 15 minutes, the morphological index kept on decreasing for a further 15 minutes, then slowly, gradually the improvement of the morphological picture took place attaining the maximum (maximum $I_m = 97$) only 90 minutes after the maximum ATP-level had been reached. At a NaF concentration of 16 mM the resynthesis of ATP went on at the same rate, in the morphological picture, however, after the initial lag period, only the rate of degeneration slowed down, but the resynthesis of ATP failed to produce any increase in the morphological index (Fig. 7).

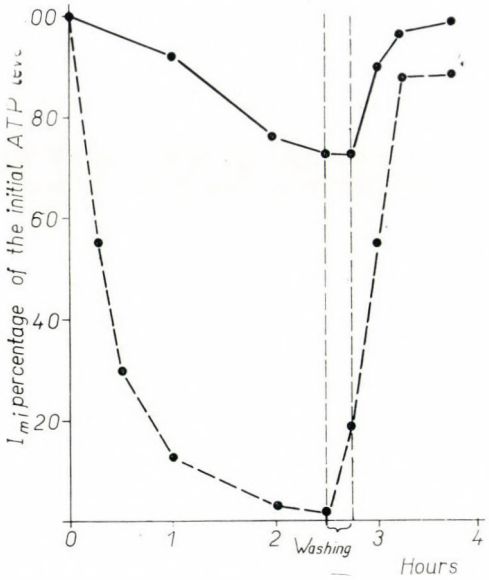

Fig. 6. Effect of fermentative ATP resynthesis on the changes of the morphological index (I_m) of human erythrocytes. For experimental details, see text; for symbols cf. Fig. 2

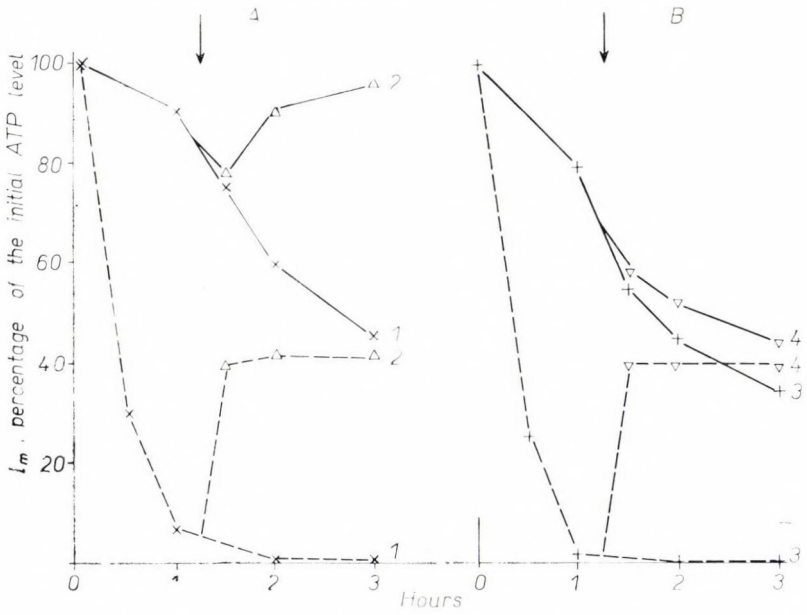

Fig. 7. Effect of 15 mM sodium pyruvate on the ATP content and morphological index (I_m) of human erythrocytes in the presence of NaF. A: 8 mM NaF; B: 16 mM NaF. Sodium pyruvate was added to the experimental system at the time indicated by the arrow. 1: and 3: NaF; 2: and 4: NaF + pyruvate. For symbols cf. Fig. 2

It can be clearly seen also in this figure, how different I_m values are assigned to the same ATP-levels in the phases of ATP-breakdown and resynthesis, respectively, and that, after the oxidative resynthesis of ATP, at a practically constant ATP-level the morphology of erythrocytes changes.

Discussion

Nakao et al. (1961) have distinguished four forms of erythrocytes: smooth disk, crenated disk, crenated sphere, and smooth sphere, and have evaluated the morphology only qualitatively using hanging drop method. Furchgott and Ponder (1940) distinguished 6 forms of erythrocytes and by the aid of appropriate multiplying factors (1.0, 0.5, 0.3, 0.2, 0.1, 0.0) made an attempt to evaluate the results quantitatively. We found the multiplying factors (1.0, 0.8, 0.6, 0.4, 0.2, 0.0) more convenient, especially in case of a more advanced degeneration, and used them in the present study.

Nakao et al. to eliminate the possible influence of the "antisphering factor", performed their experiments with suspensions of erythrocytes washed three times. In their experiments, with final concentrations of 30 per cent hematocrit and 20 mM NaF the ATP content of erythrocytes decreased by 65 per cent in 2 hours and the majority of erythrocytes was of the crenated disk form. Under the same conditions with defibrinated whole blood more than 90 per cent of the original ATP-content disappeared in 2 hours, and at the same time, the majority of erythrocytes degenerated also to the crenated disk form only. These alterations suggest that during the course of washings the erythrocyte membrane was functionally damaged and thus, due to the loss of coenzymes and to the impairment of enzymic activities, the breakdown of ATP was affected. The ATP remaining in the cells, however, cannot be utilized well for the restoration of the biconcave shape, the higher ATP-level has no favourable effect on this function. The reason for this is, in part, the structural damage caused by the washing. That is why in our experiments we used defibrinated blood, and in experiments on the fermentative resynthesis of the ATP-content of cells treated with NaF, we applied only one washing of large volume, in contrast to the four washings of Nakao et al. We considered it satisfactory to reduce NaF concentration below a critical level instead of removing NaF completely. Under such conditions the cells lost their biconcave shape only slowly and gradually following the decrease in ATP-level. In the fermentative resynthesis of ATP, however, the restoration of biconcave shape was much faster. Thus the kinetics of morphological degeneration and regeneration proved to be different.

Out of the enzymes of glycolysis, glyceraldehyde-3-phosphate dehydrogenase has been shown to be the most sensitive to IA (Lundsgaard, 1930, 1932; Lohmann, 1931; Cori et al., 1948), with higher concentrations, however, other enzymes e.g. hexokinase (Iri, 1939), inorganic pyrophosphatase (Naganna, 1948, 1950) are also inhibited. Since there was no change in the morphology of erythrocytes

with increasing IA concentrations, it should be assumed that in addition to glyceraldehyde-3-phosphate dehydrogenase no other enzymes which can be inhibited by IA play a role in the control of the shape of erythrocytes. In case of IA inhibition, 2,3-DPG gradually decomposes owing to the shift of the equilibrium of formation and breakdown, and thus it slows down the breakdown of ATP by ensuring ATP synthesis for some time in the terminal phase of glycolysis. In case of the inhibition of glycolysis with NaF this transformation cannot take place. This may explain our observation that at complete inhibition of glycolysis by IA slower ATP-breakdown and decrease of I_m were obtained than with NaF concentrations, where the inhibition of glycolysis was not yet complete. The shifts in the rates of ATP-breakdown and morphological degeneration induced by $NaHSO_3$ applied together with IA can be readily explained by the observation of Mányai and Várady (1956, 1958) that $NaHSO_3$ enhances the breakdown of 2,3-DPG. The opposite effect can be achieved when small concentrations (2 mM) of NaF are applied together with IA. This can be explained by the fact that NaF in this concentration inhibits the breakdown of 2,3-DPG by as high as 50 per cent, reducing thus the amount of ATP formed from 2,3-DPG (Clarkson, Maizels, 1952; Gárdos, 1966). The mechanism of the increased ATP-breakdown induced by adenosine applied together with IA is still unknown. The changes in the morphology of erythrocytes, however, can be satisfactorily explained by the differences in ATP-levels. As a matter of fact it has been found that varying the concentration of IA or combining it with other agents, we failed to detect any alteration in the morphology of erythrocytes which could not be traced back to the breakdown of ATP, caused by the inhibition of glyceraldehyde-3-phosphate dehydrogenase and possibly modified in its rate.

The inhibitory effect of NaF on glycolysis is essentially known: it is based on the inhibition of enolase brought about by the formation of the Mg-fluorophosphate complex (Warburg, Christian, 1942). With a NaF concentration of 5 mM the inhibition of enolase is not complete, the formation of lactic acid can still be detected, the synthesis of ATP is, however, already considerably impaired (Mányai, Székely, 1954). By increasing the concentration of NaF the inhibition of enolase becomes more and more complete and the enhancement of the rate of ATP-breakdown could be established by an ion exchange column chromatographic method. However, since NaF inhibits many other processes as well, it is impossible to single out such an ideal NaF concentration which would inhibit glycolysis completely without any other detectable effects.

In connection with the ATPases, Barron and Singer (1945) have reported the inhibitory effect of NaF on myosin-ATPase, while Garzó et al. (1952) have shown that NaF in a concentration of 5 mM has an 80 per cent inhibitory effect on partly purified swine erythrocyte ATPase. By increasing the concentration of NaF the ATPases are gradually inhibited. Thus it can be assumed that the rate of ATP-breakdown decreases and the ATP-level increases. Kramer and Straub (Straub, 1952) have actually found similar alterations in rabbit erythrocytes.

In human erythrocytes, however, we failed to detect such a shift, even when isotonic NaF was used. This fact points to the relatively higher NaF-resistance of the ATPase activities of human erythrocytes. In this way it can be explained that even if the inhibition of glycolysis is made complete by increasing the concentration of NaF in the system, there still remains a rather significant ATPase activity which results in the enhancement of ATP-breakdown in accordance with the more and more complete blocking of ATP synthesis. At the same time our experiments indicate that the enzyme system responsible for the maintenance of the biconcave shape is sensitive to NaF thus it can utilize ATP still available for the maintenance of the biconcave shape to a decreasing extent. This is proved by our following results:

1. The higher ATP-level provided by adenosine at a NaF concentration of 16 mM cannot be utilized by the cell for the maintenance of the biconcave shape.

2. At a NaF concentration of 16 mM the ATP-level of the cells is higher than that produced by arsenolysis at a NaF concentration of 8 mM; this additional amount of ATP cannot be utilized by the cell for the maintenance of the biconcave shape either.

3. The higher ATP-level produced by oxidative resynthesis can be used by the cell for the regeneration of the biconcave shape, at a NaF concentration of 8 mM, but with some delay. At a NaF concentration of 16 mM there is no morphological regeneration at all, the higher ATP-level is manifest only in decreasing the rate of degeneration.

These results show that NaF at a concentration of as low as 8 mM inhibits to some extent the utilization of ATP for the maintenance of biconcave shape and with 16 mM NaF this inhibition is very pronounced.

ATPase activity of erythrocytes is known to be bound to the membrane (Garzó et al., 1952; Clarkson, Maizels, 1952; Herbert, 1956). In our opinion, one component of the "direct membrane effect" of NaF is damaging the structure and function of the enzymic reaction chain that exerts a net ATPase activity and maintains the biconcave shape of erythrocytes. In addition to this possibility, however, the other inhibitory effects of NaF, which may have a bearing on the phenomenon observed, should be taken into account. It is to be mentioned that according to Melkin and Denstedt (1956) in erythrocytes the breakdown of inorganic pyrophosphate may also cover the energy requirement of endenergic processes. The inorganic pyrophosphatase activity of human erythrocytes is, however, already completely inhibited at a NaF concentration of 0.2 mM (Nagganna, 1948, 1950), thus the differences observed with the NaF concentrations applied by us cannot be attributed to this inhibitory effect. According to Vogt's hypothesis (1957), in the transport ATPase reaction chain of erythrocytes a lecithinase of the phospholipase-C type plays also a role. The phospholipase-C preparations known so far can be inhibited by NaF (MacFarlane, Knight, 1941). Since the most effective inhibitors of the enzyme are the agents which are known to be

effective in the induction of sphere-transformation (sodium dodecylsulphate, sodium taurocholate, digitonin, etc.), the possible role of this enzyme should also be taken into consideration.

On the basis of our experiments it can be established that in a concentration of 10 mM NaF inhibits the mechanism which maintains the biconcave shape of cells to such an extent that at this concentration it already cannot be applied for the study of the relation between ATP-content and the shape of cells, and it gives an erroneous picture of the kinetics of the change of the morphology of erythrocytes. Consequently the following experimental systems are regarded as most suitable for the study of the interrelations between ATP-content and morphological picture: 1. erythrocytes of IA-treated, defibrinated blood for study of degeneration; 2. erythrocytes of defibrinated blood pretreated with 8 mM NaF, washed once with ten volumes of 166 mM sodium chloride and then suspended in a solution containing glucose for the study of regeneration.

Acknowledgements

Thanks are due to Dr. S. Hollán, Director of the Institute, for her continuous help and support to accomplish this work, and Mrs. I. Irmai and Miss S. Keresztes for their very skilful and precise technical assistance.

References

Barron, E.S.G., Singer, T.P. (1945) J. Biol. Chem. *157* 221
Bartlett, G.R. (1959) J. Biol. Chem. *234* 469
Bücher, Th. (1947) Biochim. Biophys. Acta *1* 292
Clarkson, E.M., Maizels, M. (1952) J. Physiol. *116* 112
Cori, G.T., Slein, M.W., Cori, C.F. (1948) J. Biol. Chem. *173* 605
Dacie, J. V. (1956) Practical Haematology. Chemical Publ. Co., New York
Deutsch, A., Nilsson, R. (1953) Acta Chem. Scand. *7* 1288
Dische, Z. (1934) Naturwissenschaften *22* 855
Furchgott, R.F., Ponder, E. (1940) J. Exp. Biol. *17* 117
Gárdos, G. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 139
Gárdos, G., Straub, F.B. (1957) Acta Physiol. Acad. Sci. Hung. *12* 1
Garzó, T., Ullmann, Á., Straub, F.B. (1952) Acta Physiol. Acad. Sci. Hung. *3* 513
Gough, A. (1924) Biochem. J. *18* 202
Hamburger, H. J. (1895) Pflügers Arch. *141* 230
Herbert, E. (1956) J. Cell Comp. Physiol. *47* 11
Iri, S. (1939) J. Biochem. (Japan) *30* 217
Lennerstrand, A. (1940) Enzymologia *9* 248
Lindemann, B., Passow, H. (1960) Pflügers Arch. *271* 497
Lohmann, K. (1931) Biochem. Z. *236* 444
Lundsgaard, E. (1930) Biochem. Z. *220* 1
Lundsgaard, E. (1932) Biochem. Z. *250* 61
MacFarlane, M.G., Knight, B.C.J.G. (1941) Biochem. J. *35* 884
Mányai, S., Székely, M. (1954) Acta Physiol. Acad. Sci. Hung. *5* 7

Mányai, S., Várady, Zs. (1956) Biochim. Biophys. Acta *20* 594
Mányai, S., Várady, Zs. (1958) Acta Physiol. Acad. Sci. Hung. *14* 103
Melkin, A., Denstedt, O.F. (1956) Canad. J. Biochem. Physiol. *34* 121
Naganna, B., Menon, V.K.N. (1948) J. Biol. Chem. *174* 501
Naganna, B. (1950) J. Biol. Chem. *183* 693
Nakao, M., Nakao, T., Yamazoe, S., Yoshikawa, H. (1961) J. Biochem. (Japan) *49* 487
Norris, R. (1882) Physiology and Pathology of the Blood. Smith, Elder and Co., London
Ponder, E. (1948) Hemolysis and Related Phenomena. Grune and Stratton, New York
Prankerd, T.A.J., Altman, K.I. (1954) Biochem. J. *58* 622
Straub, F.B. (1952) MTA Orvosi Tud. Oszt. Közl. *3* 31
Teitel-Bernard, A. (1934) Le Sang *8* 298
Vogt, W. (1957) Nature *179* 300
Warburg, O., Christian, W. (1942) Biochem. Z. *310* 384

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 267—278 (1966)

On the Mechanism of Enzyme Inhibition by Antiphosphorylase

I. Effect of Substrate and Activator

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(Received May 5, 1966)

The inhibitory action on phosphorylase of antibodies formed in cocks against rabbit muscle phosphorylase-b was studied. In the presence of EDTA* the inhibitory effect of the antibody was of a non-competitive character with respect to glycogen, glucose-1-P and 5′-AMP, while in the presence of magnesium a mixed type of inhibition for 5′-AMP was observed. With respect to preincubation with substrate — activator combinations glycogen has a slightly protective and glucose-1-P a slightly sensitizing effect on the inhibition by the antibody while 5′-AMP alone has no significant effect. After pretreatment of enzyme with glycogen followed by treatment with 5′-AMP inhibition by the antibody is the lowest, and the highest after the preincubation with glucose-1-P and subsequent treatment with 5′-AMP. The effect of glucose-1-P + 5′-AMP to increase the rate of inhibition is the most marked at low activator concentrations. Glycogen neutralizes the sensitizing effect of glucose-1-P. The protective effect is the most pronounced when the antibody is added to the functioning enzyme.

These data suggest the view that the degree of inhibition by the antibody depends on the actual conformation of the enzyme.

Introduction

In an earlier study on the action of antibodies produced in roosters against rabbit muscle phosphorylase-b it was found that enzyme inhibition by the antibody is not to be attributed to aggregate formation (Jókay, 1963). To get some more information about the possible mechanisms involved in this process the influence of the substrate G-1-P, glycogen and of the activator (5′-AMP) on the enzyme inhibition by antibodies was studied.

* Abbreviations used:
EDTA = ethylenediaminetetraacetate
5′-AMP = adenosine-5′-phosphate
BSA = bovine serum albumin
K_M = apparent Michaelis-Menten constant evaluated by the method of Lineweaver and Burk
G-1-P = glucose-1-phosphate
PCMB = p-chloromercurybenzoate
NEM = N-ethylmaleimide

Experimental

Rabbit muscle phosphorylase-b was prepared by the method of Fischer and Krebs (1958) and recrystallized three times. The crystalls were dissolved in the diluent to prepare a stock solution, which was treated – similarly to the glycogen solution – with active charcoal to remove nucleotides.

For the preparation of antisera the method described in an earlier paper (Jókay, 1963) was used. The collected sera were precipitated 4–5 times with half-saturated ammonium sulphate and dialyzed against 0.15 M NaCl. This concentrated globulin solution was used in a 300 to 500 fold dilution.

Phosphorylase and antiserum were diluted with the following solution: 20 mM glycerophosphate, 2 mM EDTA, 2 mM mercaptoethanol, 0.14 M NaCl, 0.1 per cent BSA; the pH was adjusted to 6.7 with acetic acid. In some experiments NaCl was omitted from the diluent. In the experiments with magnesium only 0.2 mM EDTA was used and the magnesium acetate was mixed with the diluent except when the magnesium concentration was varied.

Phosphorylase activity in the direction of glycogen synthesis (Illingworth, Cori, 1953) was measured in a total volume of 0.8–1.0 ml at pH 6.7. The dilution of phosphorylase or the reaction time was chosen in such a way that less than 10 per cent of the substrate should be decomposed. In cases when large differences between the activities of individual samples in the same experimental series were expected the reaction time with samples of lower activities was proportionally increased.

In kinetic studies phosphorylase was preincubated with the antibody for 30 minutes at a suitable temperature and the enzyme reaction was started by the simultaneous addition of all the other necessary components (G-1-P, glycogen, 5'-AMP).

In the case of preincubation with the substrate activator combination a typical reaction was carried out in the following manner: 0.3 ml of phosphorylase was preincubated first for 5 min at 30°C with the first component, then for another 5 min with the second component, 0.3 ml of the diluent or 0.3 ml of the antibody in the dilution required was added to the reaction mixture which was again preincubated for 20 min at 30°C. The enzyme reaction was started by adding a mixture of the rest of the substrates and of the activator. The volume of the substrate-activator mixture used for preincubation was 0.1 ml, its concentration corresponded to a final concentration of 3.7×10^{-2} M of G-1-P and 0.4 per cent of glycogen in 1 ml.

The enzyme reaction was stopped with 3 ml of 5 per cent trichloroacetic acid, or in the later experiments with a mixture of 0.25 per cent sodium tungstinate and 1.5 per cent acetic acid. The inorganic P content was determined after centrifuging by the method of Taussky and Shorr (1953). Parallels were taken for each sample, and the inhibition values obtained by preincubation with the substrate-activator mixture were compared to those of the controls prepared in the same way, but without the antibody.

Results

Effect of antiphosphorylase on the kinetics of phosphorylase action. – Figs 1 to 3 show the reciprocal values of phosphorylase activity versus the reciprocal

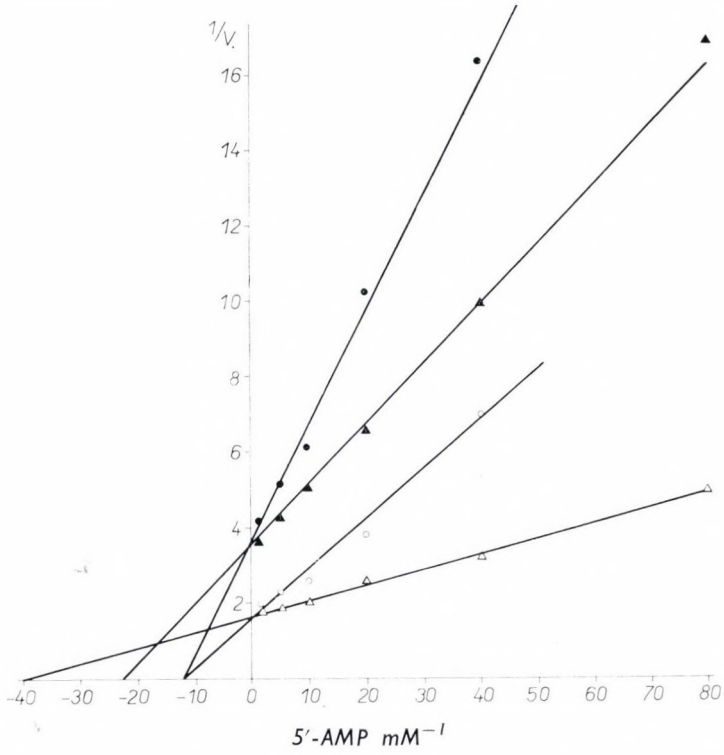

Fig. 1. Phosphorylase activity as a function of 5′-AMP concentration. Composition of the incubation mixture: 0.017 M G-1-P, 0.5 per cent glycogen, 5.3 μg phosphorylase-b, 0.075 M NaCl, 0.025 M glycerophosphate, 1 mM mercaptoethanol, 50 mg per cent BSA, 1 mM EDTA or 10 mM magnesium acetate; pH 6.7; temperature 30°C. With the inhibited enzyme: 40 μg immune serum globulin, total volume 0.8 ml. ○—○ non-inhibited phosphorylase in the presence of EDTA. ●—● inhibited phosphorylase in the presence of EDTA. △—△ non-inhibited phosphorylase in the presence of Mg. ▲—▲ inhibited phosphorylase in the presence of Mg

substrate and activator concentrations, respectively, plotted by the method of Lineweaver and Burk. Without magnesium the degree of inhibition was not affected by changing the amounts of either G-1-P or glycogen or 5'-AMP, thus the inhibiting effect of the antibody is non-competitive with any of the three

components. The results were somewhat different in the presence of magnesium. The presence of magnesium reduced the K_M value for 5'-AMP in the case of the non-inhibited enzyme to about 1/4 and that of the inhibited enzyme to not less

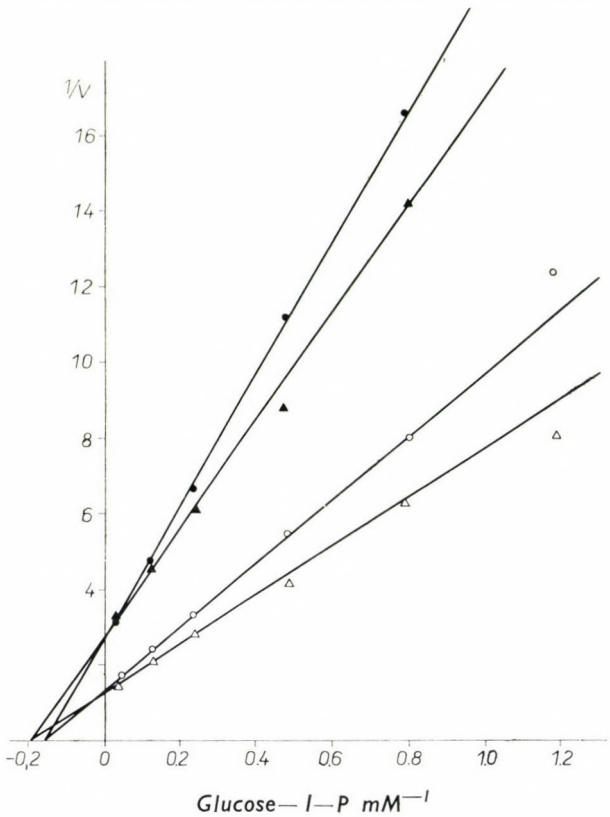

Fig. 2. Phosphorylase activity as a function of glucose-1-P concentration. Composition of the incubation mixture: 1 mM 5'-AMP; for the rest see Fig. 1. ○—○ non-inhibited phosphorylase in the presence of EDTA. ●—● inhibited phosphorylase in the presence of EDTA. △—△ non-inhibited phosphorylase in the presence of Mg. ▲—▲ inhibited phosphorylase in the presence of Mg

than 1/2. Thus a mixed type of inhibition developed with changed values of both V_{max} and K_M.

Magnesium reduced but slightly the K_M for G-1-P of the non-inhibited enzyme, but had no noticeable influence on the K_M value for glycogen. The type of inhibition was still non-competitive with respect to G-1-P and glycogen. It should be noted that while by changing G-1-P and 5'-AMP the reciprocal activity values

lie on a practically straight line, the values of changes in glycogen concentration in the presence of magnesium cannot be connected by a straight line.

Effect of preincubation of phosphorylase with different substrate-activator combinations on the inhibition by antiphosphorylase. – Table 1 shows the effect of preincubating phosphorylase with G-1-P and 5'-AMP on the inhibition by antibodies at low and high 5'-AMP levels. Preincubation with G-1-P increases

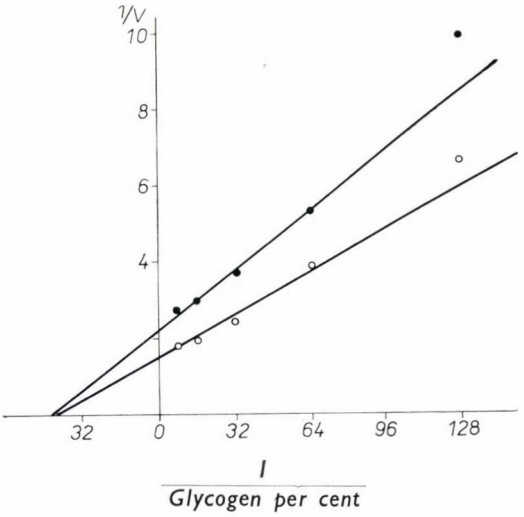

Fig. 3. Phosphorylase activity as a function of glycogen concentration. Composition of the incubation mixture: 0.033 M G-1-P, 1 mM 5'-AMP, for the rest see Fig. 1. ○—○ non-inhibited phosphorylase in the presence of EDTA. ●—● inhibited phosphorylase in the presence of EDTA

the inhibitory effect of antiphosphorylase at both 5'-AMP concentrations. While 5'-AMP alone has no significant effect it enhances the inhibitory effect of antibody, when added to phosphorylase which had been preincubated with G-1-P. The effect to increase the degree of inhibition was always greater for the order G-1-P– 5'-AMP than for the reverse. This effect of 5'-AMP prevails in this case mainly at low 5'-AMP concentrations.

Table 2 shows the effect of glycogen – 5'-AMP combinations on the same preparation under two different experimental conditions. It was found that under both experimental conditions glycogen had a protective effect which was increased by the addition of 5'-AMP, though 5'-AMP alone had no effect on the phosphorylase – antiphosphorylase reaction. The protective action of glycogen was more expressed at a low 5'-AMP level and in the presence of magnesium than without magnesium. The effect of the order of addition of glycogen and 5'-AMP to phosphorylase depends on the experimental conditions. While without magnesium at a high 5'-AMP level the same effect could be obtained independently of the

Table 1

Effect of G-1-P and 5'-AMP combinations on the inhibition of phosphorylase by antibody

0.3 ml of phosphorylase (3.5 μg) was equilibrated for 5 minutes with the first then for another 5 minutes with the second component as shown in the Table, followed by a preincubation with 0.3 ml of antibody (25 μg of serum globulin) or diluent for 20 minutes. Enzyme reaction was started by adding the rest of the substrate-activator mixture (0.2 ml). In 1 ml total volume: 0.037 M G-1-P, 0.4 per cent glycogen, 0.012 M glycerophosphate, 60 mg per cent BSA, 1.2×10^{-3} M mercaptoethanol, 1.2×10^{-3} M EDTA, pH 6,7, temperature 30°C

	1×10^{-4} M 5'-AMP		1×10^{-3} M 5'-AMP	
Preincubation of phosphorylase with	inhibition per cent	change in inhibition per cent	inhibition per cent	change in inhibition per cent
∅	40.8	∅	39.2	∅
G-1-P	43.7	7.1	42.0	7.1
G-1-P, 5'-AMP	45.8	12.3	41.4	5.6
5'-AMP, G-1-P	43.7	7.1	40.1	2.3
5'-AMP	41.2	∅	38.1	−2.8

Table 2

Effect of glycogen and 5'-AMP combinations on the inhibition of phosphorylase by antibody

The mixture contained 0.084 M NaCl, other conditions were the same as shown in Table 1

	10^{-3} M 5'-AMP, 10^{-3} M EDTA		2×10^{-5} M 5'-AMP, 2×10^{-2} M Mg	
Preincubation of phosphorylase with	inhibition per cent	changes in inhibition per cent	inhibition per cent	changes in inhibition per cent
∅	52.8	∅	61	∅
glycogen	49.5	− 6.25	52.6	−13.8
5'-AMP	53	∅	61.9	∅
glycogen, 5'-AMP	46.3	−11.5	49.0	−19.7
5'-AMP, glycogen	47	−11.0	52.7	−13.6

order of addition, in the presence of magnesium, at a low 5'-AMP level, 5'-AMP enhanced the protective action of glycogen only when added to the enzyme after glycogen.

Table 3 shows the effect of glycogen + G-1-P combinations. G-1-P alone enhances, glycogen reduces inhibition and glycogen neutralizes the sensitizing effect of G-1-P.

Table 3

Effect of glycogen and G-1-P combinations on the inhibition of phosphorylase by antibody

Experimental conditions were the same as indicated in Table 1. 5'-AMP concentration: 1×10^{-3} M

Preincubation of phosphorylase with	Inhibition per cent	Change in inhibition per cent
∅	54	∅
G-1-P	59.4	9.2
glycogen	46.7	−14.0
glycogen, G-1-P	46.8	−13.6
G-1-P, glycogen	45.2	−16.5

Table 4

Effect of preincubation with substrate-activator combinations on the inhibition of phosphorylase by antibody

For experimental conditions see Table 1, except 0.025 M glycerophosphate, 0.005 M TRIS, 0.075 NaCl, 1×10^{-3} M 5'-AMP

Preincubation of phosphorylase with	Inhibition per cent	Change in inhibition per cent
∅	59.5	0
5'-AMP	59.9	0
glucose-1-P	62.5	+ 5
glucose-1-P, 5'-AMP	71.2	+19.8
glycogen	51.5	−13.4
glycogen, 5'-AMP	41.5	−30.3
glycogen, 5'-AMP, glucose-1-P	39.5	−33.6

All these effects using another crystalline enzyme preparation are shown in Table 4, supplemented by the overall effect of the whole substrate and activator when the antibody acts on a functioning enzyme. The protective effect was found to be the greatest in the latter case.

Discussion

It has been reported for several enzymes that preincubation with the substrate or with the coenzyme protects the enzyme from the inactivating action of the antibody. This protective action is usually the greatest when the antibody acts

upon a functioning enzyme. The protective effect of the substrate or of the coenzyme has recently been explained by a change in enzyme conformation rather than by competition (Zamecnik, Lipmann, 1947; Krebs, Najjar, 1948; Cinader, 1953; Samuels, 1961, 1963; Bussard, 1963). The substrate or the coenzyme causes a change in the conformation of the enzyme (Koshland, 1958, 1963; Elődi, Szabolcsi, 1959; Szabolcsi, Biszku, 1961) and there are some data to support the assumption that in the antigen-antibody reaction conformation changes of both components of the reaction do occur (Harshman et al., 1963; Kaplan, White, 1963; Pollock, 1963). Levine (1962) has pointed out that the antigenic properties of proteins depend on their conformation. According to Monod's hypothesis (1963, 1965) the so-called allosteric enzymes may undergo various allosteric transitions due to the interaction between the subunits which also might be considered as a special type of enzyme conformation change.

Experimental data published in recent years prove that there is an interaction between the substrate and the activator binding sites of phosphorylase presumably due to a change in enzyme conformation, and that phosphorylase is also able to undergo certain allosteric transitions (Helmreich, Cori, 1964, 1964a; Lowry et al., 1964; Madsen, 1964; Morgan, Parmeggiani, 1964; Ullmann et al., 1964; Jókay et al., 1965). We believe that most of the data in this report might be explained by the ability of phosphorylase to enter under various experimental conditions different conformational states.

Our studies on the effect of antibodies produced against rabbit muscle phosphorylase led to the conclusion (Jókay, 1963) that the inhibitory effect of the antibody on the enzyme cannot be ascribed to aggregate formation. This conclusion has been recently confirmed by the experiments of Michaelides et al. (1964) with univalent antiphosphorylase fragments. These authors have studied in a comprehensive work the interaction between phosphorylase and antiphosphorylase mainly with univalent antibody fragments and in the presence of magnesium. In the present work only non-fragmented antibodies were used and contrary to Michaelides phosphorylase activity was measured in the direction of glycogen synthesis. In this way new data can be obtained with respect to the effect of G-1-P and conclusions may also be drawn from the study of the role of magnesium and of temperature.

The study of the kinetics of phosphorylase action has shown that inhibition by antibody is of a non-competitive character with respect to glycogen and G-1-P both with and without magnesium. Nevertheless with respect to 5'-AMP this is true only without magnesium, because in the presence of magnesium a mixed type of inhibition develops in which both V_{max} and K_M are changed. This mixed type inhibition observed in the presence of magnesium will be dealt with in our next paper.

As antiphosphorylase is bound practically irreversibly to phosphorylase, the strength of the enzyme-antibody bond is several orders of magnitude higher than the strength of the enzyme-substrate or of the enzyme-activator bonds and

consequently no unequivocal conclusions can be drawn from the non-competitive nature of the inhibition.

Preincubation with the substrates (glycogen, G-1-P) and the activator may influence the inhibition of the enzyme by the antibody in various ways. While G-1-P alone sensitizes the enzyme to the inhibiting action of the antibody, glycogen displays only a slight protective action. The combined effect of the substrate components and of the activator is also interesting. While 5'-AMP alone has no significant effect on the inhibition by the antibody under the given experimental conditions, when added after glycogen it decreases and after G-1-P it enhances the inhibitory effect of the antibody. Thus there is a qualitative difference in the action of 5'-AMP depending on the preincubation of the enzyme with G-1-P or glycogen, respectively.

On the other hand, while G-1-P both alone and with 5'-AMP enhances the inhibitory effect of the antibody, it has a protective action when added together with glycogen and with 5'-AMP. The inhibitory effect of the antibody is the smallest on such a functioning enzyme.

The effect of preincubations with substrate-activator combinations may be explained in all probability by the induction of conformation changes – allosteric transitions – in the enzyme by the substrate or activator which affect the degree of inhibition by the antibody. This is true primarily for G-1-P and 5'-AMP, while high molecular weight glycogen might hinder also sterically the effect of antibody.

According to Helmreich and Cori (1964) muscle phosphorylase may undergo some allosteric transitions. The data presented here suggest that the substrate components (glycogen and G-1-P) induce different allosteric transitions of the enzyme. This is manifest not only in the fact that glycogen alone has a protective and G-1-P rather a sensitizing effect with respect to the inhibition of phosphorylase by antibody, but also in the observation that the activator intensifies in both cases – in a positive or negative sense – the action on the enzyme of the previously added substrate constituent. This indicates that the effect of the activator on the steric configuration of the enzyme depends also on the actual conformation of the enzyme. It seems from the above data that this finding is valid within certain limits for both substrates investigated. The degree of protection afforded by glycogen may also be different under different conditions. This holds also for G-1-P which may enhance or reduce the inhibiting action of the antibody depending whether or not the enzyme has been preincubated with glycogen and 5'-AMP prior to the addition of G-1-P. It should be also mentioned that when crude muscle extract is used as enzyme preparation preincubated with G-1-P it results only in a slight protective action against antibody inhibition. No sensitizing effect of G-1-P was observed with crude muscle extracts.

Substrate-activator combinations had a similar effect on the enzyme as that reported in an earlier communication (Jókay et al., 1965) dealing with the inactivation of phosphorylase-b by SH reagents. In this latter case G-1-P had a definite sensitizing action, glycogen alone displayed a slight protection, while the protective

effect of 5'-AMP was more pronounced with inactivation by PCMB than with inactivation by NEM. In the case of PCMB and NEM inactivation, the effects of glycogen + 5'-AMP combination, glycogen + G-1-P, and of the whole substrate agreed qualitatively with those found in the case of antibody inhibition. The effects obtained with G-1-P + 5'-AMP combinations are different in the case of SH-reagents and antibody, respectively, but because of the different experimental procedure employed, these results cannot be compared. While inactivation with SH reagents was carried out by adding the substrate and the activator simultaneously to the enzyme (this, however, was not emphasized in the pertaining communication), in the study of the inhibitory effect of the antibody the enzyme was preincubated first with one and then with the other component. The order of addition of the agents may be significant in this case.

The sensitizing effect of G-1-P on antibody inhibition is rather interesting. It should be noted, however, that physiologically G-1-P is an end product of the reaction rather than a substrate, and this may perhaps explain the opposite effect with glycogen from this point of view. Samuels (1961) reported a similar phenomenon, namely that the phosphate ion – the competitive inhibitor of 5'-AMP deaminase – enhances the inhibition of the enzyme by the antibody.

Our results are in agreement with those obtained by Michaelides et al. (1964) on the protective action of preincubation with glycogen–5'-AMP combinations and with the whole substrate and also on the mixed type inhibition of inhibited phosphorylase for 5'-AMP. The fact that we were unable to demonstrate the change of the K_M of glycogen for the inhibited enzyme may be attributed in all probability to our working with whole instead of univalent antibody.

In summary the following explanations are suggested for the above data:

1. The effect of the substrate and of the activator on the enzyme is determined – with respect to the inhibition by the antibody – by the actual conformation of the enzyme;

2. The degree of the inhibitory effect of the antibody depends on the actual conformation of the enzyme.

References

Bussard, A. E. (1963) Ann. New York Acad. Sci. *103* 890
Cinader, B. (1953) Biochem. Soc. Symp. Cambridge, England *10* 16
Előd i, P., Szabolcsi, G. (1959) Nature *184* 56
Fischer, E. H., Krebs, E. G. (1958) J. Biol. Chem. *231* 65
Harshman, S., Robinson, J. P., Najjar, V. A. (1963) Ann. New York Acad. Sci. *103* 688
Helmreich, E., Cori, C. F. (1964) Proc. Nat. Acad. Sci. *51* 131
Helmreich, E., Cori, C. F. (1964 a) Proc. Nat. Acad. Sci. *52* 647
Illingworth, E., Cori, C. F. (1953) Biochem. Preparations *3* 1
Jókay, I. (1963) Acta Microbiol. Acad. Sci. Hung. *10* 163
Jókay, I., Damjanovich, S., Tóth, S. (1965) Arch. Biochem. Biophys. *112* 471
Kaplan, N. O., White, S. (1963) Ann. New York Acad. Sci. *103* 835

Krebs, E. G., Najjar, V. A. (1948) J. Exptl. Med. *88* 569
Koshland, D. E., Jr. (1958) Proc. Nat. Acad. Sci. *44* 98
Koshland, D. E., Jr. (1963) Cold Spring Harbor Symp. Quant. Biol. *28* 473
Levine, L. (1962) Fed. Proc. *21* 711
Lowry, O. H., Schulz, D. W., Passonneau, J. V. (1964) J. Biol. Chem. *239* 1947
Madsen, N. B. (1964) Biochem. Biophys. Res. Commun. *15* 390
Monod, J., Changeaux, J. Jacob, F. (1963) J. Mol. Biol. *6* 306
Monod, J., Wyman, J., Changeaux, J. (1965) J. Mol. Biol. *12* 88
Morgan, H. E., Parmeggiani, A. (1964) J. Biol. Chem. *239* 2440
Michaelides, M. C., Sherman, R., Helmreich, E. (1964) J. Biol. Chem. *239* 4171
Pollock, M. R. (1963) Ann. New York Acad. Sci. *103* 989
Samuels, A. J. (1961) Biophys. J. *1* 437
Samuels, A. J. (1963) Ann. New York Acad. Sci. *103* 858
Szabolcsi, G., Biszku, E. (1961) Biochem. Biophys. Acta *48* 335
Taussky, H. H., Schorr, E. (1953) J. Biol. Chem. *202* 675
Ullmann, A., Vagelos, P. R., Monod, J. (1964) Biochem. Biophys. Res. Commun. *17* 86
Wada, T., Nomura, M. (1958) J. Biochem. *45* 639
Zamecnik, P. C., Lipman, F. (1947) J. Exptl. Med. *85* 395

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 279—286 (1966)

On the Mechanism of Enzyme Inhibition by Antiphosphorylase

II. Effect of Magnesium and Temperature

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(Received May 5, 1966)

The mixed type inhibition in the presence of magnesium by antibodies formed in roosters against rabbit muscle phosphorylase-b may be split into two components, one of which is non-competitive, the other one competitive for 5'-AMP. This latter is due to the fact that at low 5'-AMP level magnesium has a more pronounced enhancing effect on the activity of the non-inhibited enzyme than on that of the partially inhibited one. Thus at a low 5'-AMP level magnesium enhances the enzyme inhibiting effect of the antibody. Inhibition is more pronounced if magnesium is added to the enzyme prior to the antibody, than if added later.

At the temperatures investigated (38 °C, 30 °C, 20 °C) in the presence of magnesium K_M for 5'-AMP decreases more markedly with decreasing temperatures in the case of the non-inhibited enzyme than in the case of the partially inhibited one. Thus in the presence of magnesium and at a low 5'-AMP level enzyme inhibition by the antibody will be greater at lower than at higher temperature.

These data suggest that the antibody bound to the enzyme forms a rigid structure and inhibits the conformation changes of the enzyme in response to the action of various factors (magnesium, low temperature).

Introduction

As stated in our previous communication inhibitory effect on phosphorylase of antiphosphorylase in the presence of EDTA is non-competitive with respect to 5'-AMP, and in the presence of magnesium this inhibition is of a mixed type (Jókay et al., 1966). As according to Madsen's observation (private communication to Helmreich and Cori, 1964) magnesium acts at a low 5'-AMP level as an activator on phosphorylase-b, it was believed that a more detailed study of the effect of magnesium on the phosphorylase-antiphosphorylase reaction may be of interest. Studies on the effect of the temperature have led us to the conclusion that in certain respects lower temperatures have a similar effect as the presence of magnesium, at least with respect to the 5'-AMP binding of phosphorylase-b and to the inhibiting effect of antibody.

The experimental procedure was the same as described in our previous communication (Jókay et al., 1966).

Results

Effect of magnesium. – As the inhibiting effect of antiphosphorylase could be influenced by changing the 5'-AMP concentration only when magnesium was present, the activity of both non-inhibited and inhibited phosphorylase was assayed at low 5'-AMP levels as a function of magnesium concentration. Magne-

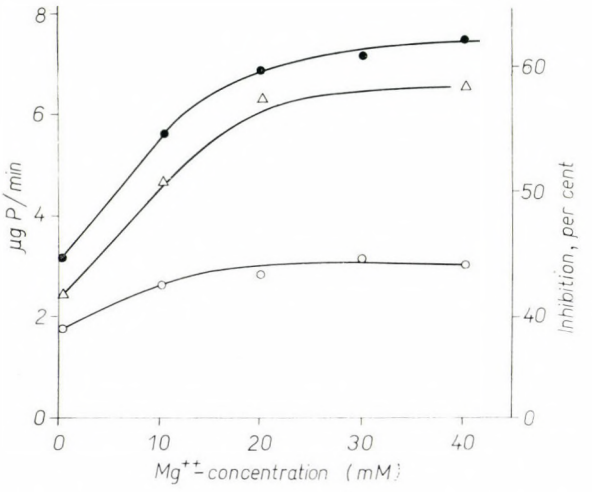

Fig. 1. Phosphorylase activity and inhibition as a function of magnesium concentration. 0.2 ml phosphorylase (6.2 μg) equilibrated with 0.2 ml magnesium acetate, and preincubated with 0.2 ml antibody (46 μg of globulin) or diluent for 20 min. The total volume of 0.8 ml contained: 0.037 M G-1-P, 0.5 per cent glycogen, 2×10^{-5} M 5'-AMP, 0.01 M glycerophosphate, 0.075 M NaCl, 1.10^{-3} M mercaptoethanol, 50 mg per cent BSA, pH 6.7, temperature 30 °C. ●—● non-inhibited phosphorylase. ○—○ inhibited phosphorylase. △—△ per cent of inhibition

sium—at a low 5'-AMP level— significantly enhanced phosphorylase activity and this increase in activity was greater for the non-inhibited enzyme than for the inhibited one (Fig. 1). Consequently the percentage inhibition caused by the antibody increased upon addition of magnesium. In the present case 20–25 mM of magnesium was found to be the optimum concentration from the point of view of antiphosphorylase inhibition.

In the following the combined effect of magnesium and 5'-AMP on the inhibition caused by the antibody was studied at low and high 5'-AMP levels (Table 1). At a low 5'-AMP concentration the presence of magnesium enhanced

Table 1

Combined effect of magnesium and 5'-AMP on the antibody inhibition of crystalline phosphorylase

0.3 ml of phosphorylase (2.9 μg) was equilibrated with the first component for five minutes, followed by equilibration with the second component for five minutes and preincubation with 0.3 ml of antibody (22 μg of globulin) or diluent for 20 minutes and equilibration for five minutes with Mg or 5'-AMP as indicated in the Table. Enzyme reaction was started with a mixture of 0.2 ml of G-1-P and glycogen. The total volume of 1 ml contained 0.037 M G-1-P, 0.5 per cent glycogen, 0.02 M magnesium acetate, 0.012 M glycerophosphate, 0.084 M NaCl, 1.2×10^{-3} M mercaptoethanol, 60 mg per cent BSA, ph 6.7, temperature 30 °C

Order of addition of the components			Inhibition, per cent	
			2×10^{-5} M 5'-AMP	2×10^{-3} M 5'-AMP
∅	AP	5'-AMP	43.7	47.5
5'-AMP	AP	∅	45.0	47.8
∅	AP	Mg, 5'-AMP	53.2	48.3
∅	AP	5'-AMP, Mg	53.2	47.4
5'-AMP	AP	Mg	53.9	47.4
Mg	AP	5'-AMP	57.8	48.8
5'-AMP, Mg	AP	∅	57.2	47.3
Mg, 5'-AMP	AP	∅	59.8	48.6

the inhibiting effect of the antibody and this effect was more marked when magnesium was added to the enzyme prior to the addition of the antibody. When magnesium was added after the antibody, inhibition was enhanced to a lesser extent, in other words, in this case the activity of the inhibited enzyme was higher than with magnesium added to the enzyme before the antibody. Under the present experimental conditions almost identical effects were obtained irrespective of the order of addition of 5'-AMP and magnesium, and the degree of inhibition was affected primarily by the order of addition of magnesium and antibody, respectively. The greater inhibition at a low 5'-AMP level could be compensated by raising the concentration of 5'-AMP.

Effect of temperature. – Figs 2 and 3 show the activity of partially inhibited and non-inhibited phosphorylase for constant quantities of enzyme and antibody vs the concentration of 5'-AMP at various temperatures. As may be seen – at the temperatures tested – the optimum temperature of the enzyme is also a function of the concentration of the activator. In this respect inhibited phosphorylase behaves differently from the non-inhibited enzyme. While at a high activator concentration a temperature decrease from 38 °C to 20 °C results in an almost identical decrease in the activities of both the inhibited and non-inhibited enzymes, at a low activator level (e.g. at 10^{-5} M 5'-AMP) the activity of the non-inhibited enzyme is higher at 20 °C than at 38 °C, while for the inhibited enzyme the reverse is true (Table 2). Consequently the percentage inhibition caused by the antibody

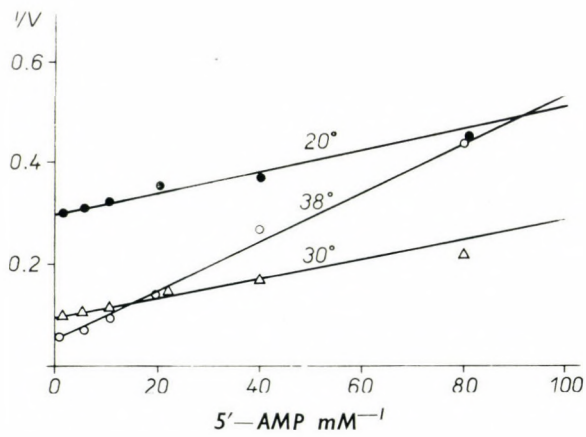

Fig. 2. Phosphorylase-b activity as a function of 5'-AMP concentration at various temperatures. 0.2 ml phosphorylase (5 μg) preincubated with 0.2 ml diluent for 30 minutes. The total volume of 0.8 ml contained: 0.067 M G-1-P, 0.5 per cent glycogen, 0.025 M magnesium acetate, 0.025 M glycerophosphate, 1×10^{-3} M mercaptoethanol, 50 mg per cent BSA, pH 6.7. Magnesium was added to the diluent. ●—● 20 °C. △—△ 30 °C. ○—○ 38 °C

Table 2

Phosphorylase activity and inhibition at 38° and 20 °C at various 5'-AMP concentrations

Experimental conditions the same as shown in Figs 2 and 3

5'-AMP concentration	Phosphorylase activity, μg P/min				Inhibition per cent	
	Non-inhibited enzyme		Inhibited enzyme			
	38°	20°	38°	20°	38°	20°
∞	18.3	3.33	10.0	1.85	43.5	44.5
5×10^{-5} M	6.67	2.9	3.63	1.38	45.5	52.3
2×10^{-5} M	3.39	2.42	1.83	1.00	46	58.6
1×10^{-5} M	1.85	1.92	0.99	0.69	46.5	64.1

at constant quantities of both enzyme and antibody and at a low 5'-AMP level will be higher at low than at high temperatures. At a high activator concentration, on the other hand, this difference caused by temperature increase is counteracted. As may be seen from Table 3 there is a very significant decrease in the K_M for 5'-AMP of phosphorylase with decreasing temperature. However, in the case of the inhibited enzyme this decrease is considerably less.

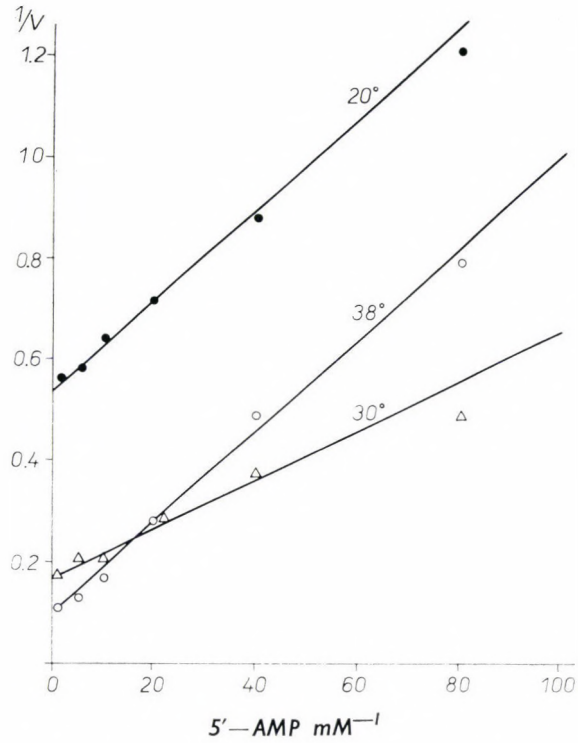

Fig. 3. Activity of partially inhibited phosphorylase-b as a function of 5'-AMP concentration at various temperatures. 0.2 ml phosphorylase (5 μg) preincubated with 0.2 ml antibody (37 μg immune serum globulin) for 30 minutes. Otherwise the same as in Fig. 2. ●—● 20 °C. △—△ 30 °C. ○—○ 38 °C

Table 3

K_M value for 5'-AMP ($M \times 10^{-5}$) of phosphorylase-b at various temperatures

Experimental conditions the same as shown in Figs 2 and 3

	Non-inhibited phosphorylase	Inhibited phosphorylase	Change in K_M for 5'-AMP in the presence of antibody
38°	8.3	9.25	1.11 ×
30°	2.2	3.0	1.37 ×
20°	0.75	1.7	2.27 ×

Discussion

Mixed type inhibition in the presence of magnesium is the result of the greater decrease in the K_M for 5'-AMP of the non-inhibited enzyme than of the inhibited one in the presence of magnesium, that is to say, at a low 5'-AMP concentration the activity of the non-inhibited enzyme is enhanced by magnesium to a higher extent than that of the inhibited one. The effect of magnesium on the non-inhibited enzyme is interpreted by a stereostructural change of phosphorylase in the presence of magnesium resulting in a capacity of the enzyme to bind 5'-AMP more strongly. To explain the effect of magnesium on the inhibition by antibodies three factors must be taken into consideration: 1. At a low 5'-AMP level the activity of the inhibited enzyme increases to a lesser extent in the presence of magnesium (enzyme inhibition is greater) when the magnesium is added prior to the antibody to the enzyme than when addition is of a reversed order. This suggests a magnesium induced change in the conformation of phosphorylase which results in a "stronger" inhibition of the enzyme by the antibody than without magnesium; 2. At a low 5'-AMP level the activity of the non-inhibited enzyme is enhanced by magnesium to a higher extent than that of the inhibited one, indicating that the antibody combines with the enzyme giving rise to a rigid structure which is no longer capable of reacting by a stereostructural change to the effect of magnesium; 3. As these effects are manifest only at low 5'-AMP level, it is suggested that the mixed type of inhibition observed in the presence of magnesium may in fact be split up into two components, namely into a component which is competitive and another which is non-competitive for 5'-AMP. The existence of this latter follows from the fact that magnesium enhances the inhibition caused by the antibody at a low 5'-AMP concentration and this enhanced inhibition can be counteracted by increasing the 5 -AMP level. The effect of temperature is also of interest. At a low temperature the capacity of phosphorylase to bind 5'-AMP is enhanced. This may serve as an explanation for the observation that under the conditions used the optimum temperature of phosphorylase-b depends also on the 5'-AMP concentration. Since with the inhibited enzyme the decrease of K_M for 5'-AMP is less than with the non-inhibited enzyme, at a low 5'-AMP level decreasing the temperature results in an increased inhibition. This greater inhibition at low temperature may be counteracted by higher concentrations of the activator, thus this inhibition is of a competitive nature with respect to 5'-AMP.

On the other hand inhibition is enhanced at a lower temperature (20 °C) if the concentration of 5'-AMP is reduced. This enhancement of inhibition may be practically counteracted by increasing the temperature (38 °C). Thus the degree of the enzyme inhibiting effect of the antibody depends on both the activator concentration (5'-AMP, magnesium) and on the temperature.

There is therefore a marked similarity between the effect of magnesium and of temperature decrease. Both factors make the enzyme-activator bond stronger. It seems that both magnesium and low temperature favour a certain stereo-

structural state – allosteric transition – of phosphorylase for which the binding of 5'-AMP is the most advantageous. The antibody on the other hand stabilizes the enzyme in another, different conformation which involves a reduced capacity for binding 5'-AMP (similarly as in the absence of magnesium). In this way a rigid structure is formed with the antibody which prevents the conversion of the enzyme under the effect of various factors (magnesium, low temperature) into an other conformation which would be more favourable for binding the activator.

Kaplan and White (1963) in their study of the interaction between lactate dehydrogenase and antibody at various temperatures have arrived to a similar conclusion, and they believe that possibly the binding of the antibody molecules to dehydrogenase results in a far more rigid structure which is less sensitive to changes of temperature.

When investigating the temperature dependence of phosphorylase-a activity Helmreich and Cori (1964) have found that "within a critical temperature range the catalyst assumes a different conformational state". According to these authors and to Lowry et al. (1964) the optimum temperature of phosphorylase-a depends on the concentration of the substrate used and on the presence of the activator.

According to Monod's theory the allosteric transitions of the allosteric enzymes may be attributed to a change in the interaction between the subunits. It seems probable that the changes in the kinetic parameters of phosphorylase-b under the effect of magnesium and low temperature may also be explained by the same theory.

References

Helmreich, E., Cori, C. F. (1964) Proc. Nat. Acad. Sci. *52* 647

Jókay, I., Damjanovich, S., Tóth, S. (1966) Acta Biochimica et Biophysica Hung. preceding paper

Kaplan, N. C., White, S. (1963) Ann. New York Acad. Sci. *103* 835

Lowry, O. H., Schulz, D. W., Passonneau, J. V. (1964) J. Biol. Chem. *239* 1947

Monod, J., Wyman, J., Changeaux, J. (1965) J. Mol. Biol. *12* 88

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 287—292 (1966)

Cross Formations in the Isotropic Band

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(Received January 22, 1966)

Cross structure has been demonstrated in the isotropic band of the muscle fibril of the frog sartorius muscle by microdensitometric method.

Introduction

Hall, Jakus and Schmitt (1946) described the axial periodicity of the substructural longitudinal formations of the striated muscle fibre. Draper and Hodge (1949) have found a regularly repeating thread system. A well pronounced cross structure system can be seen at full length in the sarcomere in Fig. 5 of the paper of Edwards et al. (1956). The importance of this structure has been emphasized by Ernst and Benedeczky (1962). Cross structure has been found by Huxley and Pepe (1964) in the isotrop band of the muscle after anti-actin treatment. The present study reports a structure system perpendicular to the longitudinal axis of the muscle fibril in the isotropic band of the cross striated muscle fibril. Some comments will also be made on the discrepancy between our finding and the sliding model.

Methods

The microdensitometer makes possible the qualitative and quantitative evaluations of electronmicrographs of high resolution. In electronmicroscopy for the intensity (I) of the electron beam falling on the photographic plate, the equation of $I = I_0 \exp(-sw)$ (Hall, 1954) is valid with certain restrictions (Zeitler and Bahr, 1952), where I_0 is the intesity of the electron beam falling on the object; w the mass thickness of the object (gcm^{-2}) at the site where the beam falls in; s the scattering cross section related to the mass unit (cm^2g^{-1}) which can be regarded as the phenomenological constant of the electron microscope (Hall, 1955).

Intensity of the electron beam falling on the photographic slide causes photographic density D (blackening) at the appropriate site of the negative. The intensity of the electron beam is greatly decreased by places of high densities

in the object, but it is hardly influenced by the places of small densities. Thus, the places of high densities in the objects have small values of photographic densities in the electron micrograph. This is registered by the densitometer.

In the experiments a KIPP (Holland) densitometer was used. The instrument continually moves the electronmicrograph perpendicularly to the scanning light beam, while the photographic density is registered automatically. The maximum

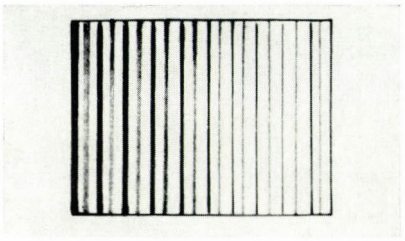

Fig. 1. Model for the densitometric evaluation of periodical structures

resolving power of the microdensitometer is 0.005 mm, in the course of our measurements it was not lower than 0.05 mm.

A line model was prepared for the microdensitometric investigations of periodical structures. The model consisted of parallel lines (Fig. 1) which followed each other at equal distances. The thicknesses and photodensities of these lines were different. Of the model microdensitometric diagrams were made. Fig. 2 illustrates the microdensitometric diagrams made of the negative of Fig. 1. The following widths were used for the illuminating diaphragm: 0.04 mm in Fig. 2a; 0.06 mm in Fig. 2b, and 0.1 mm in Fig. 2c. It can be concluded from the diagrams that the picture of the structure does not change by changing the width of the illuminating diaphragm in the given intervals.

Sartorius muscle of the frog was fixed in a 10 per cent solution of formaldehyde for 12 hours, after that in osmium tetroxide for 1 hour. It was embedded in Araldite, and photographs were taken of the longitudinal sections with a TESLA BS 242 electron microscope. The electron micrographs to be investigated were further magnified to get a final magnification of about 30,000 ×. The photographic plate was placed in the apparatus in such a way that the scanning light beam measured the photometric densities of the transversal lines between two myofilaments. As the adjustment of the scanning light beam needs great accuracy, it was performed in a binocular microscope. Densitometric diagrams of muscle fibrils were prepared by using a diaphragm-width of 0.1 mm and a diaphragm-length of 1 mm.

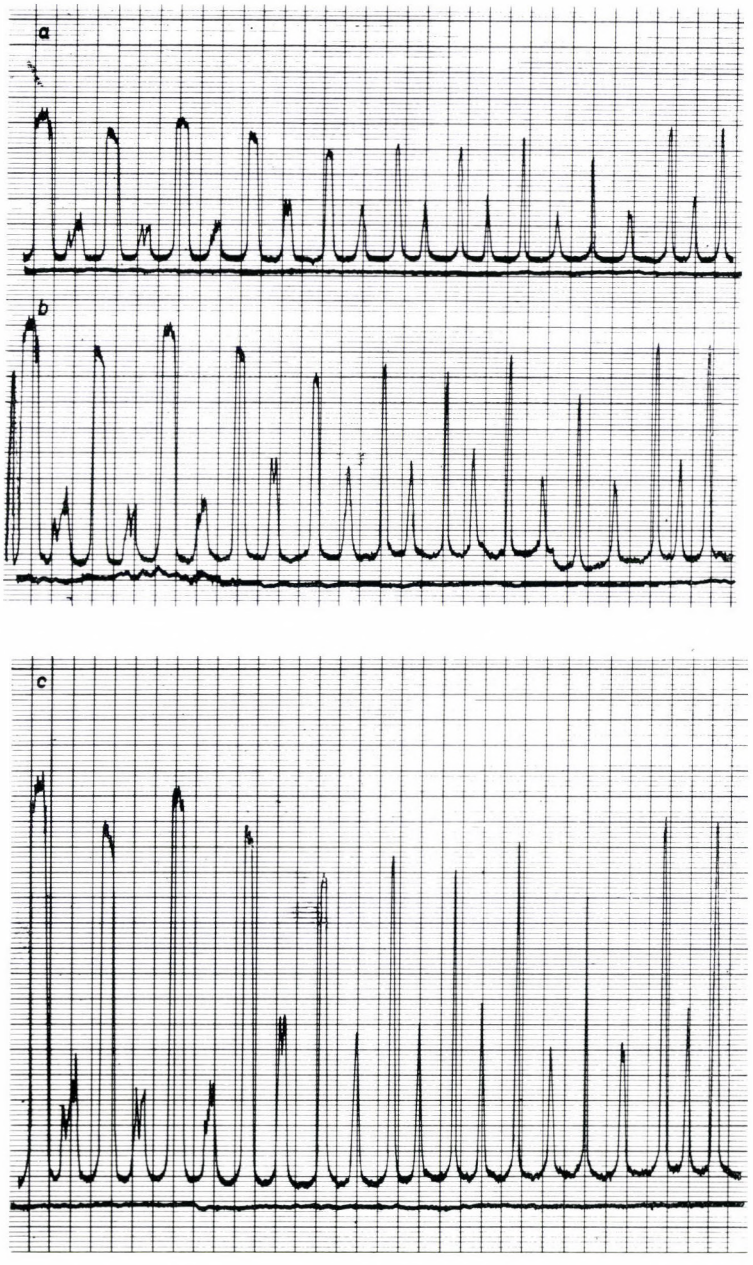

Fig. 2. The densitometric picture of the model. Widths of the diaphragms are: a. 0.04 mm; b. 0.06 mm; c. 0.1 mm

Results and Discussion

The ultrastructure of the isotrop band of the striated muscle fibril was investigated. Fig. 3 illustrates the positive picture of the micrograph evaluated by densitometry, the arrow shows the path of light. The transversal lines appearing periodically are well seen.

The microdensitometric curve obtained is shown in Fig. 4. The curves suggest the presence of a system which consists of periodically occurring lines of high densities corresponding to the cross-striations. The distance of one maxi-

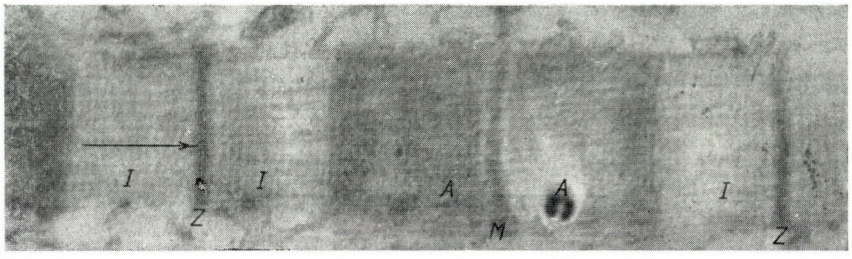

Fig. 3. Frog's sartorius muscle. Magnification 30,000 ×. The arrow shows the path of the scanning light beam of the microdensitometer

mum from the other proved to be constant. The numerical values of these can also be determined, because the microdensitometer provides a network on the recorder paper in accordance with the 0.5 mm distance of the negative. Thus, the distance of two maxima can be measured on the diagrams. The distance of the period can be calculated from this, if the magnification is known. According to our measurements the values of this is about 370 Å. In Bear's paper (1945) the value of the period length of the axial periodicity is 360 Å or 420 Å; Huxley (1953) has measured 420 Å. According to Lemazhikhin et al. (1964) the length of period changes, depending on the method of fixation for electron microscopy. The value measured by us is in good agreement with that of Lemazhikhin et al. (1964). In compliance with their measurements the length of period is 350 Å in the case of fixation with formaldehyde, and post-fixation with osmium tetroxide and phosphotungstic acid, and 380 Å in the case of fixation with formaldehyde. Our measurements give a result between these two values.

The fading of the peaks can be observed to a certain extent in the microdensitometric diagrams. The reason for this is a fading zone surrounding the transversal lines. The fading zone originates from the phase contrast, in the electron microscope and from the electron scattering effect in the photographic emulsion. However, the peaks are sharp enough in most of the cases.

The results presented in this paper indicate, that the diagram obtained represents a structural system in the isotropic band, which is perpendicular to the longitudinal axis of the muscle fibril.

Several references can be found in the literature, indicating that the myofilaments are bound to each other by a thread system in the anisotropic band of the

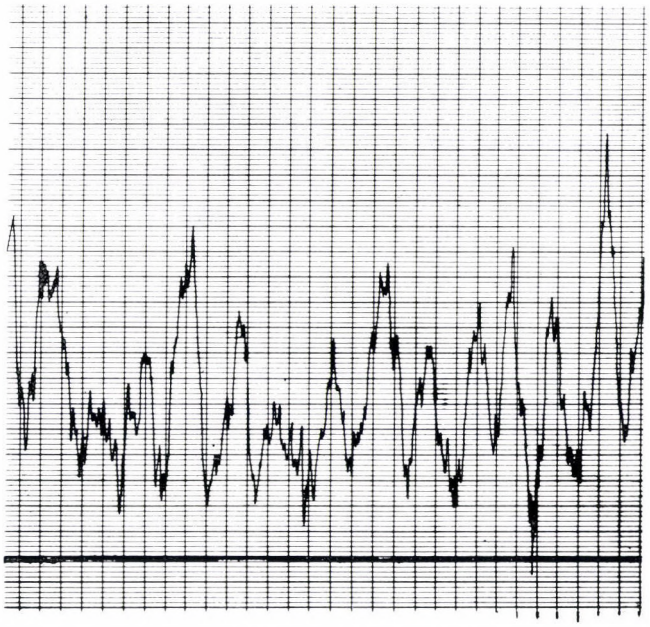

Fig. 4. The densitometric picture of the isotropic band of the muscle fibril. The width of the diaphragm is 0.1 mm; the length 1 mm

muscle fibril (Huxley, 1960; Hodge, 1956; Philpott and Szent-Györgyi, 1955; Reedy et al., 1965). Considering the data above, we demonstrated that such structures occur in the isotropic band, too. This structure makes impossible the sliding into each other of the "two different kinds of filament patterns" and this is against the "sliding" hypothesis of Huxley and Hanson (1957).

Acknowledgement

Author expresses his thanks to Professor E. Ernst for encouragement and help in this work.

References

Bear, R. S. (1945) J. Am. Chem. *67* 1625
Draper, M. H., Hodge, A. J. (1949) Nature *163* 576
Edwards, G. A., Ruska, H., de Souza Santos, P., Vallejo-Freire, A. J. (1956) J. Biophys. and Biochem. Cytol. *2* Suppl. 143
Ernst, E., Benedeczky, St. (1962) Acta Physiol. Acad. Sci. Hung. *22* 211
Hall, C. E., Jakus, M. A., Schmitt, F. O. (1946) Biol. Bull. *90* 32
Hall, C. E. (1954) J. Appl. Phys. *22* 655
Hall, C. E. (1955) J. Biophys. Biochem. Cytol. *1* 1
Hodge, A. J. (1956) J. Biophys. Biochem. Cytol. *2* Suppl. 131
Huxley, H. E., Hanson, J. (1957) Symp. Soc. Exp. Biol. *9* 229
Huxley, H. E., Hanson, J. (1960) The molecular basis of contraction, in The structure and function of muscle. Bourne, G. H. (editor), Academic Press, New York and London. Vol. 1, p. 183
Huxley, H. E. (1953) Proc. Roy. Soc. London. *141* B. 59.
Huxley, H. E., Pepe, F. A. (1964) Antibody staining of separated thin and thick filaments of striated muscle, in Biochemistry of muscle contraction. Gergely, J., Little, Brown and Co. (Editor) Boston. p. 320
Lemazhikhin, B. K., Melnikov, L. A., Razumova, L. L., Frank, G. M. (1964) Biofizika *9*, 565
Philpott, D. E., Szent-Györgyi, A. G. (1955) Biochim. et Biophys. Acta *18* 177
Reedy, M. K., Holmes, K. C., Tregear, R. T. (1965) Nature *207* 1276
Zeitler, E., Bahr, G. F. (1952) J. Appl. Phys. *33* 847

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 293—298 (1966)

Structure of the Flight Muscle Fibrils of the Bee as Seen in the Polarizing Microscope

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(Received May 2, 1966)

Polarizing microscopic observations have been combined with the densitometric evaluation of the negatives. In this way we were able to compare the relative amount of the double refraction even in the case of very complicated striation pattern and low double refraction. We could demonstrate the double refracting character of the M-line, a question discussed since a long time. Similar measurements show that the "elongation (E-) zones" of extremely stretched myofibrils still show a considerable double refraction, indicating that they represent parts of the A-band.

The polarizing microscope was the first and for a long time the single tool of the ultrastructure research of muscle (Brücke, 1858; Schmidt, 1937). Valuable results have been achieved by its use. Moreover, in the present time, even in the possession of the electron microscope, we may expect valuable informations on the fine structure and macromolecular arrangement of the myofibrils.

Some earlier results obtained on the flight muscle of insects by polarizing microscopy have been published from our institute (Ernst and Béleczki, 1956; Ernst et al., 1956). One of these papers (Ernst et al., 1956) dealt with the possible mechanism of strong elongations, mentioning that the very long I-bands may be the result of the elongation of the filaments, by which substances could have been drawn out from the A-band. A more recent work (Kerner, 1966) reported solubility differences between moderately and highly stretched myofibrils. In this paper also polarizing microscopy was used.

In the last few years we published a series of papers on the question of the changes in the length of the A-bands of the flight muscle of the bee. The "two filaments model" of Huxley and Hanson involves the termination of the primary myofilaments at the A—I junctions, a supposal, from which the absolute constant length of this kind of filaments follows. We have found that until a critical sarcomere length of 5 μ (a value close to the "no overlap point" estimated on the basis of the sliding filaments theory) the A-band length seems fairly constant, but beyond this limit it increases by the own elongation of the primary myofilaments. In the course of this work we used the electron microscope (Garamvölgyi, 1963, 1965a and b, 1966), the interference microscope (Garamvölgyi et al., 1964) combined with densitometry and the phase contrast microscope (Garamvölgyi, 1965b, 1966), but until now we unfortunately had no reliable polarizing

microscopic evidence. Our difficulties arose from the very low double refraction of single myofibrils and from the relatively small areas of the different band structures which rendered the use of the conventional method of the determination of the double refraction very uncertain.

Methods

Myofibrils were isolated from the indirect thoracic muscle of the bee, by tearing muscle fibrils with needles on slides, in a medium of Pringle's solution. For the illumination a mercury vapour lamp 220/HBO 50 was used and the polarizing microscope was equipped with an automatic exposure time regulating apparatus of Carl-Zeiss Jena. The obtained negatives were evaluated by means of a "Schnellphotometer" of the same factory. The numbers on the vertical axis of the diagrams mark the values of the galvanometer deviation corresponding to the blacking of the negatives. In this way we were able to characterize the relative amount of the double refraction of the different bands and zones related to the baseline, but we did not determine the absolute double refraction values.

Results

The fibrils of the shortest i.e. about 3 μ sarcomere length found (Garamvölgyi et al., 1964; Garamvölgyi, 1966) show non-birefringent Z-lines, or C_z bands (Fig. 1), a phenomenon known from the earlier literature (Schmidt, 1937)

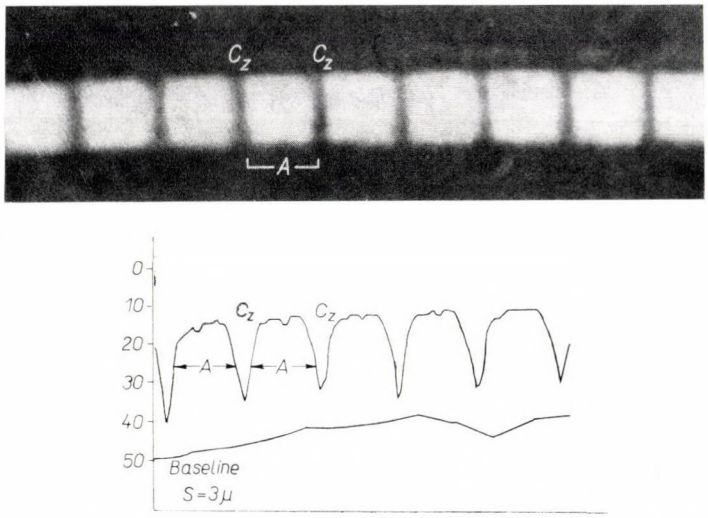

Fig. 1 *a:* Polarizing micrograph of a myofibril of a sarcomere length of about 3 μ. There are no double refracting Z-lines, the non-birefringence of their Z-lines (or C_z-bands) is in agreement with earlier observations. *b:* Densitometric curve of the same fibrils

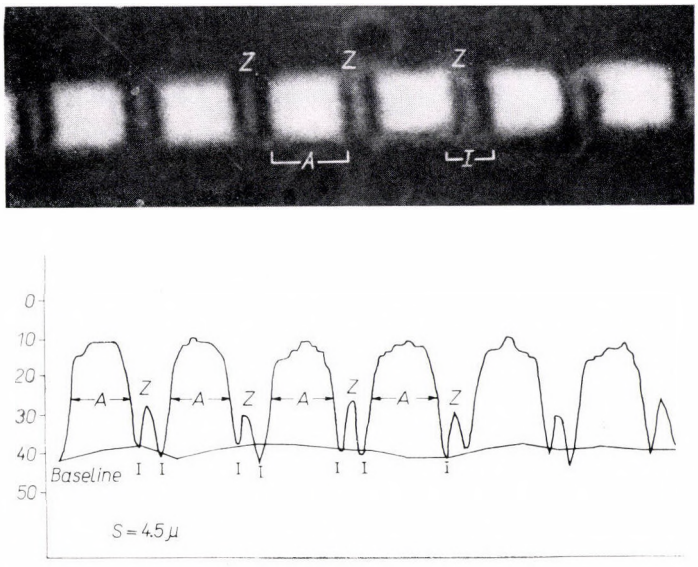

Fig. 2. *a:* Polarizing micrograph of a moderately stretched myofibril with a sarcomere length below the "no overlap point". There are double refracting Z-lines. The A-band seems quite homogeneous. *b:* Densitometric curve of the same fibrils. Note the practically missing double refraction of the I-bands

and also recently mentioned by Hodge (1955) and Hanson and Huxley (1956). Fibrils of greater sarcomere lengths, having well recognizable I-bands, show double refracting Z-lines, up to extreme sarcomere lengths (Figs 2, 3).

Fibrils of sarcomere lengths less than 5 μ show quite homogeneous A-bands, no H-zones could be recognized in the polarizing microscope. It seems that the structural differences of the different zones of the A-band are not sufficient enough to cause any visible optical effect (Fig. 2).

With respect to the question discussed above those fibrils were of particular interest which had sarcomere lengths beyond 5 μ (Garamvölgyi, 1966) and which had E-zones, regarded by us as the signs of the elongation of the A-band (i.e. of the primary myofilaments). In the polarizing microscope, too, we could recognize both kinds of E-zones, i.e. at the edges, as well as in the middle of the A-band (Garamvölgyi, 1965a, 1966) (Fig. 3). By comparing the curves belonging to the moderately and highly stretched myofibrils (Figs 2b and 3b), respectively, the character of the E-zones became quite clear. The I-bands of both fibrils show practically no double refraction, while the E-zones still exhibit a considerable birefringence (Fig. 3b). Thus we think to have excluded the possibility of confusing the I-bands with the E-zones. The relatively high double refraction of the E-zones supports our statement that the E-zones are elongated parts of the A-band and thus the I-band – E-zone limit represents the original A – I junction.

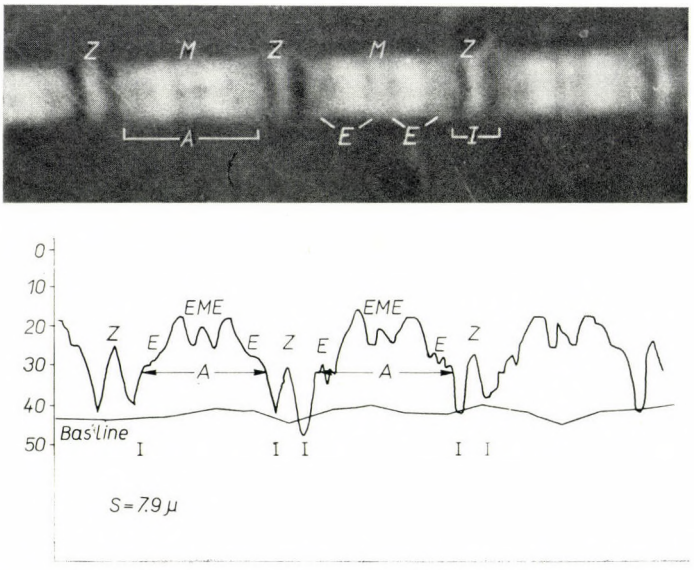

Fig. 3. *a:* Polarizing micrograph of a myofibril stretched beyond the "no overlap point". Note the missing double refraction of the I-bands, the double refracting Z-lines and the double refraction of the E-zones. There are also double refracting M-lines in the center of the A-band. *b:* Densitometric curve of the same fibril. Note the different double refractions of I-bands and E-zones

We could also observe that the M-lines of highly stretched fibrils are birefringent (Figs 3a and 3b). The optical character of the M-line was subject to discussions almost since its first description by Merkel (e.g. Häggquist', 1931) and it was many times confused with the H-zone. It can really hardly be observed in fibrils with homogeneous A-bands (Figs. 2a and 2b). In our case the determination of its optical character was due to the appearance of the central E-zones, which showed a double refraction lower than the original value for the A-band. The presence of double refracting M-lines even in highly stretched fibrils unambiguously demonstrates that the portions of the primary myofilaments belonging to the M-line are still in register and thus the decreased double refraction of the E-zones originated from the decreased diameter of the stretched primary myofilaments. This is in agreement with our earlier electron microscopic observations (Garamvölgyi, 1965a, 1966).

Discussion

The decreased, but still considerable double refraction of the E-zones and the existence of double refracting M-lines support our opinion, expressed earlier, that the constant length of the A-band can be observed until a critical sarcomere

length, but beyond this length it is the A-band which changes in length, while the length of the I-band shows a very small increase. We interpreted this fact with the continuity of the primary myofilaments along the whole sarcomere (Garamvölgyi, 1965a, b, 1966). This is also in agreement with the observations of Auber and Couteaux (1962, 1963) that the primary filaments are connected to the Z-lines and with our own observation (Garamvölgyi, 1963) that the primary filaments are continuous with the corresponding filamental portions inside the Z-lines. Therefore, we have suggested that all changes of the sarcomere length are due to the change of the own length of the primary filaments.

From this point of view the E-zones in the middle of the A-band are of increased importance, because according to the sliding model there can be only primary filaments at this site, at sarcomere lengths being beyond the "no overlap point". It is very interesting that recently a decrease of density has been observed in the medium part of the A-band in frog myofibrils stretched beyond the "no overlap point" (Page, 1965). The author came to the conclusion that these light zones are not identical with the H-zones. We fully agree with this conclusion, because our own experience also shows that the H-zones occupy practically the whole A-band about 5 μ sarcomere length (Garamvölgyi, 1965a). We assume that the zones of decreased density in highly stretched frog muscle are of the same origin, as the quite similar zones in the insect muscle, appeared as result of the same effect.

References

Auber, J., Couteaux, R. (1962) Compt. Rend. Acad. Sci. *254* 3425
Auber, J., Couteaux, R. (1963) J. Microscopie *2* 309
Brücke, E. (1858) Dkschr. math-naturwiss. Kl. *15* 69
Ernst, E., Béleczki, L. (1956) Acta Physiol. Hung. *9* 1
Ernst, E., Béleczki, L., Nagy, J. (1956) Acta Physiol. Hung. *9* 7
Garamvölgyi, N. (1963) J. Microscopie *2* 107
Garamvölgyi, N. (1965 a) J. Ultrastructure Res. *13* 409
Garamvölgyi, N. (1965 b) J. Ultrastructure Res. *13* 425
Garamvölgyi, N. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 89
Garamvölgyi, N., Kerner, J., Cser-Schultz, M. (1964) Acta Physiol. Acad. Sci. Hung. *24* 381
Hanson, J., Huxley, H. E. (1956) Symp. Soc. Exp. Biol. *9* 228
Häggquist, G. (1931) in Handb. d. mikr. Anat. d. Menschen, Möllendorf, W. von (ed), Vol. 3. part 3. Springer Verl. Berlin
Hodge, A. J. (1955) J. Biophys. Biochem. Cytol. *1* 361
Huxley, H. E., Hanson, J. (1960) in: The Structure and Function of Muscle, Bourne, G. H. (ed), Vol. 1, Acad. Press, New York
Kerner, J. (1966) Acta Biochim. Biophys. Acad. Sci. Hung. *1* 73
Page, S. (1965) J. Cell Biol. *26* 477
Schmidt, W. J. (1937) Die Doppelbrechung von Karyoplasma, Zytoplasma und Metaplasma, Bornträger Verl. Berlin

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 299—304 (1966)

Effect of Radioactive Radiation on the Activity of Isolated Frog Heart

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(Received January 17, 1966)

Frog's hearts isolated on a Straub cannula and kept in a K-free solution which has been changed several times stopped beating in 1 to 4 days. During this period the K-content of the hearts decreased significantly, and their Na-content increased. After having lost about 30 to 40 per cent of their potassium content the hearts stopped in diastole. The automatism restarted again in most of the hearts when exposed to the radiation effect of Co-60 and Ra, respectively. The dose needed for starting was about 200 rad in the case of Co-60 γ-radiation, and about 7.5 rad in the case of Ra γ-radiation, on the average. In the case of Ra-source α-radiation should also be taken into consideration. According to the experiments the effect of external radiation can also re-establish the automatism of frog hearts which have stopped beating. Our results support the opinion that radioactive radiation can have a positive biological effect in animals.

Introduction

The problem of the effect of radioactive radiation on heart activity is still a debated question nowadays although it has been investigated by several authors. According to the generally accepted view, the radioactive radiation has a negative biological effect in every case.

Zwaardemaker (1921), Vinogradov and Kowalsky (1957), Verkhovskaya and Arutunova (1953), Hoitink and Westhoff (1956), and from our Institute Ernst et al. (1959), Niedetzky and Hajnal-Papp (1963) have given an account of experiments in which solutions containing radioactive isotopes had a positive effect on isolated hearts. Zwaardemaker (1927) observed that cardiac activity of frog and eel hearts arrested by K-free solution restarted upon irradiation with α and β rays. Feenstra (1924) and Wieringa (1925) have described experiments, in which frog hearts arrested by K-rich and K-free solutions have started to beat again when placed in solutions of the same compositions but containing emanation. Tigyi (1964) demonstrated by recording simultaneously the mechanical activity and the action potential of isolated frog hearts paralysed by K-rich solution that the action potential appeared by the time the mechanical activity was not even observable. The action potential first appeared in the form of

irregular and very great negative waves. He observed very significant electric changes in the pace-maker system and in the impulse conducting system of the heart in the first few seconds after starting the activity of the heart with radioactive solution.

All the above-mentioned results were achieved by the application of radioactive solutions. This method, however, involves many uncertainties. Mechanical effects due to changing the solutions, different sensitivities of the hearts, and difficulties to prepare radioactive solutions the chemical composition of which is quite equal to the solutions used to arrest heart activity are factors which made it necessary to investigate the problem also by using external radiation sources. It is known that trace elements even in very low concentrations are able to influence the cardiac effect of radioactive radiation very significantly (Niedetzky and Hajnal-Papp, 1963). The application of an external radiation source has several advantages, e.g. the possibility of introducing unknown chemical elements (trace elements) during the change of the solutions and getting thereby erroneous results is excluded.

Haberlandt and Sandera (1927) observed the starting of activity and the quickening of pulse due to the effect of X-rays in pieces of the base of centricle of the frog heart. Tigyi (1963) described that hearts which were arrested with a K-rich solution (KCl content 0.3 per cent) restarted beating under the effect of 15 MeV electron rays in 12 per cent of the cases. No such effect was observed in the control. The dose needed to initiate heart activity was 500 rad in his experiments.

Methods

The experiments were performed on isolated frog hearts attached to Straub cannula. Blood was removed from the hearts by changing the normal Ringer solution several times after the preparation. The hearts were arrested with K-free "Ringer" solution. The composition of the solution used was the following: 0.68 g NaCl, 0.02 g $CaCl_2$ and 0.02 g $NaHCO_3$ in 100 ml double distilled water. The pH of the solution was 6.8 – 6.9. This solution was changed until the hearts stopped in diastole. The sensitivity of the hearts was very different. The hearts stopped generally after 5 changes, but in many cases 10 to 12 changes were necessary. During the night the hearts were stored in the cold room at +2 °C. Only those hearts were used for the experiments which stopped in diastole, but were still excitable: reacted with a single contraction to mechanical stimuli, to touch, and to electrical stimulus. These hearts were divided into 2 groups. One of them was irradiated with γ-rays, the other group served as a control. The experiments were performed at room temperature. The number of the hearts which restarted beating was determined simultaneously in both groups. After irradiation the hearts were cut to pieces, rinsed with distilled water, blotted with filter paper,

placed in Ni-containers, and incinerated in an electrical oven at 450 °C. The amounts of potassium and sodium were determined in the ash.

Two different radiation sources were used for the irradiation of the hearts. One of them was a Co-60 radiation source which was equal to 29 g Ra (energy yield: 1.17 and 1.33 MeV). The value of the dosage rate was 135 rad/h at the site of irradiation. The other was an open radiation source, a Ra rod weighing 1 mg, its size was 30×9 mm (energy yield: 0.19 MeV). The value of the dosage rate was 8 rad/h at the site of irradiation. In this latter case α-radiation of an energy of 4.78 MeV must also be taken into account because the hearts were placed 1 cm far from the Ra rod.

Frog hearts are very sensitive experimental objects and working with them involves several possibilities of experimental error. Most of the papers dealing with the cardiac effect of radioactivity are inadequate because in the experiments only a small number of objects was included. The effect is influenced significantly by several factors, as it is known. The question can be solved only if there are many experimental results which are evaluated by statistical methods.

Results

Hearts were arrested in diastole with K-free solution, and their excitability was investigated by mechanical stimulus. The hearts used in the experiments responded to touch with a single strong contraction. The hearts stopped after several changes of the solution, on the 1st, 2nd, or 3rd and 4th days after preparation. The K and Na contents of the hearts were determined at the end of the experiment. It was found that the K content of the hearts continuously decreased as compared to the hearts washed with normal Ringer solution. The Na content, on the other hand, continuously increased. Table 1 and Table 2 show the changes in K and Na contents.

The values for Na and K shown in the Tables are the mean values of the number of hearts given in the 2nd column. The decrease in K content compared

Table 1

Changes in K and water contents of hearts placed in K-free solutions

Solution	Number of hearts	Time (days)	Water content (per cent)	K mg/wet weight g	Decrease of K content (per cent)
Normal Ringer	33	—	85.8	1.92 ± 0.16	—
K-free solution	49	1	84.8	1.84 ± 0.70	4.2
K-free solution	53	2	85.0	1.18 ± 0.57	38.5
K-free solution	63	3	84.1	1.32 ± 0.31	31.5
K-free solution	9	4	83.7	1.26 ± 0.26	34.5

Table 2

Changes in Na content of hearts placed in K-free solution

Solution	Number of hearts	Time (days)	Na mg/wet weight g	Increase of Na content (per cent)	K/Na ratio
Normal Ringer	33	—	1.07±0.13	—	1.79
K-free solution	49	1	1.65±0.28	54.2	1.12
K-free solution	53	2	1.46±0.17	36.4	0.81
K-free solution	63	3	1.72±0.31	60.7	0.77
K-free solution	9	4	1.62±0.17	51.4	0.78

to that of the normal hearts is not significant with those which stopped in the first day, but it is significant in those which stopped in the 2nd, 3rd and 4th days. The Na content is significantly greater compared to the normal value in all of the hearts which were arrested with K-free solution. No essential change took place in the water content of the hearts, it decreased by 1 to 2 per cent only. The hearts generally stop when they lose 30 to 40 per cent of their K content, but the more sensitive hearts (those which stopped the 1st day owing to the effect of K-free solution) stopped even after a K-loss of 4.2 per cent. The K/Na ratio is 1.79 in hearts perfused with normal Ringer solution. This rate decreases significantly in the hearts arrested with K-free solution (last column of Table 2). K-loss is accompanied by a concomitant increase in Na uptake. This corresponds to the well-known reaction of the muscle according to which Na uptake takes place in the case of K-loss. Experiments concerned with the change of the K- and Na-contents were carried out on 174 hearts.

The effect of irradiation was investigated in an even larger number of hearts, altogether in 474 hearts. Table 3 and Table 4 illustrate the results of the effect of radiation on hearts which were arrested with K-free solution. Table 3 shows that 141 hearts were irradiated by Co-60 source and 117 hearts served as controls. 44 hearts (31.2 per cent) restarted from the 141 irradiated ones. From the 117 non-irradiated control hearts 7 also (5.9 per cent) restarted during the experiment. The control hearts were kept in the room where the radiation source was (because

Table 3

Effect of Co-60 γ-radiation on hearts arrested because of K-loss

	Restarted	per cent	Not started	per cent	Total
Irradiated	44	31.2	97	68.8	141
Control	7	5.9	110	94.1	117
Total	51		207		258

Table 4

Effect of Ra-radiation on hearts arrested because of K-loss

	Restarted	per cent	Not started	per cent	Total
Irradiated	38	32.8	78	67.2	116
Control	6	6.0	94	94.0	100
Total	44		172		216

of the identical temperature), behind a 5 cm thick sheet of lead where a dosis rate of 0.2 mr/h was measured during the operation of the Co source.

The statistical evaluation was performed by the χ^2 test. The value of χ^2 in this experiment is 25.1. The value of χ^2 relating to $P = 0.001$ is 10.83. Thus, the difference between the two series is significant if $P = 0.001$. The value of the dosis rate is 135 rad/h at the site of irradiation. For the starting of different hearts, different time periods were necessary. These periods varied between 1 to 2 hours and their mean values were 1.5 hours. According to this, the hearts arrested because of K-loss, restarted under the effect of about 200 rad.

The other group of the hearts which stopped beating because of K-loss (altogether 116 hearts) was irradiated by a Ra source weighing 1 mg. 100 hearts were used as a control in this series. Table 4 illustrates the results. 38 hearts (32.8 per cent) restarted out of the 116 irradiated ones owing to the effect of radiation. 6 hearts (6 per cent) restarted out of the 100 control ones during the experiment. The value of χ^2 according to these data is 22.5; and so there is a significant difference between the irradiated and control groups in the case of $P = 0.001$. Thus, the α- and γ-radiation of Ra (0.19 MeV) similarly to the γ-radiation of Co (1.17, and 1.33 MeV) brings about the automatism of the isolated frog hearts again, which stopped because of K-loss. The value of the dosis rate was 8 rad/h at the site of irradiation. Here also different time periods elapsed until the hearts restarted to beat, the average time was 1.5 hours. Consequently the γ-dose necessary for restarting the hearts is in the order of magnitude of 10 rad (mean value 7.5 rad).

Discussion

The automatism of the isolated frog hearts stopped in a K-free solution. Meanwhile the hearts lost a significant amount of potassium and took up some sodium. The decrease in K content brings about the increase in the threshold stimulus of the hearts, as it is well known. Nevertheless, a part of the hearts arrested in this way automatically started to beat again under the effect of ionizing radiation. By the method used in the present investigations the essential disturbing elements can be eliminated: first, the mechanical effect due to the changing of solutions,

and second, the possibility of contaminating the hearts. The statistical evaluation of the results obtained with a great number of hearts subjected to external irradiation showed that in some cases the automatism of the hearts was restored by radioactive radiation. These results support our earlier data obtained by using radioactive solutions (Ernst et al., 1959), and support the view that ionizing radiation can have a positive effect on biological systems. The necessary dose was 200 rad for starting in the case of applying Co-60 radiation. This result is in good agreement with Tigyi's (1963) results concerning the 15 MeV electron rays. The necessary γ-dose was 10 rad only for starting when a Ra-source was applied. This was 1 order of magnitude smaller than in the case of Co-60. The cause of this difference is that in the case of applying the Ra-source, the α-radiation of the Ra must be taken into account as a very essential factor as being responsible for the effect in the first place. The hearts were placed 1 cm far from the Ra-source. Unfortunately we were unable to measure accurately the intensity of the α-radiation, and therefore no precise value of the dose can be given. Experiments concerning the relation between the dose and the effect of the α-radiation are in progress. According to preliminary estimate the dose rate of the α-radiation was 200 rad/h on the surface of the heart. However, we have no information concerning the proportion of α-dose, which can be taken into account in restoring the automatism. The range of action of the α-radiation is only 40 μ in the cardiac muscle. In earlier experiments with radioactive solutions, about 1 rad dose was needed to bring about the restarting effect with hearts paralysed by K-rich solution. Thus, it seems possible that the β-radiation of the applied radioactive isotopes (Na-24, K-42) was responsible in the first place for this effect. This view is supported by the fact that the effect can be induced also by isotopes with only β-radiation (P-32, Ca-45).

Acknowledgement

Author expresses his thanks to Professor E. Ernst for encouragement and help in this work.

References

Ernst, E., Tigyi, J., Niedetzky, A. (1959) Acta Physiol. Acad. Sci. Hung. *16* 61
Feenstra, T. P. (1924) Arch. néerland de physiol de l'homme et des anim. *9* 279
Haberlandt, L., Sandera, R. (1927) Strahlentherapie Bd. 26. H. *3* 607
Hoitink, A. W. J. H., Westhoff, J. M. (1956) XX. International Physiological Congress, Brussels, p. 403
Niedetzky, A., Hajnal-Papp, M. (1963) Acta Physiol. Acad. Sci. Hung. *23* 315
Tigyi, J. (1963) Acta Physiol. Acad. Sci. Hung. *24* 129
Tigyi, J. (1964) MTA Biológiai Osztályának Közleményei *3* 223
Verkhovskaya, I. N., Arutunova, M. B. (1953) Izv. Akad. Nauk SSSR. Ser. biol. 88
Vinogradov, A. P., Kowalsky, V. V. (1957) Dokl. Akad. Nauk SSSR *113* 315
Wieringa, A. J. (1925) Thesis. Utrecht, 1925
Zwaardemaker, H. (1921) Ergebn. Physiol. *19* 326
Zwaardemaker, H. (1927) Pflügers Arch. f. d. ges. Physiol. *218* 354

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 305—314 (1966)

On some Technical and Dosimetry Problems of Irradiating Animals

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Uniform and proper irradiation of animals is a prerequisite for radiobiological experiments. Therefore, technical conditions which ensure the reproducibility of irradiations were worked out.

Introduction

To increase accuracy in animal dosimetry and to ensure the reproducibility of irradiations represents a current trend in the field of radiology.

The experimental work resulting in easier and more precise dosimetry is composed of a great many components including the organization of the irradiation and the consideration of the relevant physical, chemical and physiological factors. In addition to the environmental conditions, temperature, moisture-content, the atmospheric pressure to which the animals are exposed for shorter or longer periods, will affect the results. These data are, therefore, given in quantitative terms. For the sake of adequate dosage, the data connected with the dose, such as e.g. dose-rate, dose distribution, should also be specified. The specification of the type and energy of the radiation applied is also essential. To quote an example from everyday practice, e.g. if irradiation is performed with X-rays, the exciting voltage, amperage, filtering, half value layer, focus-skin or target to midline of the body distance, etc. should all be specified, although we are fully aware of the fact that both the dosimetric and the biological evaluations of these factors from the point of view of the absorbed dose are a rather difficult problem. Yet, the absorbed dose is the most suitable value to characterize the interactions involved in the biological effects of radiations.

If only possible, uniform dose distribution should be achieved in the irradiated organism. This uniformity is most difficult to realize. Actually, uniformity can at best be approached in case of low energy (5 – 100 KeV) X-irradiations. In the particular case absorbed dose values will be different within the bone and on either side of the organs interfacing the bone tissue.

In the majority of cases – whole body irradiation of animals, exposure of bacteria or homogenates – it is intended that the desired volume be irradiated

uniformly. However, only a moderately uniform irradiation might be achieved with X-rays excited within the conventional range of 80–250 kV (0.5; 1.0 mm Cu filter) even if small animals are irradiated.

On the other hand, if high energy radiation is used to irradiate thin biological objects, dose distribution will also be moderately uniform, owing to the incomplete equilibrium of secondary particles.

That is why the ICRU recommends to determine the dose in the midline of the body (center) when exposing animals to whole body irradiation. This offers the possibility to express the dose as a single figure (ICRU, 1962).

Irradiation of Small and Medium-Size Mammals

When irradiating small and medium-size mammals – even if no high energy radiation source is used – uniformity might still be increased by bilateral exposure. Bilateral exposure might be performed e.g. by the successive irradiation of both sides. Half of the dose is delivered to one side of the animal, the animal is then turned around and the second half of the dose is delivered to its other side.

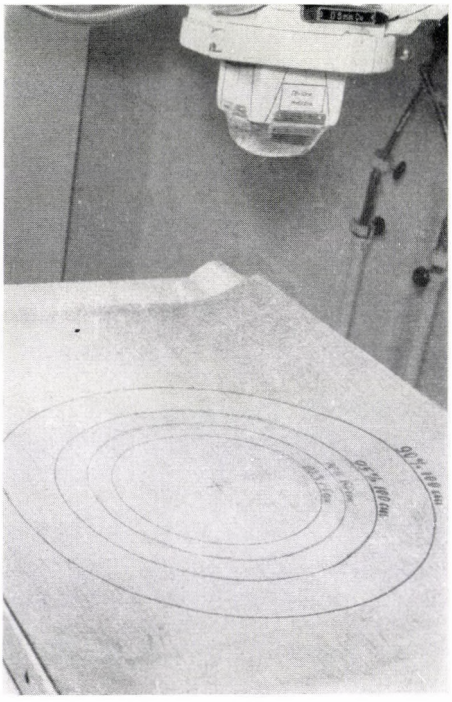
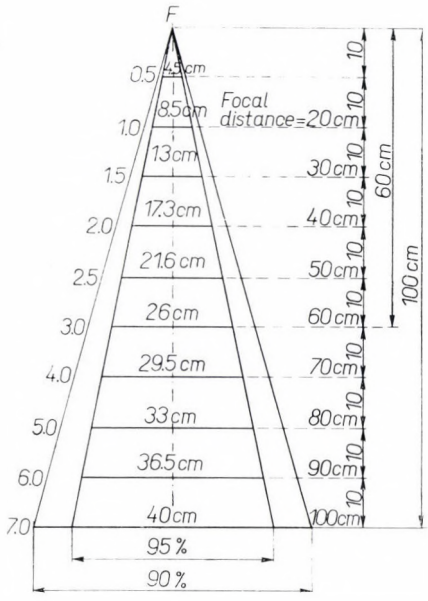


Fig. 1. Isodose curves

As emphasized by us for several years, ICRU also stresses that the most important step in achieving uniform irradiation consists in the progression from unilateral to bilateral exposure (Goodrich et al., 1965).

In order to establish reliable exposure conditions, isodose curves have been taken for the most conventional X-ray deep therapy units (Stabilivolt, THX-250), FSD 60 cm and 100 cm, respectively. The tube angle was adjusted to obtain



Fb distance (cm)	Diameter (cm)	
	95 per cent	90 per cent
120	47	65
150	57.5	81.5

Fig. 2. Diameter of the irradiation field for different FSD values

concentric circles as isodose curves. The isodose curves within and along which the animal still receives 95 and 90 per cent of the dose measured in the centre have been inscribed into a lead rubber plate put on the irradiation table (Fig. 1). The distance or area of circle within which the irradiation object still receives 95 to 90 per cent of the dose was then recorded in the diagram for varying FSD values. These isodose curves are extremely useful since they eliminate the necessity of rotating the irradiated object during irradiation, to level inhomogeneities.

Figs 3, 4, and 5 illustrate our special boxes for the exposure of mice, rats and guinea-pigs. The following characteristics should be mentioned:

1. About 15 mice can be placed into our *cylindrical* container designed for the exposure of mice.

2. The peripheral end of the container is, of necessity, farther from the tube focus than its center. To eliminate inaccuracies due to various distances from the

focus – a common source of error – the bottom of the container is such as to have a spherical surface (Fig. 6), the radius of which from the focus is at a distance of 60 cm, 100 cm, i.e. at a distance from whic hirradiation is performed in a given case.

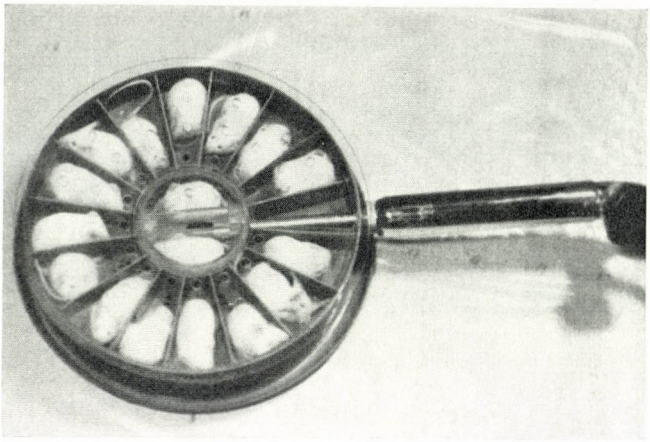


Fig. 3. Box for the exposure of mice

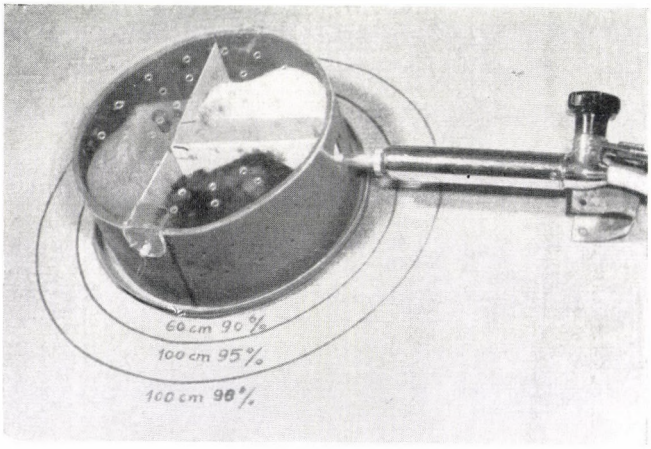


Fig. 4. Box for the exposure of rats – or guinea-pigs – 3 of each

3. Our plastic containers are made of Vinidur. Table 1 summarizes the results relating to back-scatter under the following exposure conditions: 180 kV, 10 mA, radiation filtered by 0.5 mm Cu + 2.0 mm Al filter, H.V.L. 1.00 ± 0.03 mm Cu, focus-chamber distance: 60 cm.

4. Our containers for the exposure of guinea-pigs and rats are suitable to hold 2 bigger or 3 smaller animals (Figs 4, 5) so as to ensure possibly the same scatter conditions for all the animals exposed.

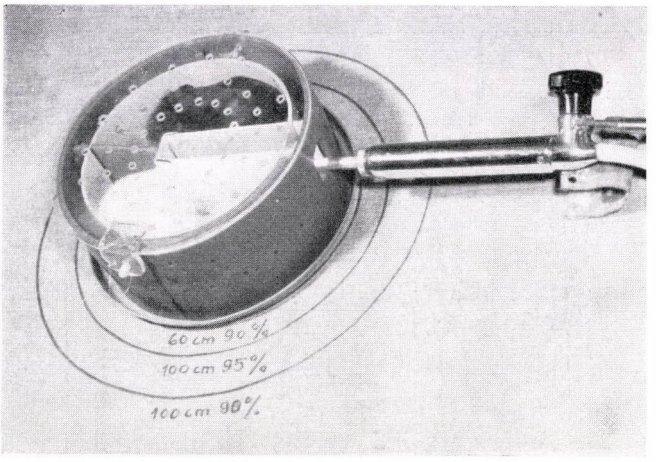


Fig. 5. Box for the exposure of rats — or guinea-pigs — 2 of each

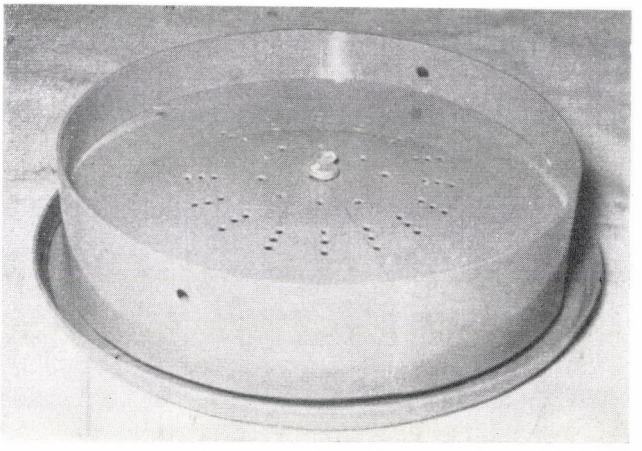


Fig. 6. The spherical surface of the bottom of our containers

5. Naturally, as it is customary, our cages are also suitable to hold sufficient backing material (at the bottom or laterally) to allow conditions of maximum back-scatter. In biological experiments the lateral placing of such scattering material involves the non-negligible drawback that means that irradiation performed from a greater distance, of necessity, reduces dose-intensity.

Table 1

Backscatter-measurements

		per cent
1.	Air dose	100
2.	Air dose with mouse phantom	99.5
3.	Air dose, surface of table 5 cm below the chamber	110
4.	Air dose, 5 cm below the chamber, surface of table with lead rubber plate	105—108
5.	Chamber placed in the centre of an empty container for mice	108—111
6.	Chamber placed close to the edge of an empty container for mice	105—107
7.	Chamber in a phantom, placed close to the edge of a container for mice, empty	102—104
8.	Chamber placed in the centre of a container for mice, with 15 mice in the container	114—115
9.	Chamber placed close to the edge of a container for mice, with 15 mice in the container	107—108
10.	Chamber in a phantom, placed close to the edge of a container for mice, with 15 mice in the container	107—109
11.	Chamber placed between two rabbits	120—134

6. The groups irradiated successively in an experimental series should comprise the same number of animals, possibly of the same weight, too. This also constitutes one of the adequate exposure conditions (Dempster et al., 1964).

Irradiation of Homogenates and Bacterium Cultures

When irradiating homogenates, bacterium cultures, etc., a higher dose is sometimes necessary. Practically this means that the dose required should be delivered within as short a time period as only possible. To this end the irradiation object should be placed rather close to the focus of the tube. Our irradiation units used with the different X-ray equipments are seen on Figs 7, 8 and 9. Since the thickness of the irradiation objects varies, adequate supports are used to ensure identical distances between focus and irradiation surface. Glass containers must not be used when irradiating micro-organisms since the higher atomic number of the glass might result in a most complex radiation pattern. We generally use containers made of polyethylene.

We should like to call the attention to the relation between the absorbed dose D, measured in rads, and exposure X, measured in R

$$D = f \cdot X$$

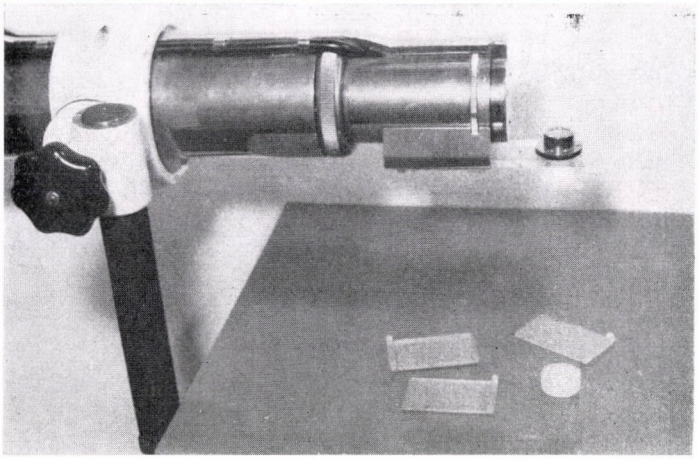


Fig. 7. Holder for the exposure of bacteria, to be used with a Dermopan apparatus

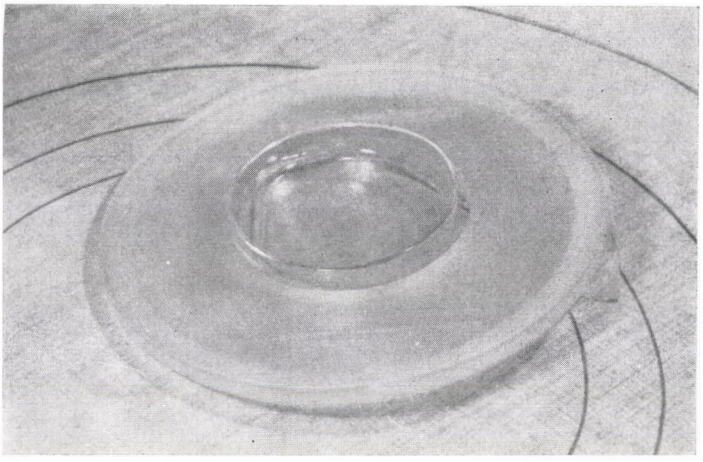


Fig. 8. Small dish with holder for the exposure of homogenates

where the value of f has to be determined for X- and gamma-rays of different qualities. For air with all qualities of radiation f, by definition, has the value of

$$0.869 \frac{\text{rad}}{\text{roentgen}} .$$

For water, the value of f varies between 0.87 and 0.97 in the photon energy range from 10 KeV to 3 MeV. In the same energy range f for muscle varies between

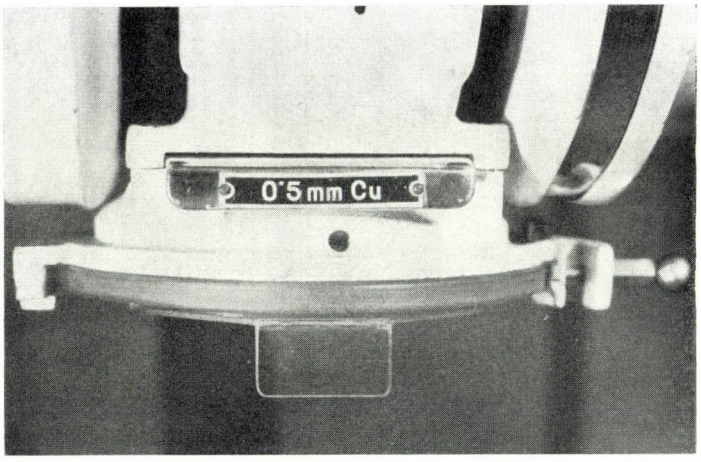


Fig. 9. Holder for the exposure of bacteria to be used with a deep-therapy apparatus

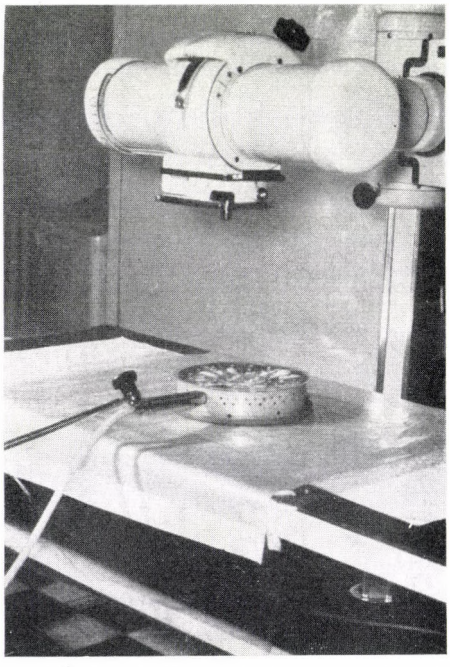


Fig. 10. Exposure of mice with continuous dose measurement

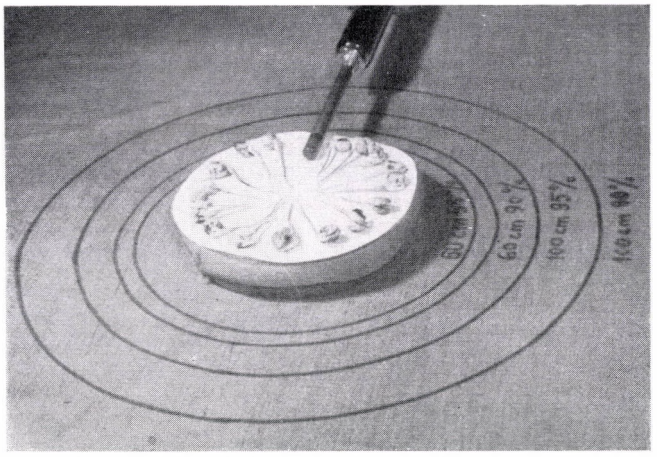


Fig. 11. Exposure of germinating beans on a wet plastic sponge cushion — with continuous dose measurement

0.91 and 0.96; f for bone has a value of 4 in the energy range of 10 KeV – 40 KeV. At higher photon energies this value drops. Above the photon energy range of 300 KeV, f values for bone and muscle are identical.

Necessity of Continuous Dose Measurement

Finally, we should like to stress that in our opinion direct dose measurement, performed simultaneously with delivering the dose — so called continuous measurement — is of primary importance. It ensures delivering the adequate dose, precisely specified in advance, even if owing to some unexpected reason (e.g. fluctuation of the potential) dose intensity is changed during irradiation. In our Institute this task is performed by Massiot's dose meter, with a chamber and accuracy of 2 R (Fig. 10).

This continuous dose meter is used also if e.g. germinating beans are irradiated on wet plastic sponge cushions (Fig. 11).

Authors take this opportunity to express their thanks to their co-worker József Pintér for his valuable technical assistance.

References

Dempster, W. J., Kapros, C., Kountz, S. (1964) The British Journal of Radiology *37* 436
Goodrich, R. M., Thomas, R. G., Wright, S. R. (1965) Health Physics *11* 531
ICRU (1962) Radiobiological Dosimetry, Report

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 315—320 (1966)

The Role of Noise in Biocybernetics

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(Received February 3, 1966)

This paper deals with the interpretation of biological noise and some of its forms of appearence in connection with irritation. We use the data of Weber's experiment for the approximation of the quantitative characterization of biological noise Attention is drawn to the possible correlations between noise level, redundancy in Weber's experiment, delay due to coding, and synaptic delays.

Introduction

The aim of our biocybernetical studies is to examine biological phenomena by a cybernetical approach (Biró and Királyfalvi, 1965). In this paper we deal with biological, in other terms, internal noise. The expression "noise" is used in the sense as employed in information theory.*

Eijkman and Vendrik (1963) have pointed out that in the sensory nerve tracts there is a neural activity of gaussian distribution which exists independently of the stimulus and can be considered as internal noise. In the organism, generally "noisy" information systems are operating and the biological noise accompanying the normal functioning of the organism can appear in several parts of the information systems (Goodwin, 1963).

In connection with the process of irritation the following phenomena, among others, can be considered as noise or as the appearance of noise effect: spontaneous activity of neurons (Rodieck et al., 1962); fluctuations in the membrane potential of the axons (Verveen and Derksen, 1965); fluctuations in the irritability of an axon and fluctuations in the stimulus threshold of the nerve fibre (the realization of this latter phenomenon has resulted in changing the classical concept of stimulus threshold) (Verveen and Derksen, 1965; Frishkopf and Rosenblith, 1956); fluctuations in the latency-period of the action potentials generated by the stimulation of an axon (Verveen and Derksen, 1965); miniature excitatory

* The biological noise mentioned above is not synonymous with the physiological noise (Ackerman, 1962) which includes the sounds originating inside the organism and being approximately at the threshold of hearing (e.g. muscle sound).

postsynaptic potentials (Eccles, 1964); miniature end-plate potentials (Fatt and Katz, 1952); spontaneous activity in the visual cortex (Lennox-Buchthal, 1960); responses to different stimuli given by examined persons at the investigation of the skin senses touch and warmth (Eijkman and Vendrik, 1963); area below the hearing threshold-curve on the audiogram (Wiener, 1957); increase of the values of $\frac{\Delta I}{I}$ in Weber's experiment in case of low stimulus intensities (Rosenblith, 1961).

Approximation of the Quantitative Characterization of Biological Noise

For the approximation of the quantitative characterization of biological noise we start from Rosenblith's paper cited above (Rosenblith, 1961). In this work in a figure about Weber's experiment the author has represented the changes of the values of $\frac{\Delta I}{I}$ as a function of stimulus intensity. According to this paper the change represented can be described by the equation $\Delta I = k(I + N_i)$ where "N_i has such a value that for low values of I, ΔI depends primarily upon N_i, while for medium and high values of I, N_i becomes negligible". Rosenblith considers N_i to correspond to the noise level of the organism.

By the conversion of the foregoing equation we obtain the following equation:

$$\frac{\Delta I}{I} = k \cdot \frac{I + N_i}{I}.$$

It follows from this equation that in case of strong stimuli – when $I \gg N_i$ – the value of $\frac{\Delta I}{I}$ almost equals the minimum value (k). Proceeding from the strong stimulus intensity towards the threshold, the value of $\frac{\Delta I}{I}$ increases, and when $I = N_i$, the value of $\frac{\Delta I}{I}$ reaches the double value of the former minimum. Therefore, if the stimulus intensity belonging to the double of the minimum value of $\frac{\Delta I}{I}$ is taken in Weber's experiment, the N_i, or rather – according to Rosenblith – the noise level of the organism can be quantitatively characterized by this value of stimulus intensity.

The data of Table 1 are compiled from those of a curve shown in Rosenblith's figure (Fig. 1) by interpreting the value of N_i as mentioned above.

Table 1

Data of Weber's experiment performed with white light

The values of I and $\frac{\Delta I}{I}$, as well as the value $N_i = 64$ have been taken from Fig. 1

I	3.1	4	10	20	30	64	100	1000	10^6
$\frac{\Delta I}{I}$	0.5	0.345	0.2	0.138	0.115	0.08	0.06	0.04	0.04
ΔI	1.55	1.38	2	2.76	3.45	5.12	6	40	$4 \cdot 10^4$
$\frac{\Delta I}{I + N_i}$	0.0231	0.0203	0.027	0.0329	0.0367	0.04	0.0366	0.0375	0.04

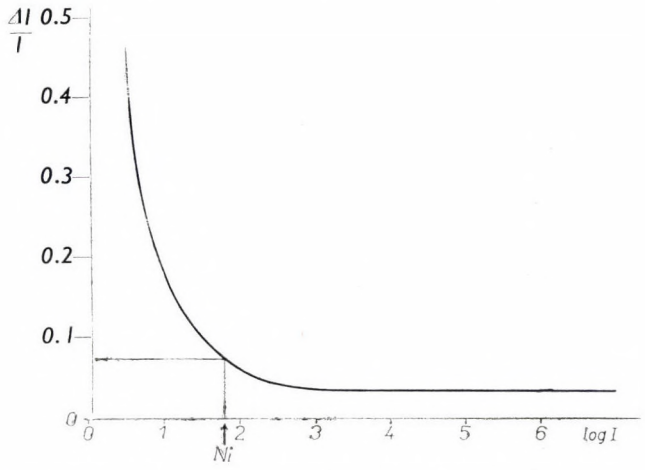

Fig. 1. The result of Weber's experiment performed with white light (Rosenblith, 1961)

In this way – in case of white light – the stimulus intensity of a value of $I \sim 64$ would correspond to N_i. (Although, the equation $\Delta I = k(I + N_i)$ given by Rosenblith does not hold exactly, as it can be seen in the last row of Table 1.)

It can be seen also in Rosenblith's figure that there is a definite connection between the values of $\frac{\Delta I}{I}$ belonging to different stimulus intensities and the values of the reaction time. Therefore, by measuring the reaction time, it would be possible to get quantitative data concerning the noise level of the organism.

Presumably, the data obtained about noise level can be used for the diagnosis of pathological lesions.

Weber's Experiment and Redundancy

According to the information theory, in order to decrease the effect of noise or to increase the reliability of the transmission, a code is applied in which the coded message consists of more signals than in a noiseless case, that is, the redundancy is increased.

On the basis of the formula concerning binary coding (Reza, 1961), it is possible to determine the frequency of the occurrence of an error:

$$M_A \leq \frac{N}{2^n} \sqrt{\frac{nq}{2\pi p}}\, p^{-nr} q^{-nq}.$$

In this formula N is the number of the messages in the source, n is the number of the signals in the individual messages, $p = P(1 \mid 1) = P(0 \mid 0)$; $q = 1 - p = P(1 \mid 0) = P(0 \mid 1)$, that is, probability of the alteration of a signal. On the basis of the above-mentioned formula it can be demonstrated that if n and consequently redundancy increase then the frequency of the occurrence of an error diminishes.

Redundancy increases also in Weber's experiment mentioned above because, in the case of the increase of stimulus intensity, the increasing frequency of the nerve impulses corresponds to the response – that is, to generator potential – due to the operation of one definite receptor, and at the same time the number of the receptors getting into irritation can also increase. Therefore, the number of the signals – that is, the nerve impulses – going through the information system toward the central nervous system can increase in two different ways. In Weber's experiment the increase of the number of the signals means also an increase in redundancy because the signals always deliver the same amount of information, that is, the just noticeable increase of stimulus intensity.

Synaptic Delays and Noise

The following formula can be given for the rate of transmission of information:

$$C = \frac{W}{2\pi} \log \frac{2Wa^2}{\varepsilon^2}.$$

In this formula W stands for the frequency band-width, a^2 for the spectral density of the information source, and ε^2 for the total energy of noise of a steady spectral density (Fenyő and Frey, 1964). It can be seen that if the energy of the noise increases the rate of transmission of information decreases.

When redundancy also comes into being, it "necessarily causes a delay in transmission and reception. That is, a higher rate of transmission can be obtained at the expense of a longer delay" (Reza, 1961).

If the delays occurring in the synapses can be brought into connection with a coding process (Ernst, in press) the above aspects possibly can be taken into consideration also in connection with biological noise, especially, with synaptic noise (Eccles, 1964). In this case the question may arise whether the different synaptic delays (Table 2) (Eccles, 1964; Furshpan and Potter, 1959) are caused – among others – by the fact that the volume of the noise (or else the demand for the reliability of transmission) is different in the synapses located at different sites in various organisms.

Table 2

Synaptic delays

Eccles, 1964; Furshpan and Potter, 1959

Synaptic types	Synaptic delay (msec)
Motoneurone mammal 38°C	0.3
Motoneurone frog	1.0 – 1.2
Motoneurone toad	1.5
Giant synapse Loligo	1 – 2
EPP frog with muscle	0.6 – 1.0
EPP mammalian muscle 38°C	0.2
Cat nictitating membrane	6 – 10
Guinea pig vas deferens	
Neuromuscular synapse of the isolated rat diaphragm	0.217±0.004
Giant motor synapse of the Crayfish	0.12 ±0.01

Acknowledgement

We express our thanks to Professor E. Ernst for having called our attention to the aforesaid problems.

References

Ackerman, E. (1962) Biophysical science. Engelwool Cliffs, N. J. p. 14
Biró, G., Királyfalvi, L. (1965) MTA Biol. Oszt. Közl. *8* 157
Eccles, J. C. (1964) The physiology of synapse. Springer-Verlag, p. 35
Eijkman, E., Vendrik, A. J. H. (1963) Biophys. J. *3* 65
Ernst, J. Bevezetés a biofizikába (in press)
Fatt, P., Katz, B. (1952) J. Physiol. *117* 109
Fenyő, I., Frey, T. (1964) Matematika villamosmérnököknek. Budapest, p. 778

Frishkopf, L. S., Rosenblith, W. A. (1956) in: Symposium on information theory in biology. Pergamon Press, p. 153
Furshpan, E. J., Potter, D. D. (1959) J. Physiol. *145* 289
Goodwin, B. C. (1963) Temporal organization in cells. A dynamic theory of cellular control processes. Acad. Press, London
Lennox-Buchthal, M. A. (1960) Acta Physiol. Scand. *50*, suppl. 175, 94
Reza, F. (1961) An introduction to information theory. New York, p. 164, 165
Rodieck, R. W., Kiang, Y.-S., Gerstein, G. L. (1962) Biophys. J. *2* 351
Rosenblith, W. A. (1961) in: Biophysical science — A study program. Oncley, J. C. (editor) New York, p. 485
Verveen, A. A., Derksen, H. E. (1965) Kybernetik, *2* 152
Wiener, N. (1957) Cybernetics, New York, p. 104

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 321—328 (1966)

Excitation as an Electron Process*

(Theoretical paper)

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(Received February 15, 1966)

1. The theory of excitation, even though not generally accepted nowadays yet very wide-spread, is based on three findings: 1. Changes in the permeability of the membrane of the excited unit (Bernstein, 1902); 2. The overshoot experimentally found and described by Burdon-Sanderson and Gotch (1891); 3. The Na-K exchange experimentally found and described by Ernst and Scheffer (1928). These early years indicate that the basic concepts of the contemporary ion-theory of excitation date back to a period of exact sciences when the theory of electrolytes had come into prominence and was quite current also in biology.**

Since then, however, electron physics and electron chemistry have become the leading concept of exact science, and electron biology is also gaining ground day by day. That is why the question arises how to explain that the membrane-electrolyte theory of excitation – though untimely – is widely accepted even today.

Apart from the early date of the discoveries furnishing the basis for this theory another circumstance may also play a role in its being wide-spread. Namely the fact that electric processes have been used for stimulation for some two hundred years. Thus *electrotonus* has become a very general concept of electrophysiology, according to which certain functional changes could be found in organs and tissues treated with electric current in different experiments.

But in the processes of life stimuli are not of electric nature. The stimulus for vision is light; and though internal electrons are accepted in physics as playing a role in the photo-conductive effect, many papers dealing with the sensation of light use the phrase of membrane polarization even today. The situation is similar concerning the sensation of warmth caused by heat; biological literature does not mention thermoelectrons in this case either though thermocurrent was described in biology very early (Hermann, 1871).

* From a lecture delivered at the invitation of the Eötvös Loránd Physical Society, Veszprém, 1965 (Ernst, 1965).

** Consequently the author, in opposition to the contraction-theory based on the orthodox chemical concept of the glycogen $\rightleftarrows$ lactic acid reaction, worked out the concepts of electrostriction (volume diminution) and Na-K exchange accompanying muscle activity (Ernst, 1928, 1963).

2. The lack of an electron concept in the description of sensations brought about by mechanical stimuli is still more conspicuous. Therefore one mechanoreceptor will be dealt with in this paper to show how some physical data may permit to describe the excitation of these receptors as an electron process.

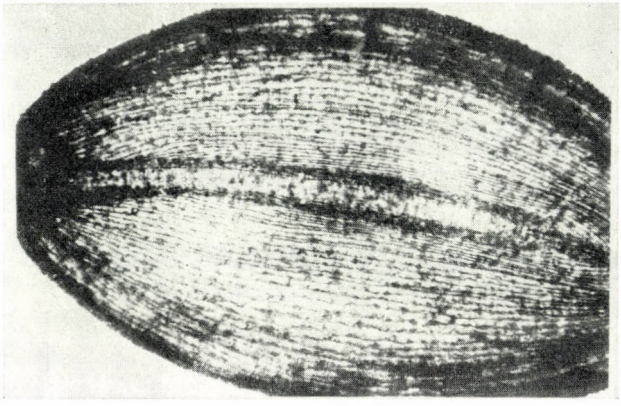

Fig. 1. Pacinian corpuscle; longitudinal section (×200) (Hubbard, 1958)

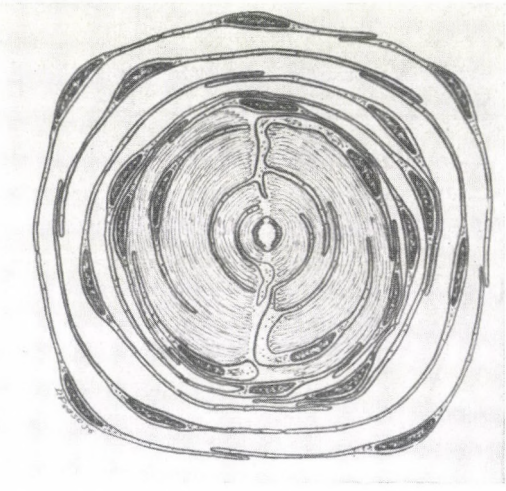

Fig. 2. Cross section of the Pacinian body (× 2000) (Pease and Quilliam, 1957)

The Pacinian corpuscle* is about 1 mm in extension; Fig. 1 demonstrates its longitudinal section. The core is the single nerve fibre encircled by about 100 lamellae denser in the vicinity of the nerve fibre. Fig. 2 demonstrates a cross

* Organ of touch and pressure, common in the animal kingdom.

section of the Pacinian corpuscle, the magnification of this drawing is $\times 2000$. In an experiment mechanical impression of even 1μ depth brings about excitation, the electric sign of which is shown in Fig. 3. The mechanical impression is marked on the upper line serving, simultaneously, as a time signal for 1 msec each; the

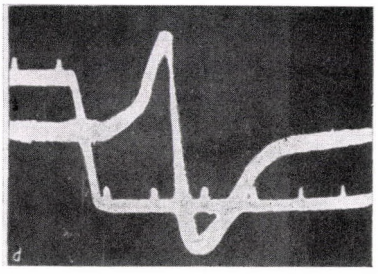

Fig. 3. The receptor and action potentials of a Pacinian body (Gray and Sato, 1953)

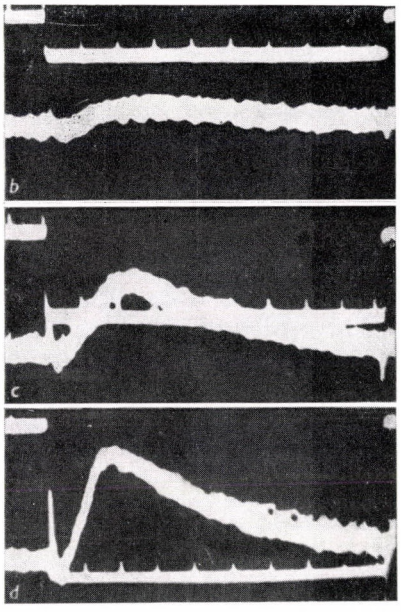

Fig. 4. Receptor potentials, the amplitudes depend on the intensities of the stimuli; the action potentials are eliminated by treating the Pacinian body with procaine. (Gray and Sato, 1953)

lower trace demonstrates the receptor potential continuing in the diphasic action current of the nerve.

In Fig. 4 the receptor potentials depending on the intensity of the stimuli can be seen; no action potentials are present since the preparation has been treated with procaine.

3. Summarizing the tribo-, contact- and piezoelectricity in the notion of *mechano-electricity* in these electrical phenomena the mechanoelectrons seem to play a role similar to that of the photo- and thermoelectrons in the respective processes. The situation can perhaps be described by saying that all changes in the mechanical state of a material system are accompanied also by electric processes, namely by changes of electric charge i.e. by shift of electrons.

a) Contact electricity is known to occur not only between metals but also between solid or fluid insulators, and the substance of greater dielectric constant gives electrons to that of smaller dielectric constant. Now, ferroelectric substances have very high dielectric constants; e.g. for the Seignette-salt $\varepsilon = 10^3 - 10^4$, for methylamids $\varepsilon \sim 100 - 200$.* Important is the fact that between two substances of different dielectric constants one can become an electron donor and the other an electron acceptor, if the distance between them diminishes to $10^{-6} - 10^{-8}$ cm.

b) Besides the ferroelectricity of Seignette-salt its piezo- and pyroelectricity should be mentioned; according to the latter, electric potential difference occurs at a temperature difference. This characteristic is always accompanied by piezoelectricity,** hence biological tissues being – as mentioned above – pyroelectric, they can be supposed to be piezoelectric also. That is indeed the case, e.g. the deformation potential in muscle was described long ago (e.g. Schenck, 1895; de Meyer, 1921).*** From the biological point of view the fact seems to be very important that the development of free radicals and their migration due to mechanical action occur in wool and silk fibers (Windle and Wiersema, 1964).

In general, a cylindric body bent in one direction suffers, on the side of bending, packing of its material i.e. negative dilatation and on the other side positive dilatation. This changing of density is accompanied by an electron migration from the side of negative dilatation to that of positive dilatation.****

c) As a continuation of the discussion about ferroelectricity, KH_2PO_4 should be mentioned, the ferroelectricity of which changes to antiferroelectricity when K is replaced by NH_4. Such a transformation is accompanied by changes in the dielectric constants and by an electron shift. Especially the ferroelectric $KNbO_3$ seems to be an important example, because $NaNbO_3$ – after K has been

* In connection with these data one should be reminded of Maxwell's law concerning dielectric constants specified for systems of inhomogeneous composition (Wagner, 1914; Güntherschulze, 1927; Brown, 1956). Furthermore, dielectric constants as high as 2100 and 400 are described for biological systems (Sugiura and Koga, 1965).

** The inverse statement would not be true.

*** In recent literature of physics see e.g. Kramer (Kramer, 1949), Sujak (Piróg, et al, 1965) etc.

**** Considering these facts established in physics (Jones, 1956) one is reminded of the excitation brought about by certain hairs deformed by mechanical action. E.g. the hairs of the Ctenophora may be mentioned; these hairs are bent by changing the position of the statolith when the animal changes its own position (see Ernst, 1965).

replaced by Na – becomes antiferroelectric the process being accompanied by volume diminution* (Forsbergh, 1956; Martin, 1964).

4. On the basis of what has fragmentarily been described in the preceding chapter the receptor potential – due to mechanical stimulation of the mechanoreceptors – is, in this paper, supposed to be the result of mechanoelectrons. Consequently the action potential, being the continuation of the receptor potential, should also be looked upon as an electron process. Fig. 5 shows a series of action potentials of the nerve fibre (Hensel and Zottermann, 1951) which is the continuation of the core of a Pacinian corpuscle. Fig. 6 demonstrates the close relation

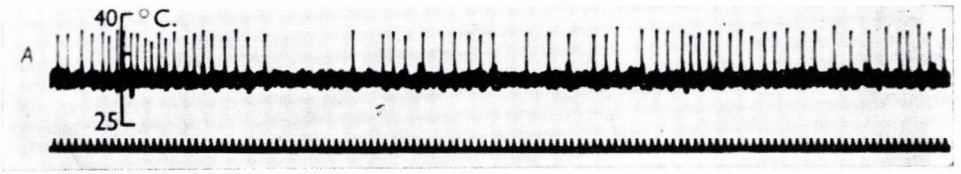

Fig. 5. Action potentials of the nerve belonging to a Pacinian corpuscle; time: 50 cyc/sec (Hensel and Zottermann, 1951)

of both potentials; according to this figure (Hodgkin, 1948), when a sensory nerve is stimulated with electric currents (the relative strength of which is written on the traces) the generator potential is continued by action potentials of different frequencies.

Just this phenomenon, viz. that the receptor or generator potential of the nature of d.c. continues in action potentials of different frequencies, seems to be of outstanding importance from the point of view of electron biology.** For this phenomenon means no less than a biological organ or tissue is capable of frequency modulation, a characteristic of semiconductors functioning of course by electron processes*** (see e.g. Ernst, 1956).

* In the light of these facts volume diminution and K-Na exchange – described by the author as phenomena accompanying muscular activity – are to be interpreted with due circumspection.

** A century ago this sentence was published (Frey, 1883): "Es muss also dem Nerven die Fähigkeit zugetheilt werden, den stetigen Verlauf des constanten Stromes in getrennte Erregungsstösse umzusetzen . . ." ("The ability should be ascribed to nerves to transform the constant course of d. c. into separate impulses of excitation . . ."). Not much later the opinion was expressed that living plasma is able to transform a constant stimulus into rhythmical excitation (Biedermann, 1895). In this context the question seems to be of great interest where the frequency modulation occurs when the muscle – whose nerve is stimulated with d. c. – shows a tetanic contraction (e.g. Biedermann, 1888). – By means of a blinking arrangement a constant process can be transformed into a rhythmical one also in an inanimate system.

*** Electronic processes occurring in $n-p$ semiconductors have been compared to contact potentials (Pohl, 1957).

This frequency modulation could – if further speculation on that topic is wanted – be expressed in the language of information theory. Viz., this process may be looked upon as coding or code modulation, and one cannot arrive at this stage of the theory but by way of electron concepts.

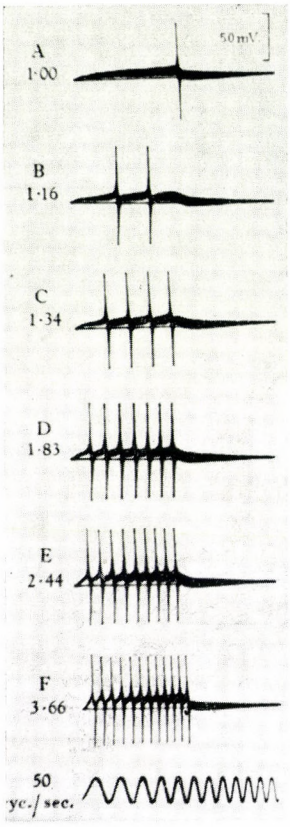

Fig. 6. Generator and action potentials (Hodgkin, 1948)

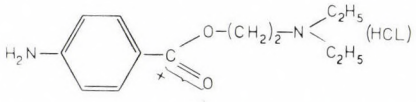

Fig. 7. Chemical structure of procaine

Furthermore, the theory can go still deeper along these lines. The action potentials i.e. the frequent impulses (the sign of excitation) – as described above – vanish if the preparation of a Pacinian corpuscle has been treated with procaine. Thus the question arises what kind of a reaction could explain this effect of procaine. Its chemical structure is shown in Fig. 7 demonstrating also that this

compound contains several π-electrons. Hence the suggestion does not seem to be too bold that an electron process between the compound and some content of the nerve may occur and explain the effect.

This concept may perhaps be supported by the further fact that thiocaine – arising after carbonyl O has been replaced by an S atom – is six times as effective as procaine. From the electron formula of the S atom

$$1s^2\ 2s^2\ 2p^6\ 3s^2\ 3p^4$$

the outermost p-electron jumping to a d orbit behaves as a π-electron and may explain the increased effect.*

References

Bernstein, J. (1902) Arch. Ges. Physiol. *92* 251, 542
Biedermann, W. (1888) Sitzungsber. Akad. Wien, *97* 115
Biedermann, W. (1895) Elektrophysiologie. Gustav Fischer, Jena, S. 269
Brown, W. F. jr. (1956) Encyclopedia of Physics. Springer Verl. Berlin, XVII. p. 130
Burdon-Sanderson, J., Gotch, F. (1891) J. Physiol. *12* 43
Ernst, E. (1928) Arch. Ges. Physiol. *220* 672
Ernst, E. (1956) Biofizika *2* 296
Ernst, E. (1963) Biophysics of the Striated Muscle. Publishing House of the Hung. Acad. Sci. Budapest.
Ernst, J. (1965) Towards Electronbiology. Értesítő, 1966. p. 109.
Ernst, E., Scheffer, L. (1928) Arch. Ges. Physiol. *220* 655
Forsbergh, P. W. jr. (1956) Encyclopedia of Physics. Springer Verl. Berlin, XVII. p. 264.
Frey, M. (1883) Du Bois-Reymonds Arch. Physiol. p. 43
Gray, J. A. B., Sato, M. (1953) J. Physiol. *122* 610
Güntherschulze, A. (1927) Handb. Physik. Verl. J. Springer, Berlin, XII. p. 510.
Hensel, H., Zottermann, Y. (1951) J. Physiol. *115* 16
Hermann, L. (1871) Arch. Ges. Physiol. *4* 163
Hodgkin, A. L. (1948) J. Physiol. *107* 165
Hubbard, S. J. (1958) J. Physiol. *141* 198
Jones, H. (1956) Encyclopedia of Physics. Springer Verl. Berlin. XIX. p. 253.
Kramer, J. (1949) Z. Physik. *125* 739
Martin, H. J. (1964) Die Ferroelektrika. Akad. Verlagsges. Leipzig.
Meyer, J. (1921) Arch. Internat. Physiol. *16* 193
Pease, D. C., Quilliam, T. A. (1957) J. Biophys. Biochem. Cyt. *3* I, 331
Pirog, M., Kowalczyk, R., Sujak, B. (1965) Acta Physica Polonica *28* 681
Pohl, R. W. (1957) Elektrizitätslehre. Springer Verlag Berlin.
Schenck, Fr. (1895) Arch. Ges. Physiol. *61* 494
Sugiura, Y., Koga, Sh. (1965) Biophys. J. *5* 439
Windle, J. J., Wiersema, A. K. (1964) J. Polymer Sci. *8* 1531

* In this context the effect of some compounds used for radiation protection and containing an S atom may be mentioned; and also the increasing of their effect when the S-atom has been replaced by a Se-atom of the electron structure $1s^2\ 2s^2\ 2p^6\ 3s^2\ 3p^6\ 3d^{10}\ 4s^2\ 4p^4$, where the outermost p electron becomes a π-electron, which is still more mobile than that in the S-atom. Thus the effect of a compound which contains Te instead of S or Se may be of some interest.

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 329—332 (1966)

The Response of Ribonuclease Level to Cellular Injury in Tobacco Leaf Tissues

(Preliminary Report)

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(Received July 29, 1966)

In plant tissues the synthesis of a number of enzymes appears to be repressed under a variety of conditions. Derepression can be achieved e.g. by treating the tissues with hormones such as gibberellic (Varner, Chandra, 1964) or indoleacetic acid (Südi, 1964; Venis, 1964). Increased enzyme levels in diseased or injured plant tissues (cf. Solymosy, Farkas, 1963) might also be due to derepression of enzyme protein synthesis evoked by substances produced upon injury. The pertaining evidence, although incomplete, is in line with the derepression hypothesis (Click, Hackett, 1963; Edelman, Hall, 1965; Minamikawa, Uritani, 1965; Farkas, Stahmann, 1966). In the present paper we report on a system which appears to be ideal for studying the biochemical mechanism responsible for the increase in enzyme levels in damaged plant tissues. The work by Diener (1961) and Shinde et al. (1964) on ribonucleases (RNase) in injured plant tissues served as a basis for the present investigations.

Leaf tissues of *White Burley* tobacco plants responded to mechanical damage with a rapid increase in RNase level. Although the method generally adopted in similar studies (gentle rubbing of the leaf surface with carborundum) gave satisfactory results it was found that the most reproducible and subtle way to induce cellular injury (stress) is the rapid infiltration of leaf disks with water in Thunberg tubes followed by the evaporation of the excess of water. The kinetics of the increase in enzyme activity in infiltrated tissues is shown in Fig. 1. It may be seen that there is a rapid increase in RNase activity which lasts for about 2 to 3 hours. It is preceded by a lag period of 1 to 2 hours. The increase in activity levels off in about 4 to 5 hours. The high enzyme level is maintained for more than 24 hours.

To shed some light on the nature of increase in RNase activity inhibitors of different modes of action were fed to the tissues by vacuum infiltration and the RNase levels were compared to those in leaf disks infiltrated with H_2O and in control tissues. Inhibitors of nucleic acid and protein synthesis equally prevented the rise in RNase activity in the infiltrated tissues. Most inhibitors not only prevented the increase in RNase activity but also reduced the RNase level well below that of the controls (Table 1). The effect of other, less specific, inhibitors

of protein synthesis (10^{-4}M 2,4-dinitrophenol, 10^{-3}M KCN) and that of the anaerobic conditions was also tested. These treatments also inhibited the increase in RNase level, although to a lesser extent.

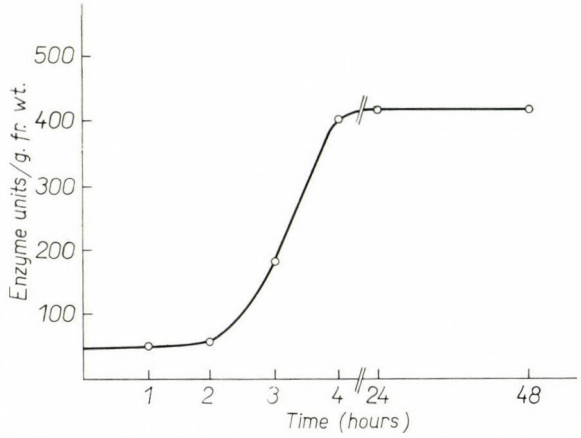

Fig. 1. Time course of the increase in ribonuclease activity in tobacco leaf tissue injured by rapid vacuum infiltration of water. RNase activity was assayed as described earlier. One enzyme unit corresponded to an increase in optical density of 0.010 at 260 mμ over the zero time control

Table 1

Effect of inhibitors of nucleic acid and protein synthesis on the increase in RNase activity in tobacco leaf tissues infiltrated with water

Disks were cut out from White Burley tobacco leaves, vacuum infiltrated with inhibitors or water and assayed 3 hours after infiltration for RNase activity.

Compound	Concentration	RNase activity in enzyme units/1 g fr. wt		
		Untreated	Tissue infiltrated with	
			H_2O	Inhibitor
8-Azaadenine	1.5×10^{-2}	192	252	52
Actinomycin D	5×10^{-5}	112	216	128
Puromycin	10^{-4}	156	320	118
p-Fluorophenylalanine	2×10^{-2}	192	256	88
Chloramphenicol	7×10^{-3}	112	216	80

The results indicate that the rapid rise in RNase activity in water-infiltrated leaves is probably due to enzyme protein synthesis. The experiments with actinomycin D suggest that DNA-dependent RNA synthesis is involved. To test the specificity of actinomycin D action the effect of mitomycin, another inhibitor known to form complexes with DNA, was also investigated. Mitomycin which is supposed to block DNA reduplication was but slightly effective in inhibiting the increase in RNase level.

There are three points which deserve special attention:

(a) The system governing the RNase level in *White Burley* tobacco leaves is apparently unique as far as its fast response to cellular injury is concerned [cf. the significantly more sluggish response of other enzymatic systems investigated so far: Click and Hackett (1963), Edelman and Hall (1965), Minamikawa and Uritani (1965), Farkas and Stahmann (1966)].

(b) The rise of RNase level is exceptionally sensitive to inhibitors of nucleic acid and protein synthesis both as far as the speed and extent of inhibiton is concerned. The speed of inhibition is particularly crucial since in the case of fast efficiency the side effects of the inhibitors are minimized.

(c) The inhibitors lowered the RNase level of infiltrated leaves well below that of the control in less than three hours. In some cases, upon 24-hour treatments with 5×10^{-5}M actinomycin D, the RNase activity almost completely disappeared from the tissues. This is suggestive of a very rapid turnover of the RNase molecule.

In addition to the above peculiarities, which make the RNase system of the *White Burley* tobacco leaf very suitable for studying the regulation of enzyme level, some properties of the RNase investigated also deserve attention. Thus, the estimation of the molecular weight of the partially purified RNase by molecular sieving on calibrated Sephadex G-100 columns, as described by Andrews (1964), gave a value of 32,000. In co-chromatography pancreatic RNase (Armour) completely separated from the partially purified tobacco enzyme.

A detailed report of the results presented is in press in Phytochemistry.

References

Andrews, P. (1964) Biochem J. *91* 222
Click, R. E., Hackett, D. P. (1963) Proc. Natl. Acad. Sci. U. S. *50* 243
Diener, T. O. (1961) Virology *14* 177
Edelman, J., Hall, M. A. (1965) Biochem. J. *95* 403
Farkas, G. L., Stahmann, M. A. (1966) Phytopathology *56* 669
Minamikawa, T., Uritani, I. (1965) J. Biochem (Tokyo) *57* 678
Shinde, B. G., Chandrasekher, B. K., Santilli, V. (1964) Phytopathology *54* 908
Solymosy, F., Farkas, G. L. (1963) Virology *21* 210
Südi, J. (1964) Nature *201* 1009
Varner, J. E., Chandra, G. R. (1964) Proc. Natl. Acad. Sci. U. S. *52* 100
Venis, M. (1964) Nature *202* 900

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1. pp. 333—344 (1966)

Proceedings of the Fourth Conference on Biophysics

Abstracts

Budapest, 1966

Magnetic Susceptometer for Studying Biological Substances

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To investigate some processes on biological objects as the molecular level an improvement of the Theorell's microbalance was constructed. The sensitivity of the apparatus is $0.0003 \cdot 10^{-6}$ cgs.

The construction of the apparatus and the measurements made on gelatin solutions of different concentrations (1 to 6 per cent) are reported. It is concluded that the Wiedemann's law is valid for gelatin solutions. The mass susceptibility of the gelatin is $-0.571 \cdot 10^{-6}$ cgs. The susceptibility of the ferric salt-gelatin solution is less than that of ferric salt dissolved in water. This is apparently due to the formation of ferric-gelatin complex.

Investigation of the Ultrasound Absorption Coefficient of the Dielectrics as a Function of Temperature

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Sound absorption in the case of gases is interpreted on the basis of the relaxation of the molecular degree of freedom. In the case of fluids the structure relaxation also contributes to this. In the case of solid bodies a whole series of causes may play a part. As it is known, the ultrasound waves are propagated in the form of pressure fluctuations in the available space. The molecules may get nearer to each other owing to the effect of induced change of pressure in fluids or solids. This nearing may disrupt the intramolecular bands and may cause errors in the electron shells of molecules. The errors of the electron shells of molecules are in relation to the polarizability of the molecules and the high ultrasound absorption coefficient of some substances can be explained by this. Therefore, the ultrasound absorption coefficients of different dielectrics were measured in the case of frequencies of 836 and 1100 kHz and investigated as a function of temperature.

The above-mentioned polarizability and the absorption of the ultrasound energy obviously occur at the irradiation of the tissues of living organisms, too, therefore experiments will be carried out in this field as well.

The Role of Model Experiments in Biophysics

L. TÓTH

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The phenomena occurring on bodies in nature (specimens) are generally very complicated. Therefore we usually choose the most characteristic parameters of the processes under investigation and create such conditions under which the processes can be well reproduced (models).

Model experiments are of a special importance in biophysics and biology. A well chosen model can promote not only the presentation of the problem, but the scientific investigation of the phenomenon, too.

In this short communication first the classification of the models is surveyed: there are geometrical, physical and mathematical models. The basic problems in connection with them will be enumerated, e.g. the application of the dimension theory and the modelling by computer. Following this the models which can be applied in the teaching of biophysics and in scientific investigations will be discussed and an experiment will be demonstrated (transistor models for the demonstration of the nerve activity).

Thermistor Thermometer Functioning on the Basis of Null Method

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The thermistor is generally used to the measurement of temperature in incompensated Wheatstone bridges. If the greatest accuracy of the thermistor is required, then the more expensive is its production, because considerable stability must be achieved.

The advantage of the thermistor thermometer constructed by the null-method is that the compensation is independent of the supply-voltage and so the application of an expensive zero instrument can be avoided.

The bridge is fed by a transistor audio-frequency generator. Headphone, loud-speaker, tuning indicator-tube and oscilloscope can be used as indicator with the help of an appropriate. The bridge is compensated by a potentiometer and every position of the potentiometer is an unambiguous function of the temperature.

The thermometer can be applied on a wide scale, among others in the medical practice, too, both as fever thermometer and as skin thermometer.

Polarizing Microscopic Studies on Insect Flight Muscle Fibrils

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Following the earlier trend of our Institute, in order to compare small differences in double refraction polarizing microscopic observations were combined with the photometric evaluation of the micrographs. Our main conclusions are as follows:

1. The E- (elongation) zones which appear beyond a sarcomere length of 5 μ exhibit a double refraction which is decreased in relation to the original double refraction of the A-band, but which is still well recognizable. In this way E-zones can be easily distinguished from the I-bands.

2. Double refracting M-lines occur in extremely stretched fibrils, a fact which shows that the primary myofilaments could not slide parallel to each other to a considerable extent.

These observations, compared with other results, show that beyond a critical sarcomere length the A-bands do not preserve their constant length and that the appearing E-zones result from the elongation of the primary myofilaments. The importance of these phenomena are discussed from the point of view of the functional mechanism of the sarcomere.

Cross Striation in the Isotropic Band

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Electron micrographs blacken according to the densities in the investigated object. This makes possible the microdensitometric investigation of substructural formations.

We observed a transversal structure in the electron microscopic picture of the isotropic band of the sartorius muscle of the frog. This structure was investigated by microdensitometry. The transversal structure of the isotropic band makes questionable that the actin and myosin filaments are able to slide on each other. Our conclusion opposes the "sliding" hypothesis by Huxley and Hanson.

Effect of Ultrasound on the ^{24}Na Exchange of the Isolated Frog Muscle

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The ^{24}Na exchange of the isolated frog muscle (sartorius) was investigated at different temperatures. Activation energy was calculated by the penetration time constants obtained from these experiments. The activation energy decreased as compared to the control in the case of irradiation by ultrasound. From this the conclusion can be drawn that the ultrasound activates the part process of metabolism controlling the penetration of the isolated frog muscle and in this way the active Na-transport has its effect.

Attempts were made to estimate that part of the free enthalpy which is necessary for 1 mol ^{24}Na to efflux from the cell, and the entropy change of the irreversible process in connection with the ^{24}Na exchange was calculated as well.

Changes of the Resting Potential of the Muscle in Case of the Constancy of Potassium Concentration

J. TIGYI

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According to the original interpretation of the membrane theory, the resting potential of the nerve and muscle is the potassium concentration potential of the internal and external potassium milieu of the fibre. Some experimental data (e.g. Koketsu and Kimura, 1960) do not support this interpretation, or rather, they can be reconciled with this theory only with an additional explanation (e.g. Kernan, 1964).

In our experiments on the separation of irritation and contraction a phenomenon was observed which seemed to contradict the membrane theory. If the sartorius muscle is kept for 60 minutes in a solution which has a salt content equal to that of the normal Ringer solution but is made 4-fold hypertonic by sucrose, the resting potential measured by micro-

electrodes decreases from the original 97 ± 3 mV to 52 ± 3 mV. At the same time the potassium content of the muscle does not decrease, or exhibits only a minimum change. The change of the resting potential is in a good approximation reversible, which makes any argument about the whole destruction of the membrane improbable.

According to our experiments, the resting potential cannot be interpreted as the potassium concentration potential.

Use of Ramp Impulse for Diminishing the Electrode Polarization at the Unipolar Recording of the Action Potentials

G. Biró, L. Királyfalvi, J. Örkényi

Biophysical Institute, Medical University, Pécs

In our experiments on the kinetics of the electrical activity of the muscle it is necessary to stimulate the muscle by placing the stimulating electrodes to both ends of the muscle. In such cases the recording electrode is between them. When the stimulation amplitude is great a very large artefact appears because of the polarization of the electrodes. This is especially large when there is a unipolar recording. For the reliable measurement of the latency, the diminishing of this artefact is necessary. — A stimulation method will be reported in which before the stimulating square wave impulse, a slowly rising ramp (linearly rising current) impulse of little amplitude and of the opposite sign is applied without a stimulating effect. This way of stimulation together with a suitable recording ensure a significant diminishing of the artefact.

Investigation of the Density of Muscle Water

S. Pócsik

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Experiments on the density of muscle water were continued. The density of muscle water was determined by measuring the mass and volume of the gradually drying up muscle. The volume of the muscle was determined by a hydrostatic method and the data were evaluated by a statistical method. The results have shown that in the gradually drying up muscle water density increases. This indicates that muscle water is being bound at an increasing rate.

Preliminary Experiments for the Interpretation of the Root Pressure by Thermoosmosis

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Biophysical Institute, Medical University, Pécs

Hypotonic fluid production against pressure can be explained by the operation of thermoosmosis. If root pressure is considered to be a hypotonic fluid production against pressure, the above mechanism can be used for its interpretation. According to our measurements the concentration of the bleeding sap of 3—7 weeks old sunflower seedlings is 5 or

6 times less than that of the sap in the root cells. It has been investigated how the concentration and quantity of the bleeding sap change as a function of time, temperature and concentration of the solution in which the roots are kept 2.4 ± 0.3 is the Q_{10} of the quantity of the sap. The greater the quantity of the bleeding sap, the smaller its concentration. These results support the view that root pressure is connected with the operation of thermoosmosis. Thus the hitherto unknown factor called "active pressure of plasma" gets a concrete meaning.

Some Problems in the Analysis of the UV Irradiation of the T7 Phages

I. TARJÁN, GY. RONTÓ

Institute of Medical Physics, Medical University, Budapest

A mathematical model is presented for the interpretation of the inactivation dose effect curves obtained after UV irradiation of the T7 phages in the case when host cell reactivation was completely inhibited. The supplied model was used to determine from the experimental curves the probability of the event with which an absorbed photon ($\lambda = 254$ nm) causes a damage on an intact phage ($\alpha = 2.5 \cdot 10^{-4}$), as well as the probability of reactivation ($\gamma = 1.9 \cdot 10^{-5}$) provoked by an absorbed UV photon. The model is suitable for the description of other authors' one hitlike dose effect curves as well.

Effect of Visible Light on the Activity of Isolated Frog Hearts

T. LAKATOS, A. KOLLÁR-MÓROCZ

Biophysical Institute, Medical University, Pécs

Isolated frog hearts arrested with K-free and Ca-free Ringer solutions were illuminated by several ten thousands lux. Later, the original solution was changed to a solution containing Na-eosin in concentrations of 10^{-5} and 10^{-6}, respectively. The hearts were then illuminated again. Most of the hearts which were arrested with K-free solution restarted to beat if illuminated in the presence of eosin, but no such effect was observed with the hearts arrested with Ca-free solution.

In our opinion the results of the experiments are in relation with the electron excitation effect of the light.

Investigation of Scintillation Induced by the β-Radiation of Tritium in the Nerve Tissue and in the Transparent Parts of the Eye

N. KÁLLAY, P. RÁCZ

Biophysical Institute, Medical University, Pécs and Ophthalmic Clinic, Medical University, Pécs

Scintillation effect by incorporated H-3 was studied in the nervus ischiadicus of frog. The light impulses obtained were counted by a photomultiplier. There was a 20 to 30 per cent scintillation effect (in case of $P = 0.001$) with fresh nerves under physiological conditions as compared to different controls. This effect increased linearly with the amount of incorpo-

rated H-3 within the limit of error in case of an activity of 0.1—0.4 mC/ml H-3. The calculated efficiency for the incorporated H-3 was 0.05 per cent.

This efficiency could be highly improved by employing optically clearer biological objects (crystalline lens, cornea), and by creating direct optical connections. When whole eyes of rabbits were incubated for 16 hours in 0.1 mC/ml H-3 solution, the cornea gave 50 to 70 per cent, and the crystalline lens 90 to 120 per cent more impulses than the control. The investigation of these scintillations should give a possibility of the localization of materials in living cells.

Scintillation Induced by Tritium β-Radiation in Cross Striated Muscle

L. KUTAS

Biophysical Institute, Medical University, Pécs

According to the investigation of our Institute the muscle in certain respects behaves in a way characteristic for the semiconductors, and several of its features can be interpreted by the supposition of the semiconductor mechanism. It is supposed that this can be utilized for the demonstration of radiation by investigating the electric conducting mechanism or the scintillation of the muscle.

In our experiments the latter was investigated on the abdomen muscle of the frog, incubated in Ringer solutions containing tritium of different activities (0.2 to 2.0 mC/ml). Scintillation was registered by a photomultiplier equipped with a one channel analyser.

According to our experimental results, the number of the light impulses coming from the muscle kept in the solution of the lowest activity, was almost the double of the control's kept in inactive Ringer solution. By muscles kept in solutions of increasing activities, the impulse number was generally proportional to the activity. Considering the sensitivity spectra of the photocathode, the wavelength of at least part of this light must be between 4000—6000 Å. As the upper limit of the energy of the radiation is 18 keV, the outcoming light cannot be the result of the Cherenkow effect.

The above discussed phenomena suggest that a semiconductor mechanism is operating and that this may play a very significant role in the further analysis of the primer mechanism of biological radiation effect.

Effect of Ionizing Radiation on Sinus Pieces of the Frog Heart

M. HAJNAL-PAPP

Biophysical Institute, Medical University, Pécs

Earlier experiments performed in our Institute showed that arrested hearts re-started to beat in part of the cases if placed in a radioactive solution. To elucidate the process, the effect of radiation on isolated sinus pieces was also investigated. The sinus of several frog's hearts were cut into 3—5 pieces. These sinus pieces regularly contract in normal Ringer solution for a day. In K-free solution 77 sinus pieces stopped after 44 minutes on the average while in Na-24 solution of a similar composition this average was 58 minutes (71 pieces). 51 pieces (40 per cent) restarted out of the 77 sinus pieces stopped in K-free solution if placed in radioactive solution. None of the pieces started if the radioactive solution was replaced by an inactive solution.

Effect of β-Radiation on the Lifetime of Sperms

A. NIEDETZKY

Biophysical Institute, Medical University, Pécs

Testes of frogs were suspended in Holtfreter solution. The suspension was divided into two parts. One of them was irradiated by a Sr^{90}-Y^{90} source of 100 mC activity. The other part was used as a control. The irradiation was performed through a mica window of 5.5 mg/cm^2 thickness. The dosage was measured by the Fricke method. The moving sperms were counted in the counting chamber (Bürker) before irradiation, and following the irradiation daily. The period during which the number of the moving sperms decreased to their half was measured (T). The mean value of T was 17.1 hours in suspensions irradiated by 20 to 1220 rad, and 30.2 hours in the control. — β-radiation caused a significant decrease in the value of T as shown by the t-test. There was a positive correlation between the dose measured in rad and the decrease of T.

Investigations on the Relative Biological Effects of Various Types of Ionizing Radiations

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Public Health Service of the Hungarian People's Army
and "Frédéric Joliot-Curie" National Research Institute
for Radiobiology and Radiohygiene, Budapest

The effect of different doses of 180-kvp X-rays, Co^{60} gamma rays and mixed neutron gamma radiation of the atomic reactor was investigated on the haematopoiesis of mice. The actual state of erythropoiesis was estimated on the basis of a 72-hour incorporation of radioactive Fe^{59} isotope by the erythrocytes and the most important depot organs — such as bone marrow, spleen and liver. The injection was given intravenously 24, 72 and 120 hours after irradiation, respectively. It was established that the changes of these indices of the iron metabolism do not depend only on the type and dose of the radiation but also on the interval between the radiation and the injection of radioactive iron. For quantitative comparison the determination of iron utilization by the erythrocytes seemed to be more appropriate than the measurement of the relative specific activity of the mentioned organs.

Factors Influencing the Accuracy of the Measurement of Incorporated Radioactive Substances

L. BOZÓKY

National Oncological Institute, Budapest

After a short description of the whole body counting apparatus constructed in the National Oncological Institute, experiments to establish the role of certain factors influencing the accuracy of measuring incorporated radioactive substances are reported.

Such factors are the effect of the fresh, from decay products of radon filtered and through the chamber perfused air as compared to the unfiltered air; the effect of the temperature increase induced by the body temperature of the examined person and by the illumination; the role of electric disturbances; the effect of the movement of external radioactive radiation sources; the role of the self-absorption of the standard KCl solution and other problems of standardization.

Investigation of the γ-Energy Dependence of the G. M. Tube Dose Meters

B. KANYÁR, J. NAGY

Institute of Medical Physics, Medical University, Budapest

The use of low-energy ($E_\gamma < 150$ keV) γ-radiation isotopes has recently gained ground in both medical and biological isotope practices. The measuring of contamination, which may appear has turned up particular problems. The instruments with GM-tube used for the measurements of the dose intensity in this energy range show a strong energy dependence, the deviation can be several hundreds per cent. This energy dependence can be decreased by the use of different absorbents.

In our measurements the so-called GK-4 type EMG 1862 and the Gamma Transrate portable dose meters, generally used in our country were investigated. By knowing the activity and the dose constant of the low-energy γ-radiation isotopes (^{57}Co, ^{241}Am, ^{125}I) used by us, we determined the counts per min. values obtained for 1 mr/hour dose intensity without absorbents and with absorbents of different qualities, thicknesses and geometries. In the case of an adequate compensation absorbent these dose meters can be used for practical purposes in the mentioned γ-energy range (perhaps in the suitable X-ray range, too).

Application of ^{85}Kr in Investigations on the Circulatory System

GY. FARKAS, GY. MOLNÁR

Third Department of Medicine, Medical University, Budapest

The advantages of the application of radioactive noble gases are pointed out. Recent experiments on the anastomoses of the portal and systemic venous circulation on dogs are reported as a continuation of the investigations discussed at the former general meeting.

^{85}Kr gas had been absorbed in physiological salt before and, in the course of the investigations was detected in the expired air. Experiments carried out on dogs have shown, that after intravenous administration the isotop appears in the air in about 5—8 seconds, in the case of the administration into the spleen, the appearance-time was about 30—35 seconds. An artificial shunt (vena lienalis — vena renalis) was made, the time of appearance was practically the same as after intravenous administration of ^{85}Kr.

According to the authors' opinion this method is suitable for human examinations, too and it is especially significant in the demonstration of anastomoses developing in liver diseases.

Technical and Dosimetric Problems of the Irradiation of Animals

K. DÓSAY, GY. KOCZKÁS

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

The unambigous and proper performance of the irradiation of animals is an essential point in radiation biology. Therefore some technical and dosimetric conditions have been worked out which will make irradiation experiments reproducible.

Convenient stocks and distance setting rods were made for the irradiation of the animals. The appropriate isodose curves were plotted. The continuous dose measurement

was introduced in the irradiation experiments and the returning dispersion values were also determined according to the irradiation conditions.

The greater dose intensities needed for the irradiation of homogenates and bacterium cultures were also recorded with different types of radiation.

Post-Irradiation Heparinemia

S. GYÖRGYI, J. GAZSÓ, J. NAGY

Institute of Medical Physics, Medical University, Budapest

Some factors playing a part in the development of coagulation disturbances caused by ionizing radiation were investigated on rabbits irradiated with a whole body dose of 1800 rad. A method was worked out for the determination of the time changes of the "heparin level" present in the circulatory system. It was found that upon irradiation the platelet count and the platelet resistance did not decrease significantly but the heparin level of the blood significantly increased, and reached its maximum on the fourth day after irradiation. The time changes of the thrombin inactivation values measured at the same time supplemented the data obtained for the heparin levels. Our results show that the prolongation of the coagulation time is caused by the accumulation of heparin or rather, by other heparin-like anti-coagulation substances.

Effect of Caffeine on the Host Cells of the T7 Phages

T. POZSONYI, GY. RONTÓ

Institute of Medical Physics, Medical University, Budapest

Treating E. coli B host cells with caffeine at different concentrations (50 to 800 mg per cent) resulted in the inhibition of host cell reactivation if the cells were infected with UV irradiated T7 phages. The plaque forming ability was unaffected even with the highest caffeine concentration used when the host cells were infected with non-irradiated phages. For the analysis of this effect of the caffeine, the developmental cycle of the T7 phages of E. coli B host cells was investigated.

According to our measurements the characteristic data of the developmental cycle of the non-irradiated phages (the adsorption velocity constant, the latency) change as a function of the caffeine concentration according to the prolongation of the cycle.

Agar Gel Diffusion Tests on Bacteriophages

GY. TAMÁS, GY. RONTÓ, T. POZSONYI

Institute of Medical Physics, Medical University, Budapest

A method has been developed for measuring diffusion coefficients of the T2 and T7 phages of E. coli B in agar gels at different concentrations.

The diffusion coefficient measured in free liquid was determined by the help of a regression equation, calculated with the least square method and was found to be in good agreement with the value of the diffusion coefficient calculated on the basis of phage adsorption.

The size of the spherical T7 phage particles was calculated from the value of the diffusion coefficient.

The resistance of the agar gel at different concentrations to the diffusion of phage particles was determined. It was found that this diffusion resistance depends very much on the size of the diffusing phage particles and on the concentration of the agar gel.

Possibilities of Using Informations Obtained from Ultrasound Holograms for Diagnostic Purposes

D. KAMOCSAY, P. GREGUSS

First Obstetrical Clinic, Medical University, Budapest

Ultrasounds have been applied for a long time for structure analyses but their use in medical diagnostics has been very restricted up to now. This is mostly so because by ultrasounds it is generally very difficult to obtain picture-like informations, similar to X-ray pictures, especially when the person to be examined is accessible from only one side. The so-called B and C photographic methods known in structure analysis, based on a dot-like scanning of the area to be examined, have certain diagnostical possibilities, but cannot substitute the informations gained by X-ray because they are far too complicated and expensive.

By developing D. Gábor's (Imperial College, London) holographical theory we found that there is no fundamental difficulty to obtain picture-like informations by using ultrasound holograms for diagnostic purposes, even in case of reflexion. A short communication is given about the first experiments bearing on this topic and about the equipment used.

Side Effect of Docaquosum (Deoxycorticosterone-glycoside) on the Activity of the Thyroid Gland

K. GÓLIÁN-BARTHA

Institute of Medical Physics, Medical University, Budapest

Studies on the side effect of Docaquosum on the activity of the thyroid gland were undertaken because of its well-known catabolic effect.

This suggestion was supported by in vivo measurements. Curves representing iodine accumulation showed significantly ($P < 0.02$) lower iodine uptake at doses of 5 mg/kg/day and 10 mg/kg/day. The amount of the precursors of the thyroid hormone (diiodotyrosine and monoiodotyrosine) significantly decreased; inorganic iodide content increased to about its double and the amount of triiodothyronine also showed a strong increase. The results were significant at $P < 0.01$.

The composition of the iodine containing hormone of the plasma significantly changed at a dose of 10 mg/kg/day: the diiodotyrosine and monoiodotyrosine contents increased by 20 to 30 per cent while the thyroxine content decreased by more than 50 per cent. The amounts of iodide and triiodothyronine remained unchanged.

It is suggested that the drug effecting primarily the production of ACTH, decreased the rate of TSH production in the hypophysis and this caused the observed decreased activity of the thyroid gland.

Printed in Hungary

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

A kézirat nyomdába érkezett: 1966. VII. 16. — Terjedelem: 11,75 (A/5) ív, 67 ábra

66.62653 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

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21. XI. 1966 Index: 26.030

VOLUME 1
NUMBER 4
1966

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)

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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).

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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.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 345—354 (1966)

Application of Gel-filtration in the Study of the Fluorescent Substances of Sclerotic Human Aortic Wall

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(Received February 9, 1966)

1. Fractions of different molecular weights found in elastase-extracts of atherosclerotic and normal human aortas were separated by gel-filtration and the fluorescence excited at 385 mμ was studied in a Spektromom 201-type spectrophotometer. It has been established that the fluorescent substance which accumulates in atherosclerosis is to be found in the high molecular weight fractions.

2. In the high molecular weight fluorescent fractions not the desmosins — which play an important role in the cross-linkage formation in elastin — are accumulated, but a so far unknown substance. The low molecular weight fractions which contain the desmosins exhibit negligible fluorescence.

3. During hydrolysis the fluorescence characteristic of atherosclerosis is considerably diminished. Since desmosins can be isolated from such hydrolysates it follows that the fluorescence investigated in the present study was not due to desmosins.

Introduction

Several groups of investigators have been concerned with the fluorescent substance of elastin (Kärkelä, Kulonen, 1959; LaBella, 1961, 1962; Loomeijer, 1958, 1961; Partridge et al., 1963; Sinex, Faris, 1962; Walford et al., 1961). The main interest has been focused on the question, whether the problem is to be solved at a molecular or macromolecular level. If the fluorescence is due to the cross-linkages within the polypeptide chains of elastin, we deal with a molecular biological problem, if, however, some other component outside the polypeptide chains of the complex-built elastic fibre is responsible for fluorescence, the problem can be solved at the macromolecular level. Recent papers (Partridge, 1962; Partridge et al., 1963; Thomas et al., 1963) have pointed to the fact that in the polypeptide chains of elastin, in addition to hydrogen bonds and other electrostatic bondings known so far, there is an other crosslinking substance, a new amino acid, called desmosin. This substance has been isolated from the yellow peptide of elastin. Its structure proved to be tetracarboxy-tetraamino acid. It is encountered in two isomeric forms (desmosin and isodesmosin).

It has been shown in our previous investigations (Banga et al., 1966) that in the elastin of atherosclerotic aortic walls the amount of fluorescent substances

increases and there is a strict correlation between the initial stage of atherosclerosis and the enhancement of fluorescence. The aim of our present work was to establish whether or not the fluorescent substance which accumulates in atherosclerosis was identical with the desmosins. After the enzymatic digestion of elastin, desmosins appear in the 1000 molecular weight polypeptide fraction (Partridge et al., 1963). Desmosins isolated after hydrolysis have a molecular weight of 550 and an absorption maximum at 275 mμ. On the basis of these data it was evident to infer that the gel-filtration of the elastolysates of aortas, by means of which polypeptides of different molecular weights can be separated, might be a suitable method for answering the above question. The results obtained give an answer also to the question, whether one or more fluorescent substances exist in the aortic wall.

Experimental

The powder of aortas examined was prepared as described earlier (Banga et al., 1966). Three extracts were prepared from the aorta-powder by solubilization with elastase, then the extracts were gel-filtered separately and the fractions obtained were analyzed.

Preparation of extracts. 1.5 g of the aorta-powder was incubated with 15 mg of crude elastase (E. U./mg = 16) in 15 ml N/20 Na_2CO_3-HCl buffer, pH 8.8, at 37°C for 16 hours. After centrifugation the supernatant was decanted and the aortalysate thus obtained was referred to as extract I. The remainder was suspended in 15 ml of the above buffer, boiled, then 15 mg of crude elastase was added. The mixture was kept at 37°C for 24 hours. The supernatant obtained after centrifugation formed extract II. The remainder of extract II was treated as that of extract I, and in this way extract III was obtained. The prolonged incubation time was necessary for the solubilization of all components of the aorta wall. The volumes of the extract were about 10 ml, each.

Gel-filtration. The extracts were gel-filtered separately. A 30 cm long Sephadex G−25 (Pharmacia, Uppsala) column 2 cm in diameter was used, equilibrated with N/20 Na_2CO_3-HCl buffer, pH 8.8. Fractions of 5 ml were collected (12 tubes). The flow rate was 1 ml per minute. The first three tubes (i. e. 15 ml) did not contain protein and failed to show any fluorescence. Therefore in the Tables data are presented only from the 4th tube.

The I−III extracts and the gel-filtration tubes were separately examined for both fluorescence and protein content without and after hydrolysis with 5 N HCl.

The data presented were derived from two normal adult aortas and four human aortas with severe atherosclerosis. The values were averaged and only the averages are indicated in the tables. The difference in fluorescence between normal and atherosclerotic aortas was so clear-cut that neither more data nor significance calculation were necessary for the comparison.

Fluorescence was measured as described previously (Banga et al., 1966), with the modification that a Spektromom 201 spectrophotometer was used, with 385 mμ activation, and that the concentration of the chinine-sulphate-standard was 50 μg/ml instead of 10 μg/ml. The calculations were changed accordingly. The measurements were controlled in a spectrofluorimeter, too. It has been established that by the used activation maximum at 385 mμ the fluorescence maximum was at 400–405 mμ. With the extracts of atherosclerotic aortas and also with the tubes of 4, 5 and 6 serial dilutions were made, partly because in some cases the fluorescence was so high that it could not be measured directly, and partly because the real value of fluorescence is given by the hyperbolic dilution curve. With the I–III extracts and that all of the gel-filtrated tubes of normal aortas maximum fluorescence could be measured also in the undiluted samples. Therefore the dilution curves did not give different results. With the atherosclerotic aortas the measured fluorescence values were multiplied by the dilution factors.

Hydrolysis. 2–3 ml of the fractions were put into bomb-tubes, then equal volumes of concentrated HCl were added, and the samples were hydrolyzed at 110°C for 16 hours. The hydrolysates were three times evaporated in a water bath, and the dry remainder was dissolved in the buffer used by preparing elastolysates.

The protein content was assayed as described in our previous paper (Banga et al., 1966).

Results

The data of aorta-extracts obtained by elastase-treatment are shown in Table 1.

The average values of fluorescence in the sclerotic aortas ranged from 1997 to 6454, while in normal aortas they ranged from 235 to 414 (Table 1, columns B and C). The difference is thus about 5–27 fold. Consequently, the amount of fluorescent substances increased owing to sclerosis. This is in accordance with our earlier findings (Banga et al., 1966).

The specific fluorescence was also considerably higher in the extracts of sclerotic aortas than in those of normal aortas (Table 1, columns D and E). The values were the lowest in extract I and gradually increased up to extract III. The difference in fluorescence between sclerotic and normal aortas was 5–16 fold (Table 1, columns F and G).

Comparing the solubilization with elastase of sclerotic and normal aortas, it was found that the sclerotic aortas dissolved somewhat better than normal ones. The lower solubility of normal aortas was shown also by the fact that extract III of normal aortas contained twice as much protein as that of sclerotic ones.

The yellow chromophoric group was found to occur in both kinds of aortas to about the same extent. The yellow substance appeared in extracts I and II, i.e.

Table 1

Data on the elastase extracts of aortas

(The average values of 4 sclerotic and 2 normal aortas are indicated)

Extract	Fluorescence measured		Specific fluorescence		Ratio of specific fluorescence		Per cent soluble protein	
	sclerotic	normal	sclerotic	normal	sclerotic	normal	sclerotic	normal
	aorta		aorta		aorta		aorta	
A	B	C	D	E	F	G	H	J
I	2579	414	75	15	5	1	64	59
II	6454	235	323	20	16	1	32	33
III	1997	405	848	89	9	1	4	8

in the more soluble fractions. The fluorescence thus could not be due entirely to the yellow chromophoric substance, since extract III, though it exhibited the strongest fluorescence, was colorless.

The data of the fractions obtained by gel-filtration are shown in Tables 2 and 3, and in Fig. 1. The fluorescence of sclerotic aortas was constantly higher than that of normal aortas (Table 2, columns C and F). The greatest difference in fluorescence between sclerotic and normal aortas was measured in tubes 4, 5, and 6, i.e. the fluorescent substance accumulated in the high molecular weight peptide fraction.

The values of specific fluorescence (Table 2, columns D and G) were also higher in the sclerotic aortas than in the normal ones. The markedly high values of tube 4, especially in sclerotic aortas, were remarkable. These values gradually increased from extract I to extract III (Fig. 1). Thus it appears that most of the specific fluorescence examined by us is bound to the largest molecular weight peptide and that the fluorescent substance is localized in the less soluble fractions. Table 3 as well as Fig. 1 show that among all fractions obtained by gel-filtration, the greatest difference in the specific fluorescence between sclerotic and normal aortas was found with fraction 4. The specific fluorescence of fraction 5 showed characteristics similar to those of fraction 4, but the values were lower and the differences in fluorescence between sclerotic and normal aortas were also smaller (Tables 2 and 3, and Fig. 1). In fractions 6 and 7 relatively high specific fluorescence values were still measured, but from tube 8 the values considerably decreased attaining a final minimum value. Here the differences in the specific fluorescence between sclerotic and normal aortas became blurred (Table 3 and Fig. 1).

The distribution of the protein content in gel-filtration tubes of normal and sclerotic aortas is shown in Table 2, columns E and H. It can be seen that the majority of proteins was found in tubes 5–8.

Table 2

Data obtained by the gel-filtration of extracts

(The average values of 4 sclerotic and 2 normal aortas are indicated.)

Extract	Tube No.	Sclerotic aorta			Normal aorta		
		fluorescence measured*	specific fluoresc.	soluble protein per cent	fluorescence measured*	specific fluoresc.	soluble protein per cent
A	B	C	D	E	F	G	H
I	4	337	569	3.4	40	10	4.5
	5	572	65	18.4	98	18	14.8
	6	505	19	19.4	88	16	15.5
	7	379	39	18.4	83	12	18.5
	8	190	25	12.7	75	10	15.0
	9	85	17	9.3	53	9	11.0
	10	72	16	7.7	45	10	7.9
	11	31	12	6.4	13	4	7.6
	12	37	12	4.5	12	2	5.2
II	4	1615	2176	4.4	58	135	2.8
	5	4608	868	10.8	143	59	15.3
	6	3880	404	19.7	115	35	16.8
	7	630	89	21.6	58	17	15.5
	8	200	84	17.2	38	10	13.4
	9	100	25	9.3	20	7	9.6
	10	77	31	6.4	4	2	7.1
	11	64	45	6.9	43	14	12.1
	12	61	34	3.7	15	8	7.4
III	4	1068	3090	10.8	98	483	3.2
	5	966	1653	19.0	215	219	18.0
	6	893	1158	18.0	140	107	22.6
	7	156	288	12.6	48	50	13.2
	8	41	85	9.7	20	28	11.9
	9	15	28	7.7	10	21	7.4
	10	16	20	10.0	5	7	8.2
	11	8	17	8.7	1	2	10.3
	12	2	3	3.5	1	2	5.2

* Calculated for undiluted material.

The yellow chromophoric substance accumulates in the aorta wall with age (LaBella, Lindsay, 1963). In our investigations we failed to find any correlation between the yellow color and the fluorescence of the fractions obtained by gel-

Table 3

Specific fluorescence ratios in the tubes obtained by gel-filtration

Tube No.	Fluorescence ratio of sclerotic to normal aortas			Average of extracts I–II–III
	I	II	III	
	extracts			
4	57 : 1	16 : 1	6 : 1	26 : 1
5	3 : 1	15 : 1	8 : 1	9 : 1
6–12 average	1 : 1	5 : 1	7 : 1	4 : 1

filtration. According to our observation tube 4 was colorless, consequently the highest molecular weight peptide did not bind any colored substance. Since the fluorescence of this tube was found to be the highest, this provides an indirect evidence that we were not dealing with the fluorescence of the yellow substance. The largest amounts of the yellow component appeared in tubes 5 and 6.

It can be stated that the yellow substance and the high fluorescence measured by us are in no connection with each other. After centrifugation of the freezed tubes a colorless and a yellow layer could be separated and their fluorescence was measured. There was no difference in fluorescence between the two layers, thus it can be established that a colorless peptide must serve as the source of fluorescence and that the yellow substance does not influence it.

On gel-filtration on Sephadex G-25 column, in the initial fractions (tubes 4–6) there were polypeptides of a molecular weight of about 4–5000. These fractions did not contain desmosin in free state, since the molecular weight of the latter is 550 (Partridge et al., 1963). This was supported also by the analysis of the hydrolysates of the fractions obtained by gel-filtration. As it has been mentioned in the introduction, desmosins were isolated from yellow polypeptides of a molecular weight of 1000 after acidic hydrolysis (Partridge et al., 1963; Thomas et al., 1963). Evidently, the desmosins are not destroyed during acid hydrolysis. On the contrary, in our experiments the fluorescent substance was either destroyed on hydrolysis in all three extracts (in tubes 4, 5, 6 and 7), or its amount was considerably reduced. These results are summarized in Table 4. As it is to be seen, the specific fluorescence of both sclerotic and normal aortas was significantly decreased upon hydrolysis. This conveys additional evidence for the independence of the observed high fluorescence and desmosins. Further investigations are needed, however, to reach a final conclusion.

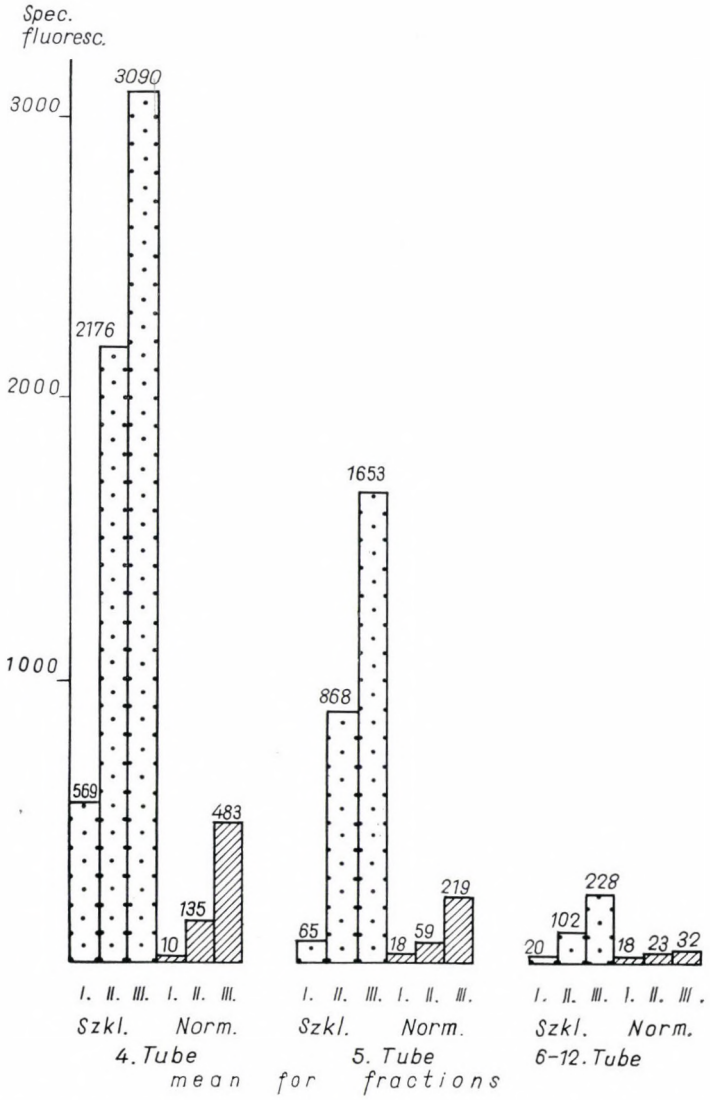

Fig. 1. Comparison of specific fluorescence values in the 4, 5 and 6—12 gel-filtrated tubes of extracts I, II and III of sclerotic and normal aortas, respectively. I, II, III: number of extract. Szkl: sclerotic aorta. Norm: normal aorta

Table 4

The effect of hydrolysis on the fluorescence of sclerotic and normal aorta walls

Aorta	Extract No.	Tube No.	Specific fluorescence	
			before hydrolysis	after hydrolysis
Normal	I	4	20	2
		5	14	10
		6	15	10
	II	4	221	146
		5	94	76
		6	53	28
	III	4	786	360
		5	255	146
Sclerotic	I	4	1580	2
		5	105	75
		6	86	40
		7	64	36
	II	4	810	140
		5	260	190
	III	4	3300	430
		5	2600	10
		6	1560	80
		7	300	2

Discussion

The fluorescent substance which accumulates in atherosclerotic aortas could be related to the so-called old-age pigment, a colored substance known for a long time to accumulate in the tissues with age. All properties characteristic of that substance (Deane, 1958) can be recognized in pure elastins isolated from aortas as well as from other tissues. Consequently, elastin also contains the yellow age pigment. This pigment is strongly bound to peptides which show a fluorescence maximum at 340 mμ when activated at 290 mμ (LaBella, 1962). On the basis of our studies we cannot decide conclusively whether the fluorescent substance demonstrated by us is identical with the age pigment. At any rate, two facts contradict the view of their being identical: first, the fluorescence did not disappear when the yellow pigment was separated from the colorless polypeptide, moreover, the colorless extracts III and its fractions obtained by gel-filtration

exhibited the highest specific fluorescence; second, in our case the fluorescence maximum was at 400 – 405 mμ when activated at 385 mμ.

On the basis of indirect evidence our results give an unequivocal negative answer to the question raised in the introduction, i.e. whether desmosins which play a role in the formation of cross-linkages in elastin are identical with the fluorescent substance examined by us. This, however, does not exclude the possibility that the accumulated substance also participates in the cross-linkages of elastin. Recently a substance, referred to as X_4, has been isolated from elastin (Franzblau et al., 1965). It was identified as N-(5 amino 5 carboxypentanyl)-lysine (Lysinonorleucine) as a new crosslinking amino acid (Franzblau et al., 1965a). Theoretically this substance – having a saturated aliphatic chain containing no conjugated double bonds – could not show any fluorescence.

As to the problem raised in the introduction, we cannot tell definitely whether a macromolecular or molecular biological solution is to be expected. The colorless fluorescent substance examined by us may play a role in the formation of cross-linkages in the elastin molecule but may also belong to such muco- or lipo-proteid components which are necessary only for holding together elastic fibrils.

The present investigations confirm our earlier conclusion (Banga et al., 1965) that there are more kinds of fluorescent substances in elastin.

In the study of the fluorescent substances of elastin the isolation of desmosins is in the center of interest. Our investigations call the attention to another fluorescent substance independent of the desmosins. By the aid of our method the accumulation of this substance which appears to be significant in the development of atherosclerosis, can be readily demonstrated.

References

Banga, I., Mayláth-Palágyi, J., Jobbágy, A. (1965) Acta Physiol. Acad. Sci. Hung. *26* 305
Banga, I., Mayláth-Palágyi, J., Jobbágy, A. (1966) Acta Physiol. Acad. Sci. Hung. *30*, 79
Deane, H. W (1958) Intracellular Lipides: Their Detection and Significance. In: Frontiers in Cytology, S. L. Palay, ed., Yale University Press, New Haven, p. 227
Franzblau, C., Sinex, F. M., Faris, B. (1965) Nature *205* 802
Franzblau, C., Sinex, F. M., Faris, B., Lampidis, R. (1965a) Biochem. Biophys. Res. Commun. *21* 575
Kärkelä, A., Kulonen, E. (1959) Acta Chem. Scand. *13* 814
LaBella, F. S. (1961) Arch. Biochem. Biophys. *93* 72
LaBella, F. S. (1962) J. Geront. *17* 8
LaBella, F. S., Lindsay, W. G. (1963) J. Geront. *18* 111
Loomeijer, F. J. (1958) Nature *182* 182
Loomeijer, F. J. (1961) J. Atheroscler. Res. *1* 62
Partridge, S. M. (1962) in Advances in Protein Chemistry, Anson, M. L. et al. (eds.), Acad. Press, New York, London, Vol. 17, p. 290
Partridge, S. M., Elsden, D. F., Thomas, J. (1963) Nature *197* 1297
Sinex, F. M., Faris, B. (1962) Fed. Proc. *21* 408
Thomas, J., Elsden, D. F., Partridge, S. M. (1963) Nature *200* 651
Walford, R. L., Moyer, D. L., Schneider, R. B. (1961) Arch. Path. *72* 158

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 355—360 (1966)

Reoxidation and Reactivation of Reduced Insulin

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(Received 25 July, 1966)

The recovery of insulin activity following the oxidation of chemically reduced insulin chains varied between 0—50 per cent according to the conditions of the reoxidation. Highest yields were obtained in presence of a copper-versene complex or 1 M LiBr. It is pointed out that the ratio of disulfide bonds to other types of interactions between different amino acid residues influences the ease with which the native conformation is formed.

Reduction of disulfide bonds of a number of proteins leads to the loss of biological activity. In some cases the reduced protein is spontaneously reactivated following the oxidation of the sulfhydryl groups (Anfinsen, Haber, 1961; White, 1961; Levinthal et al., 1962).

Insulin, which contains two inter-chain and one intra-chain disulfide bridges, is practically inactivated after reduction (Fraenkel-Conrat, Fraenkel-Conrat, 1950). The separated chains show some residual activity in several biological tests.

Reactivation experiments with reduced insulin chains have yielded variable results. Dixon and Wardlaw (1960), Wilson et al. (1962) observed the recovery of only a few per cent of the original insulin activity. If it is assumed that random combination of two cysteine residues occurs with equal probability, such a low percentage of the original activity may be expected to arise through spontaneous oxidation. The results of Dixon and Wardlaw, as well as of several later authors (Meinhofer, Brinkhof, 1963) accordingly, differ from the findings with ribonuclease, lysozyme or alkaline phosphatase. It appeared therefore, that the primary structure in itself does not determine the conformation of the insulin molecule. Anfinsen (1965) suggested that insulin may not be the direct result of proteosynthesis, instead a single chain polypeptide may be formed first which is then secondarily transformed into the final product by limited proteolysis, as chymotrypsinogen is activated to chymotrypsin. In this case reduced insulin cannot be expected to be fully reactivated. Experiments by Humbel (1965) have, however, proved that the two chains of insulin are synthesized separately.

During the past few years Tsou and co-workers (Du Yu Chang et al., 1961; Tsou et al., 1963; Du Yu Chang et al., 1965) reported more than 40 per cent reactivation of insulin from the reduced and separated chains.

The present experiments while confirming these results, were devised to study the appropriate conditions of increasing the yield of reactivated insulin.

Methods

Insulin (Richter) was a crystalline Zn-insulin assaying 22 units/mg*. Some critical experiments were performed with a preparation of pure porcine insulin. It gave the same results as those obtained with the commercially available mixed porcine and bovine insulin samples.

Disulfide bridges were broken by the use of either of two methods, sulfitolysis or electrolytic reduction.

Sulfitolysis. Insulin was dissolved in 8 M urea, and Na_2SO_3 and $CuSO_4$ were added. The final concentration of reactants was: 25 mg/ml insulin, 0.4 M Na_2SO_3, 0.08 M $CuSO_4$ and 8 M urea.

The mixture of S-sulfo-insulin chains was thoroughly dialysed against 1×10^{-3} M versene and then centrifuged in the cold. The S-sulfo-A-chain remained in solution, whereas the S-sulfo-B-chain was the main component of the precipitate. The precipitate was washed in the centrifuge tubes three times with distilled water and then the B-chain was dissolved in the reaction mixture. The separated chains were then mixed in the original ratio.

Electrolytic reduction of insulin was carried out as described by Markus (1964). The appearance of SH groups was followed by the method of Ellman (1959).

Insulin activity was assayed by the mouse convulsion test. In every case native and reactivated insulin samples were tested side by side at different levels to find out and to compare the appropriate insulin activity causing convulsion in about 50 per cent of the experimental animals.

Results

1. *Reactivation experiments using sulfitolyzed insulin*

In preliminary experiments the optimum conditions of the reactivation were studied by varying the concentration of S-sulfo-insulin, the concentration of added mercaptoethanol and the pH. In 0.05 M Tris-buffer the pH optimum was found to be 7.5—8.5, the optimum protein concentration 0.3 mg S-sulfo-insulin per ml, and the optimum amount of mercaptoethanol 1—3 mol per insulin-SH equivalent.

Under the best conditions 10—25 per cent of the original insulin activity reappeared, if the mixture was left to stand at room temperature for 18—24 hours.

* We are indebted to the firm Kőbányai Gyógyszerárugyár, Budapest for supplying us with the crystalline insulin.

Table 1

Reactivation of insulin from S-sulfo insulin chains

0.3 mg/ml S-sulfo-insulin (or equivalent amounts of S-sulfo-A and B chains) in 0.05 M Tris-buffer of pH 7.5, in presence of a 1×10^{-3} M versene and 1×10^{-5} M 2-mercaptoethanol

Reactivation form	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	dose[1]	response[2]	dose	response	dose	response	dose	response
S-sulfo-A-chain	25 30	0/5 0/5	30	0/20	3.0 4.5	0/10 0/10		
S-sulfo-B-chain	25 30	0/5 0/5	30	0/20	4.5 6.75	0/10 0/10		
S-sulfo-A-chain + S-sulfo-B-chain[3]	15	5/10	3.5 7.5 15	0/20 7/20 15/20	2.6 5.2 7.8	0/5 4/10 8/10		
S-sulfo-insulin[4]	15	6/10	3.0 7.5 15	0/20 11/20 15/20	3.3 6.6 8.2 9.9	0/5 5/10 7/10 8/10	3.75 5.6 7.5 15	4/18 4/5 6/6 18/18
native insulin	1.0 1.5	1/10 3/10	1.0 1.5	3/20 9/20	1.0 1.4	4/10 7/10	1.0 1.5	0/11 9/11

[1] μg/mouse, [2] number of animals displaying convulsion per number of mice in the group, [3] chains separated and mixed in the original ratio, [4] chains not separated.

In some experiments with longer incubation periods further increase in the activity was observed. Table 1 is a summary of such a series of experiments under the following conditions: 0.3 mg/ml S-sulfo-insulin in 0.05 M Tris-buffer pH 7.5 and 5×10^{-5} M mercaptoethanol.

2. *Reactivation experiments using electrolytically reduced insulin*

For the electrolytic reduction a 5 mg/ml insulin solution was used. Reduction was followed by SH determination until the number of SH groups reached the plateau value of 6 per mol.

The reduced insulin solution was diluted to an insulin concentration of 0.3 mg/ml in presence of 0.05 M Tris-buffer, pH 7.5 and various additions as

Table 2

Reactivation of insulin after electrolytic reduction

0.3 mg/ml reduced insulin in 0.05 M Tris-buffer of pH 7.5, in presence of 1×10^{-3} M versene and 1×10^{-5} M 2-mercaptoethanol. Other additions as indicated

Exp. No.	Additions		Reactivated insulin		Control native insulin	
	1×10^{-3}M Cu^{++}	1 M LiBr	dose[1]	response[2]	dose	response
1.	+	−	15	4/9	3.0	5/10
2.	+	−	20	9/10	2.0	5/10
3.	+	−	7.5	5/10	1.5	5/10
4.	+	−	4.5	3/10	1.5	4/10
5.	−	+	5.0	5/10	1.5	3/10
	−	+			3.0	6/10
6.	−	+	3.0	2/8	1.0	0/8
	−	+	6.0	5/8	1.5	4/8
7.	−	+	5.0	5/10	3.0	6/10
8.*	+	−	2.0	8/20	1.0	9/20
	+	−	3.0	15/20	1.5	15/20
9.*	−	+	3.0	3/10	1.0	1/10
	−	+	4.0	4/10	2.0	5/10
	−	+	6.0	7/10	3.0	7/10

* In the last two experiments insulin was reduced in a concentration of 0,3 mg/ml, whereas in all the previous experiments reduction was performed at an insulin concentration of 10 mg/ml. — [1] μg/mouse, [2] number of mice displaying convulsions per total number of mice in the group.

listed in Table 2. The solutions were left to stand at room temperature exposed to air for 24—28 hours.

With no additions, electrolytically reduced insulin was reactivated only to a few per cent. Addition of 5×10^{-5} mercaptoethanol increased the recovery of active insulin to about 10 per cent. The same extent of insulin reactivation was achieved if Cu^{++} ions were added in a concentration of 10^{-7} to 10^{-8} M (in absence of mercaptoethanol).

Higher levels of reactivation were observed if 1×10^{-3} M Cu^{++} and 1×10^{-3} M versene were added together with 5×10^{-5} M mercaptoethanol: the reactivation reached 10—25 per cent in these cases.

The yield could be further improved when insulin was electrolytically reduced in a concentration of 0.3 mg/ml and this solution was supplemented with Cu^{++} and versene (1×10^{-3} M each) and mercaptoethanol (5×10^{-5} M). The extent of recovery of insulin activity after reoxidation was between 20 and 50 per cent of the original insulin activity.

Several other ions and salts were tested which did not give any results comparable to the effect of copperversene. However, positive results were achieved

with LiBr. 30 – 50 per cent of the insulin activity could be recovered after oxidation, if the electrolytically reduced insulin was incubated in presence of 1×10^{-5} M mercaptoethanol + 1 M LiBr. The extent of reactivation by LiBr was independent of the concentration at which the insulin was reduced in contrast to the previous observation with Cu^{++} + versene.

Discussion

The results presented in this paper confirm those obtained by Tsou et al. that a substantial part of the insulin activity may be recovered during the spontaneous oxidation of the inactive reduced insulin chains.

It appears that the conditions of reoxidation determine the extent to which the correct pairing of cysteine residues into the disulfide bonds of insulin is achieved. Whereas ribonuclease is spontaneously reactivated in dilute salt solutions, the same conditions do not ensure the formation of the native conformation of the insulin chains.

The model experiments in which we were able to increase the yield of the presumably native conformation of insulin may be interpreted in the following way. Copper ions form chelate complexes with the polypeptide chain. On the other hand, 1 M LiBr is known to have an effect on proteins and polypeptides which is reflected by a strong decrease of levorotation. Both of these agents decrease the freedom of rotation of the CONH bonds, and their effect in increasing the yield of active insulin may be attributed to such structural influences.

The observation that reduced insulin cannot be reactivated in dilute buffer solutions does not contradict the presently accepted hypothesis that the conformation of a protein is determined by its primary structure.

A number of interactions take place between different residues of a polypeptide chain: a certain set of interactions occur in a certain medium. As a result of these interactions the SH groups may become correctly paired in each other's vicinity, thereby allowing the formation (directly after a disulfide-sulfhydryl exchange reaction) of disulfide bonds. Due to the cooperative nature of these forces, the greater the number of other forces (hydrophobic bonds, hydrogen bonds, electrostatic interactions) in relation to the number of disulfide bonds, the easier will be the formation of the correct disulfide bridges. Reduced ribonuclease contains 8 SH groups in a polypeptide of 124 amino acids, reduced insulin has 6 SH groups in the two chains of altogether 51 amino acids. The ratio of cysteine to total amino acids is 1 : 8.5 for insulin, and 1 : 15.5 for ribonuclease. This alone may explain the failure of the reduced insulin to assume its native conformation in dilute salt solutions. Such artificial influences as exerted by the presence of copper ions or LiBr on the conformation of the insulin chains appear to be needed to force the polypeptide chain into a more advantageous conformation.

The in vivo newly synthesized insulin chains are subject to environmental influences which are certainly not completely simulated by a dilute buffer solution

of pH 7.5. The lipids of the endoplasmatic reticulum or other factors may restrain the rotation of the peptide bonds and force the nascent polypeptides in vivo into a conformation favourable for the formation of the correct disulfide bonds.

References

Anfinsen, C. B. (1965) Proc. Nat. Acad. Sci. U. S. *53* 684
Anfinsen, C. B., Haber, E. (1961) J. Biol. Chem. *236* 1361
Dixon, G. H., Wardlaw, A. C. (1960) Nature *188* 721
Du Yu Chang, Chang Yu Shang, Lu Zi Xian, Tsou Chen Lu (1961) Scientia Sinica *10* 84
Du Yu Chang, Jiang Rong Quing, Tsou Chen Lu (1965) Scientia Sinica *2* 239
Ellman, G. L. (1959) Arch. Biochem. Biophys. *82* 70
Fraenkel-Conrat, J., Fraenkel-Conrat, H. (1950) Biochim. Biophys. Acta *5* 89
Humbel, R. E. (1965) Proc. Nat. Acad. Sci, U. S. *53* 853
Levinthal, C., Singer, E. R., Fetherolf, K. (1962) Proc. Nat. Acad. Sci. U. S. *48* 1230
Markus, G. (1964) J. Biol. Chem. *239* 4162
Meinhofer, J., Brinkhof, O. (1963) Nature *199* 1095
Tsou Chen Lu, Du Yu Chang, Yu Geng Yun (1963) Scientia Sinica *12* 4522
White, F. H., Jr. (1961) J. Biol. Chem. *236* 1353
Wilson, S., Dixon, G. H., Wardlaw, A. C. (1962) Biochim. Biophys. Acta *62* 483

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 361—368 (1966)

Quantitative Starch Gel Electrophoresis of Rat and Rabbit Sera by Dissolution of the Gel

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(Received March 25, 1966)

The simple and easy method of Habermann and Szopa (1959) is adapted for quantitative starch gel electrophoresis, without rendering the gel transparent and without using a scanner. After staining, the sliced and dried strips are dissolved in hot formic acid and the extinctions of the solutions are determined in a photometer. Data on the number and proportion of protein fractions in rat and rabbit sera, respectively, are given.

Since starch-gel electrophoresis was introduced by Smithies (1955) many attempts have been made to apply this method in quantitative determinations. The usual way to achieve this was to record the absorbancy of the transparent gel strips by a convenient scanner (Fine, Waszczenko, 1958; Pert, Kutt, 1958; Pert et al., 1959; Smithies, 1959; Vesselinowitch, 1958; Kunio Matsui, Kei Yaeno, 1963).

If gel strips are stained in methanol containing solutions they become opaque and have to be rendered transparent for photometry. This can be achieved by using benzyl alcohol, glycerol or by covering the gel strip with an agar layer (Smithies, 1959). In neither case are the gel strips easy to handle and the operation requires some manual skill.

Gel strips can be stained in aqueous solutions, too, to avoid opacity. However, in this case differentiation will set in much later (in about some days) and the strips break most easily.

With direct photometry some problems still remain in evaluating the absorbancy of different protein peaks (Smithies, 1959).

In view of the problems mentioned above and in want of a scanner, we adapted Habermann and Szopa's (1959) special method which eliminates direct photometry. Determination of the protein content in the separated fractions is performed by dissolving the sliced gel strips in hot formic acid and reading the absorbancy of the protein-bound dissolved dye in a photometer. Data on protein ratios in rat and rabbit sera determined by this method are given below.

Materials

Chemically pure potato starch was hydrolyzed according to the method of Smithies (1955). Hydrolysis generally required about 90 minutes.

A tris-EDTA-borate buffer, originally suggested by A. C. Peacock for acrylamide gel electrophoresis in a personal communication to Ferris (1964) and recommended by the latter to the author of this paper, was used with a slight modification for preparing the gel as follows. 10.8 g tris (tris/hydroxymethyl aminomethane), 0.8 g EDTA (ethylendiamine tetraacetic acid) and 5.5 g boric acid were dissolved in distilled water. About 25 ml of N NaOH were added to adjust the pH to 8.6. The mixture was finally filled up to 1 liter. This stock solution was diluted 1 : 2 with distilled water before use. This buffer solution was preferred to Poulik's (1957) tris buffer, because it contained less of the expensive tris and gave more uniform results in a series of runs.

In the electrophoresis apparatus and for the "bridge" solution the borate buffer of Moretti et al. (1957) was used.

Serum pools were obtained by centrifuging the mixed blood of 5–12 rats. Rabbit sera were prepared from the blood of individual animals. Blood was withdrawn by heart puncture with rats and from the ear vein with rabbits.

Apparatus

Two, 220 mm long, 120 mm wide and 10 mm thick trays made of "Plexiglass" sheets – fitted together according to Moretti et al. (1957) with some modifications served as gel trays (Fig. 1). Each of the trays is constructed as follows. A 2 mm thick plexi sheet forms the bottom. A 1 mm plexi frame with two, parallel, 195 mm long, 35 mm wide openings is stuck on the bottom (A). Further two, 2 mm thick, detachable plexi frames – both of them with two openings of the same dimensions as above – are placed on top of the first two sheets (B). A further, 1 mm thick identical plexi frame is placed on top of the others and finally a 2 mm thick lid (C). This latter has only four slots (35×10 mm) which are located above the ends of the grooves formed by the frames fixed together. The role of these slots is to hold filter paper bridges between the gel and the buffer containers. Practically, all these sheets are fixed tightly together by two bars which protrude from the bottom sheet and which fit into the respective holes close to both longitudinal ends of each plexi sheet and keep them firmly in place. With this arrangement in our system the total thickness of the trays is 10 mm, the depth of the grooves holding the gel is 6 mm.

The advantage of these gel trays is that they can be varied according to special wishes. Dimensions of the trays and grooves, form, size and thickness of the plexi layers might be changed if needed. Still, it is not advisable to increase the thickness of the gel over 8–10 mm (Smithies, 1955).

An LKB Power Supply, type 3371 B, was used to supply constant current.

The absorbancy of the dissolved gel strips at 6300 Å was determined by a UNICAM Sp 600 spectrophotometer.

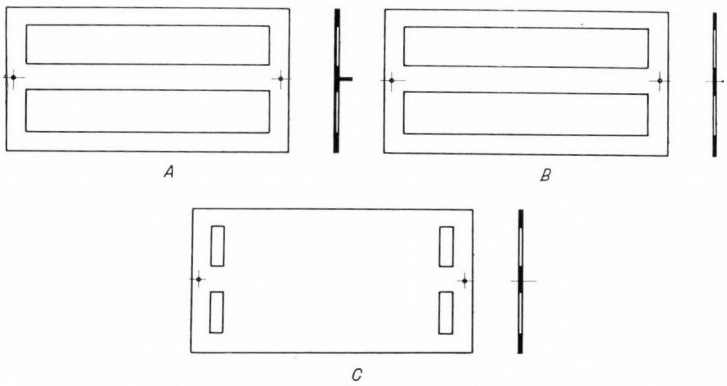

Fig. 1. Design of the plexi sheets forming the gel tray

Dimensions of the gel tray: 220×120×2 mm plexi sheet forms the bottom. A one mm thick frame is stuck on the bottom sheet, with two, 195×35 mm openings (A). Additional two, 2 mm and one, 1 mm thick, detachable frames of the same dimensions (B) are put on the bars protruding from the bottom sheet. The 2 mm thick lid of the gel tray (C) has the same dimensions as the bottom sheet, with two 35×10 mm slots at both ends to hold filter paper wicks. The plexi sheets and frames form a tray 10 mm thick, with two grooves 6 mm deep for holding the gel.

Methods

Preparation of the gel and insertion of the sample

The method of Smithies (1955) was applied using the modified buffer of Peacock mentioned above. 18 g hydrolyzed starch are mixed with 124 ml buffer. After boiling, the air bubbles are expelled by a vacuum pump and the gel is poured into the grooves of the tray. The gel is left to cool for about 5 hours at room temperature.

The gel under the lid's slot is cut and lifted with a spatula. Two, 6 mm deep, 35×10 mm large openings are thus formed for the insertion of filter paper bridges. The insertion of the serum sample is performed as follows. The lid is lifted for a few minutes and a cut is made through the whole width of the gel with a sharp metal spatula at 4 cm to the cathode end. Three 5×30 mm filter paper strips (Macheray–Nagel 214) are fitted together, soaked in the serum to be examined and slightly dried on filter paper to eliminate excess liquid. The paper strips are then placed into the cut formed with the spatula. The liquid content of 3 paper strips represents 0.12 ml. Thus, four serum samples might be introduced into the grooves. If more parallels are needed, a single serum can be examined in all the grooves, or four different sera in one run.

Electrophoresis

The trays are put into the apparatus vertically. Connection between gel and buffer solution is made by 6 layer strips of filter paper soaked in borate buffer. The ends are folded two or three times and pressed into the corresponding slot of the tray.

Electrophoresis is carried out at 300 to 330 V and 17 to 18 mA for 17 hours at room temperature. Under these conditions albumin migrates about 8 to 9 cm. This migration distance was found optimal to obtain sharp bands without remarkable diffusion.

Staining and washing

After the respective plexi sheets had been dismantled, 1 mm thick layers are cut off the surface of the gel with a wire and discarded to eliminate possible distortions; additional two, 2 mm thick gel strips are obtained from each groove and stained. The 1 mm thick gel layers left on the bottom of the grooves are discarded. Thus, eight strips result from each run, since two gel trays with two grooves each, are used. Strips 2 mm thick were used because under the experimental conditions photometry of the bands gave convenient extinction values. They were neither too high, nor too low to be read in the photometer.

The eight gel strips are stained for 30 minutes with the methanol-containing acetic acid-water amidoblack solution of Smithies (1955); but the concentration of the dye is lower: 0.2 per cent. Half a liter of this solution is used to stain the gels from ten runs. Differentiation with the same composition of solvent without dye is accomplished in 24 hours. The solvent can be reused two or three times after decolourizing it with charcoal, but not more because its methanol content decreases. Prior to any quantitative determination fresh solvent is poured on the strips which are then left to stand for at least 48 hours.

Dissolution of the gel and photometry

The gel strips are sliced according to the well distinguishable protein bands with a sharp knife or razor blade. If trailings or distortions are seen on either side of the gel strip, the strip is to be discarded.

In each experiment several protein-free parts are cut from the gel and used for the determination of the correction factor for blanks.

Both the protein-containing and the protein-free slices are left do dry at room-temperature for at least two days. The dry separated slices are weighed on an analytical balance with a mg accuracy. Each slice is put into a separate test tube. 3 ml (to the albumin containing slice 6 ml) of chemically pure, 98 per cent formic acid are added by a convenient automatic pipette or burette to each test tube and the tubes with the slices are left covered overnight. Next day the tubes with the swollen slices are placed for 10 min in a boiling water bath and shaken

while hot to ensure perfect dissolution. After cooling, 3 ml (6 ml to the albumin containing slice) of N NaOH are added. The absorbancy of the blue solution is determined in the spectrophotometer in a 20 mm cuvette at 6300 Å against distilled water. The absorbancy of the stained albumin fraction is determined in a 5 mm cuvette and the value multiplied by eight.

Calculation

Extinction values corresponding to the protein content of each slice are calculated according to the formula (Habermann, Szopa, 1959):

$$Ext_{prot} = Ext_t - \frac{Ext_{blank}}{W_{blank}} \cdot W_{prot}$$

where

Ext_{prot} : extinction corresponding to the protein content of the slice;
Ext_t : total extinction of the protein containing slice as read in the photometer;
Ext_{blank} : extinction of the protein-free slice (mean value);
W_{blank} : weight of the protein-free slice in mg (mean value);
W_{prot} : weight of the protein containing slice in mg.

In the same experiment (same gel, electrophoretic run, staining procedure) instead of the $\frac{Ext_{blank}}{W_{blank}}$ ratio, a correction factor, calculated from several E_{blank} and W_{blank} values, respectively, is used.

The aim of calculating the correction factor is to eliminate the value of gel-bound dye in the protein-free parts of the gel slice. Thus, several protein-free parts are cut off the gel (generally one part out of each of the eight strips to be determined). After drying, weighing and dissolving them, their extinctions are measured in the photometer. To obtain correction factors, extinction values should be divided by mg weights. The mean of several correction factors is taken for the correction of the extinctions obtained with the protein containing slices. Each mg weight of a protein containing slice is multiplied by the correction factor and this value is substracted from the total extinction of the protein containing slice. This makes it possible to eliminate background staining independently of the width of protein bands.

For the illustration of what has been said above, data on the determination and evaluation of the mean of the correction factors of several gel strips and values of a single gel strip are presented in Tables 1 and 2. Data in Table 2 were corrected by the mean correction factor indicated in Table 1. The mean correction factor may be used for a series of gel strips of the same experiment.

In a series of experiments rat serum pools and individual rabbit sera were examined according to the method described. Under our experimental conditions

Table 1

Calculation of the mean correction factor

Determination of extinction values of protein-free gel bands to eliminate background staining. Out of each of 8 gel strips of a single electrophoretic run, protein-free parts were cut, their weights and extinctions measured. The mean value of the correction factor is calculated from 8 determinations

Number	W_{blank} mg	Ext_{blank}	$\frac{Ext_{blank}}{W_{blank}}$
1	326	0.18	0.00055
2	324	0.17	0.00053
3	255	0.13	0.00051
4	264	0.13	0.00049
5	229	0.11	0.00048
6	239	0.13	0.00054
7	284	0.15	0.00053
8	337	0.16	0.00048

$$\text{Correction factor} \left[\frac{Ext_{blank}}{W_{blank}}\right] \text{ mean} = 0.00051$$

Table 2

Protein distribution in a gel strip

In every gel strip originating from an electrophoretic run ten protein bands were cut, weighed, dissolved and measured in the photometer. Total extinctions were corrected by the mean correction factor of Table 1. In this way the the extinction of the background was eliminated. The corrected extinctions represent true protein content

Number of fractions	Weight of slices mg	Ext_t at 6300 Å	Ext_{prot}	Distributions of fractions, per cent
1	220	0.23	0.12	1.9
2	85	0.35	0.31	4.8
3	60	0.24	0.21	3.2
4	64	0.26	0.23	3.6
5	86	0.18	0.14	2.2
6	79	0.19	0.15	2.3
7	91	0.47	0.42	6.5
8	116	0.52	0.46	7.1
9	91	0.28	0.23	3.6
10	215	4.32*	4.21	65
			6.48	100.2

* Calculated from the extinction read (see text).

Table 3

Percentage distribution of serum proteins in starch gel

Rat and rabbit sera of normal and X-ray irradiated animals were subjected to starch gel electrophoresis. The difference in the percentage distribution of protein fractions between normal and treated animals was estimated
* = ± deviation; ** = ± deviation in per cent;

Sample	Number of determinations	Fraction number										
		1	2	3	4	5	6	7	8	9	10	11
Rat serum pool No. 17/1 (normal)	13	1.7 *0.44 **26.0	9.7 1.8 18.6	6 0.8 12.9	8 0.88 11.0	5.5 1 18.2	11.5 1.3 11.2	2.6 0.4 16.4	2.6 0.5 20.0	4.2 0.7 15.8	47 5.5 11.6	1.5 0.24 16.0
Rat serum pool No. 17/3 (treated)	10	1.6 0.33 21.0	8.4 0.13 15.1	6.6 0.11 16.0	8.5 0.10 12.1	4.4 0.6 13.8	9.1 0.9 9.9	2.4 0.38 15.8	3.1 0.37 12.0	4.5 0.87 19.3	51 2.8 5.5	1.2 0.20 16.7
Rabbit serum No. 19 (normal)	12	1.4 0.28 20.0	5.6 0.43 8.0	3.3 0.35 10.6	4.2 0.46 11.0	2.3 0.23 10.0	2.3 0.23 10.0	7.5 0.44 5.9	7.1 0.32 4.5	3.1 0.30 9.6	64 1.1 1.7	—
Rabbit serum No. 19/a (treated)	10	1.7 0.30 17.6	5.4 1.06 19.6	3.2 0.58 20.4	5.1 0.69 13.5	4.0 0.46 11.5	4.2 0.38 9.1	11.8 1.39 11.8	7.9 0.61 7.7	3.3 0.29 9.8	54 4.3 8.0	—

rat and rabbit sera were generally separated into 10 to 11 fractions. Sometimes, if the bands were not sharp enough, two or three fractions could not be determined separately. The composition of rat and rabbit sera is somewhat different. No prealbumin was found in rabbit sera. Percentage distribution is given in Table 3 and the nomenclature of fractions according to Smithies in Fig. 2.

The changes induced by treatment in the percentage distribution of proteins in rat and rabbit sera are easy to follow. Determinations were made in the sera from blood pools both from normal and treated* rats. As regards rabbits, blood was withdrawn prior to and after experiment from the same animal.

In quantitative determinations deviations from the mean value are normally about ± 10 per cent. In some cases, in particular when fractions are very small, deviations amount to about ± 20 per cent.

The method provides useful information on experimental and pathological changes in the composition of serum proteins.

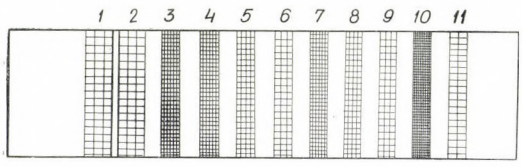

Fig. 2. Nomenclature of rat serum protein fractions according to Smithies
1. γ globulin, 2. γ globulin, 3. β lipoprotein, 4. S α_2, 5. $\alpha\beta$ (haptoglobin region), 6. post β (α_2), 7. β globulin (transferrin), 8. F α_2, 9. post albumin, 10. albumin, 11. prealbumin (α_1, orosomucoid)

Acknowledgement

I wish to thank Dr. B. Dalos, head of the Department, for encouragement and helpfu l advice.

References

Ferris, T. G., Easterling, R. E., Budd, R. E. (1964) Anal. Biochem. *8* 477
Fine, J. M., Waszczenko, Z. E. (1958) Nature *181* 269
Habermann, E., Szopa, B. (1959) Ztschr. ges. Exp. Med. *131* 520
Kunio Matsui, Kei Yaeno (1963) Anal. Biochem. *6* 491
Moretti, J., Boussier, G., Jayle, M. F. (1957) Bull. Soc. Chim. Biol. *39* 593
Pert, J. H., Kutt, H. (1958) Proc. Soc. Exp. Biol. Med. *99* 181
Pert, J. H., Engle, R. E., Woods, K. R., Sleisinger, M. H. (1959) J. Lab. Clin. Med. *54* 572
Poulik, M. D. (1957) Nature *180* 1477
mithies, O. (1955) Biochem. J. *61* 629
mithies, O. (1959) Adv. Protein. Chem. *14* 65
Vesselinowitch, S. D. (1958) Nature *182* 665

* These experiments will be reported later

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 369—378 (1966)

Steroid Biosynthetic Studies with Rabbit Adrenal Tissue

Metabolism of Progesterone-4-^{14}C, Deoxycorticosterone-1,2-^{3}H, Corticosterone-1,2-^{3}H and 11-Dehydrocorticosterone-1,2-^{3}H

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(Received July 19, 1966)

Incubation of rabbit adrenal slices with progesterone-4-^{14}C, corticosterone-1,2-^{3}H, 11-dehydrocorticosterone-1,2-^{3}H and deoxycorticosterone-1,2-^{3}H yielded numerous metabolites. The main biosynthetic sequence leading to aldosterone was progesterone → deoxycorticosterone → corticosterone → 18-OH-corticosterone → aldosterone. In addition 11-dehydrocorticosterone-1,2-^{3}H was converted to 18-OH-11-dehydrocorticosterone and aldosterone, proving the existence of alternative pathways leading to aldosterone. Corticosterone-1,2-^{3}H was converted to 11-dehydrocorticosterone. The results indicate that steroids with an 11-keto configuration may undergo further transformations and can serve as precursors to 18-oxygenated corticosteroids.

Introduction

In a previous paper (Fazekas, 1961) we have described the *in vitro* metabolism of 11-dehydrocorticosterone by rabbit adrenal tissue. According to the results 11-dehydrocorticosterone, occurring in significant amounts in the adrenal cortex of the rabbit is not an end product, but can serve as biosynthetic precursor to various polyhydroxylated corticosteroids. In recent years we have extended our studies using labelled steroid precursors and investigated their conversion into various corticosteroids in the rabbit adrenal. The present paper is dealing with our results obtained by the *in vitro* incubation of rabbit adrenal slices with progesterone-4-^{14}C, deoxycorticosterone-1,2-^{3}H, corticosterone-1,2-^{3}H and 11-dehydrocorticosterone-1,2-^{3}H.

Materials and Methods

Steroids

The labelled steroids were obtained commercially from the Radiochemical Centre, Amersham (progesterone-4-^{14}C) and New England Nuclear Corp. (tritiated steroids). Steroids were made up in stock solutions, with specific activities as follows: 1. Progesterone-4-^{14}C: 1.4 μC/μM; 2. Deoxycorticosterone-1,2-^{3}H: 62.7 μC/μM; 3. Corticosterone-1,2-^{3}H 71.4 μC/μM; 4. 11-dehydrocorticosterone-

1.2-^{3}H: 68 μC/μM. The radiochemical purity of the steroid substrates was checked by paper chromatography. The non-radioactive steroids (progesterone, deoxycorticosterone, corticosterone, 11-dehydrocorticosterone and aldosterone) used for carrier dilution techniques and as reference substances, were of commercial origin. 18-hydroxycorticosterone, 18-hydroxy-11-dehydrocorticosterone and 6-β-hydroxy-11-dehydrocorticosterone were generously gifted by Dr. R. Neher, of Ciba, Basle, Switzerland.

Adrenal tissue

Adrenals for various incubations were obtained from 2 rabbits. As the steroid transformations, apart from slight quantitative differences, were uniform, results obtained in one series of experiments with the adrenals of the same animal are described here. After killing the rabbit, adrenals were removed immediately, and cut into slices 0.3—0.5 mm thick.

Incubations

Four separate incubations were carried out simultaneously. 100 mg of adrenal slices in each flask were incubated in the presence of labelled steroid substrates as follows:

Flask N°	Steroid Substrate	Quantity
1	Progresterone-4-^{14}C	110 μg, 0.5 μC
2	Corticosterone-1,2-^{3}H	100 μg, 21 μC
3	11-dehydrocortico-sterone-1,2-^{3}H	100 μg, 20 μC
4	Deoxycorticosterone-1,2-^{3}H	100 μg, 19 μC

Steroids were dissolved in 0.1 ml of propylene glycol in the different flasks prior to incubation.

Adrenal slices were incubated in Krebs—Ringer-phosphate solution, 3 ml/flask, containing 200 mg per cent glucose, 10^{-3} M NAD, 10^{-3} M NADP, 10^{-3} ATP and 10^{-3} M Na-fumarate. (Light, BDH), at pH 7.4, 37°C for 3 hours.

Extraction

After incubation steroids were extracted by the addition of 2 vols of acetone. The acetone was filtered, distilled off and the aqueous phase extracted 3× with methylene chloride. The methylene chloride fractions were pooled, dried over anhydrous sodium sulphate and evaporated to dryness. The dry residue was partitioned between 20 ml each of 70% aqueous ethanol and n-hexane. The ethanol layer was evaporated to dryness and the residue was used for chromato-

graphic fractionation. All evaporations were carried out in a water bath with a temperature not exceeding 45°C.

Paper chromatography

Steroids were run on strips of Whatman N°1 paper, with descending method at room temperature. The following chromatographic systems were used:
1. A, B_1, B_3, B_5 systems of Bush (1952)
2. Formamide: benzene system of Zaffaroni (1953)

Column chromatography

For the purification of several acetylated derivatives 1.5 g silica-gel (Davison, grade 12) columns were used. The following solvent sequence was used:

1. 20 ml of petroleum aether
2. 20 ml of 1 per cent ethylacetate in petroleum ether
3. 20 ml of 5 per cent ethylacetate in petroleum ether
4. 20 ml of 10 per cent ethylacetate in petroleum ether
5. 20 ml of 20 per cent ethylacetate in petroleum ether
6. 20 ml of 50 per cent ethylacetate in petroleum ether

By applying this solvent sequence, the steroids in question were eluted with fractions 3, 4 and 5 whereas the apolar contaminants with fractions 1 and 2. For quantitative measurements a blank column was run parallel.

Quantitative measurement of steroids

Steroids on the paper chromatograms were detected in UV light (Chromatolite). Spots were eluted with ethyl acetate : methanol 1 : 1 (Smith, 1960). The recovery of elution was 100% as checked by cortisol-4-^{14}C.

The quantity of UV absorbing corticosteroids was determined in a Unicam SP 500 spectrophotometer by measuring the O.D. at 240 mμ in methanol. The purity of the steroid was controlled by taking readings at 225 and 255 mμ.

Specific activity of isolated steroids

Steroids from the incubates were isolated by the addition of non-radioactive carriers. Purification was continued until the specific activity after at least 2 consecutive derivatives and chromatographies differed by less than 10 per cent. The quantitative measurement of the radioactivity of the ^{14}C labelled samples was performed on alumina planchettes at infinite thinness with a thin end window tube at 6 per cent efficiency. The measurement of tritiated samples was performed on copper plates, in a windowless gas flow counter at 30 per cent efficiency. All

radioactive counting was carried out with a statistical error of less than 5 per cent. The radioactive areas on the paper chromatogram were detected by running through a radiochromatogram scanner (Nuclear Chicago, Actigraph II.)

Microchemical reactions of steroids

The microchemical reactions used for derivative formation were carried out in glass-stoppered tubes. Stercids were acetylated by the method described elsewhere (Fazekas, 1961). Hydrolysis of steroid-acetates was achieved by the method of Meyer (1953). Oxidations with chromic acid were carried out according to Lieberman et al. (1953).

For the oxidation of aldosterone 18-21-diacetate to aldosterone 18-lactone-21-monoacetate the same procedure was used, except that the time of oxidation was 10 min. (Mattox, Mason, 1956).

Results

After the incubation, steroids were extracted as described and the extracts were chromatographed in solvent system B_5. The chromatograms were run through the radioscanner and the distribution of radioactivity was recorded (Fig. 1).

Analysis of Progesterone-4-^{14}C incubate

The radioactive area, near the end of the strip was eluted and rerun in system B_3. Four radioactive spots were separated, three of which migrated with authentic corticosterone, 11-dehydrocorticosterone and deoxycorticosterone, respectively. The fourth substance, near the solvent front run with authentic progesterone. Corticosterone, 11-dehydrocorticosterone and deoxycorticosterone were acetylated and the acetates run in system B_1, where they migrated with the authentic acetates. Progesterone was rerun in the Bush A system. The steroid acetates were then purified on a silica-gel column and their specific activity was measured. After this step the acetates were hydrolyzed back to the free steroid alcohols and run again through a silica-gel column, where the specific activities remained constant. Results are summarized in Table 1.

The specific activity of progesterone-4-^{14}C, isolated from the incubate was 293 cpm/μg. The quantity of the diluting, endogenously synthesized non-radioactive progesterone was calculated by using the formula of isotope dilution. This indicates an intensive progesterone synthesis (440 μg/g/3 hrs) in rabbit adrenal.

Aldosterone ($R_f = 0.39$) was isolated by the carrier dilution technique. 100 μg of non-radioactive aldosterone carrier was added to the eluate and the steroid was run in the formamide: benzene system for 24 hrs. Radioactivity

migrated with the carrier. After acetylation, aldosterone 18,21-diacetate was run in system B_3. Specific activity remained constant after oxidation with chromic acid and running in system B_1. 18-hydroxy-corticosterone was isolated from the radioactive area with $R_f = 0.13$. After the addition of 50 μg of carrier it was acetylated and run in system B_1, hydrolyzed back to the free steroid alcohol and rerun in system B_5. Specific activity remained constant.

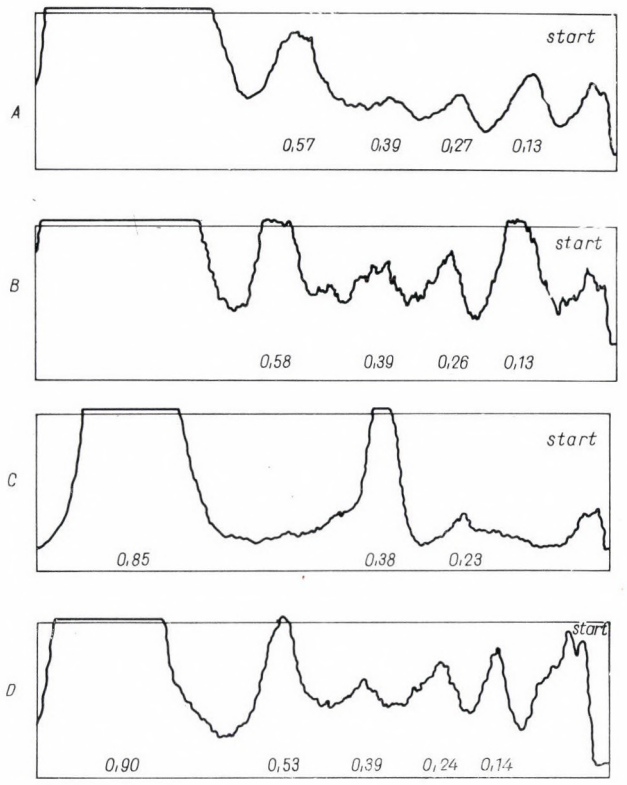

Fig. 1. Radioscannograms of the chromatograms from the different precursor-incubates. A. Deoxycorticosterone-1,2-^{3}H incubate, B. Progesterone-4-^{14}C incubate, C. 11-dehydrocorticosterone-1,2-^{3}H incubate, D. Corticosterone-1,2-^{3}H incubate, Solvent system: Bush B_5, The numbers show the R_f values of different compounds

The steroid with $R_f = 0.26$ was rerun in system B_5 with cortisol, where the radioactivity separated from the carrier. Regarding that the counts incorporated were not enough for exact identification the compound was tentatively identified as 6-β-hydroxy-11-dehydrocorticosterone based on the running properties.

The radioactive metabolite with $R_f = 0.58$ having no reference compound, was not identified.

Table 1

Specific activities (SA) and total cpm incorporated into various steroids from progesterone--4-^{14}C by rabbit adrenal slices in vitro

Compound	μg carrier	Derivative	SA cmp/μg	Total cmp incorp. / total incubate
Corticosterone	—	Acetate Free steroid	333 322	11,016
11-dehydrocorticosterone	—	Acetate Free steroid	310 315	4,158
11-deoxycorticosterone	—	Acetate Free steroid	305 310	4,650
18-hydroxycorticosterone	50	21-monoacetate Free steroid	6 6.2	310
Aldosterone	100	18—21-diacetate 18-lactone-21-monoacetate	1.9 2.0	200

Table 2

Identification of radioactive metabolites obtained by incubation of corticosterone-1,2-^{3}H with rabbit adrenal slices in vitro

Compound	μg carrier	Derivate	SA cpm/μg	Total cpm incorp. / total incubate
Aldosterone	100	18—21-diacetate 18-lactone-21-monoacetate	365 360	36,000
18-hydroxycorticosterone	50	21-monoacetate Free steroid	1,010 1,080	54,000
6-β-hydroxy-11-dehydro-corticosterone	100	6—21-diacetate Free steroid	469 480	48,000
11-dehydro-corticosterone	—	Acetate Free steroid	161,000 165,000	480,000

Analysis of corticosterone-1,2-^3H incubate

From this incubate, 18-hydroxy-corticosterone and aldosterone were both isolated by the identification sequence described above. In addition, 6-β-OH-11-dehydrocorticosterone was identified as an important metabolite, migrating with $R_f = 0.24$ in solvent system B_5. After the addition of non-radioactive carrier and acetylation the acetate was run in system B_1, and the specific activity measured. Hydrolysis of the acetate yielded the free steroid, which maintained its specific activity.

A major part of corticosterone-1,2-^{3}H was converted into 11-dehydrocorticosterone.

The compound with $R_f = 0.53$ was not identified.

Analysis of 11-dehydrocorticosterone-1,2-^3H incubate

As it can be seen on the scannogram the most prominent peak of radioactivity is with $R_f = 0.38$. From this area 18-hydroxy-11-dehydrocorticosterone and aldosterone were identified as follows: the area of the radioactive spot was eluted and after the addition of 100 μg each of authentic 18-hydroxy-11-dehydrocorticosterone and aldosterone carriers it was evaporated to dryness and the residue run in the formamide: benzene system for 24 hrs. Radioactivity migrated with the carriers. After elution and acetylation the acetates were run in system B_3. Two UV absorbing spots were obtained, that of aldosterone-diacetate and of 18-hydroxy-11-dehydrocorticosterone-21-monoacetate. The bulk of radioactivity migrated with the carrier 18-hydroxy-11-dehydrocorticosterone-acetate. The two spots were eluted and the specific activities measured. After this step, aldosterone-diacetate was oxidized with chromic acid and 18-hydroxy-11-dehydrocorticosterone acetate hydrolyzed back to the free steroid alcohol. After running in systems B_1 and B_5, respectively, specific activities were measured.

In addition, 6-β-hydroxy-11-dehydrocorticosterone was isolated from this incubate.

An attempt was made to identify corticosterone from the radioactive area next to the solvent front. This area was eluted and steroids were run in system B_1. Two radioactive spots were obtained, one, which migrated with authentic 11-dehydrocorticosterone, the other having the mobility of corticosterone ($R_f = 0.24$). The latter was eluted, subjected to the acetylation procedure, and rerun again in system B_1 parallel with authentic corticosterone-acetate. As the polarity of the metabolite has not changed ($R_f = 0.24$) whereas authentic corticosterone-acetate migrated with $R_f = 0.75$, the compound was not corticosterone, having no group to be acetylated. Further attempts were made by the addition of non-radioactive carrier corticosterone but the isolated counts were negligible. Accordingly 11-dehydrocorticosterone was not converted into corticosterone in the rabbit adrenal.

Table 3

Identification of steroids isolated from the 11-dehydrocorticosterone-1,2-^3H incubate

Compound	μg carrier	Derivative	SA cpm/μg	Total cpm incorp. / total incubate
18-hydroxy-11-dehydro-corticosterone	100	21-monoacetate Free steroid	1,327 1,392	278,000
Aldosterone	100	18—21-diacetate 18-lactone-21-monoacetate	102 106	21,000
6-β-hydroxy-11-dehydro-corticosterone	100	6—21-diacetate Free steroid	118 120	24,000

Analysis of deoxycorticosterone-1,2-^3H incubate

As the scannogram is showing from deoxycorticosterone numerous derivatives were formed. The steroid with $R_f = 0.13$ was 18-hydroxy-corticosterone, 36,000 cpm/total incubate. 6-β-hydroxy-11-dehydrocorticosterone had $R_f = 0.27$, 27,000 cpm/total incubate. Aldosterone run with $R_f = 0.39$, 22,000 cpm/total incubate. Corticosterone was synthesized in large amounts, 610,000 cpm/total incubate. 11-dehydrocorticosterone was also isolated in a quantity of 152,000 cpm/total incubate.

All these steroids were isolated and identified by the methods described above.

Discussion

The main steroid secreted by the adrenal cortex of the rabbit is corticosterone. This compound is synthesized from deoxycorticosterone according to the biosynthetic scheme of Hechter and Pincus (1954). Our results, obtained by incubations with progesterone-4-^{14}C and deoxycorticosterone-1,2-^3H point to the same biosynthetic pathway in the rabbit adrenal. Corticosterone is converted into 11-dehydrocorticosterone at a significant rate, while the reverse of this transformation is practically nil, as 11-dehydrocorticosterone-1,2-^3H was not converted into corticosterone.

The biosynthesis of 18-hydroxy-11-dehydrocorticosterone from 18-hydroxy-corticosterone by beef zona glomerulosa tissue was first demonstrated by Sandor and Lanthier (1963a). In these experiments 18-hydroxy-11-dehydrocorticosterone was synthesized by the oxidation of the 11-β-hydroxyl group of 18-hydroxy-corticosterone like the corticosterone→11-dehydrocorticosterone transformation. More recently, Sharma et al. (1966) have studied in detail the conditions necessary for this transformation.

In the present experiments we have shown the direct hydroxylation of 11-dehydrocorticosterone at carbon 18. The quantity of 18-hydroxy-11-dehydrocorticosterone synthesized from 11-dehydrocorticosterone is surprisingly high as compared to the quantity of 18-hydroxy-corticosterone synthesized from corticosterone. Regarding that the concentrations and specific activities of the two substrates were nearly identical, this shows a more intensive (4×) hydroxylation of 11-dehydrocorticosterone at carbon 18. This observation is furnishing some information on the mechanism of 18-hydroxylation, e.g. that the enzyme is more effectively hydroxylating the 18-angular methyl group if the steroid is having an 11-keto centrifugation. As the steric configuration of the 11-hydroxy group is β, it is probable that the steroid substrate is linked to the surface of the enzyme by its β-side and the 11-keto group, by occupying less space and being less polar, is promoting the enzyme-steroid association.

From 11-dehydrocorticosterone-1,2-^{3}H aldosterone was also synthesized indicating the existence of alternative biosynthetic pathways leading to aldosterone. The conversion of progesterone-4-^{14}C to aldosterone was first detected by Chen et al. (1958) in perfused calf adrenals and later confirmed by Travis and Farrell (1958) with beef adrenal capsule scrapings. Raman et al. (1964) described the synthesis of 18-hydroxy-corticosterone and aldosterone from progesterone-4-^{14}C in mouse adrenals in vitro. Mulrow and Cohn (1959) found that human adrenal slices converted corticosterone-4-^{14}C into aldosterone. Based on these results corticosterone proved to be the most important precursor to aldosterone. Recently, Pasqualini (1964) reported the conversion of tritiated 18-hydroxy-corticosterone to aldosterone in an aldosterone secreting human adrenocortical tumour and adjacent cortex. Consequently the main biosynthetic pathway of aldosterone formation is progesterone → deoxycorticosterone → corticosterone → 18-OH-corticosterone → aldosterone. This sequence is supported by the present results, since we have isolated both 18-OH-corticosterone and aldosterone from the corticosterone incubate.

Ulick and Vetter (1962) have shown, that in all cases where aldosterone secretion was increased in man there was a similar increase in the secretion of 18-hydroxy-corticosterone. Sandor et al. (1963b) in chicken and duck, Raman et al. (1964) in mice and Sandor et al. (1964) in the turtle reported similar findings. After this, the role of 18-hydroxy-corticosterone as immediate precursor to aldosterone was firmly established. Nicolis and Ulick (1965) in a recent paper have shown that corticosterone is converted to aldosterone 18–81 times more effectively in frog, beef and human adrenals than tritiated 18-hydroxy-corticosterone incubated under identical conditions. These findings are explained by the authors by the existence of 18-hydroxy-corticosterone mainly in the 18–20 cyclic hemiketal form, which is relatively more resistant to oxidation.

We should like to emphasize the significance of steroids with an 11-keto configuration in the adrenal cortex. Brode et al. (1962) described the synthesis of 11-β-OH-progesterone in an aldosterone secreting tumour and we have demon-

strated (Fazekas eta l., 1966) the formation of 11-ketoprogesterone in a similar tissue. Accordingly 11-keto-steroids are constantly formed from the 11-β-OH analogs and they can undergo further transformations as indicated by the present results.

The appearance of 6-β-OH-11-dehydrocorticosterone points to the existence of an effective 6-β-hydroxylating enzyme system in the rabbit adrenal cortex.

Further investigations are needed to clarify the full significance of 11-keto-steroids in the adrenal cortex, because they may have an important role in the biogenesis of 18-oxygenated corticosteroids.

References

Brode, E., Grant, J. K., Symington, T. (1962) Acta Endocr. *41* 411
Bush, I. E. (1952) Biochem. J. *50* 370
Chen, P. S., Schedl, H. P., Rosenfeld, G., Bartter, F. C. (1958) Proc. Soc. Exp. Biol. *97* 683
Fazekas, A. G. (1961) Acta Physiol. Hung. *18* 253
Fazekas, A. G., Webb, J. L. (1966) in preparation
Hechter, O., Pincus, G. (1954) Physiol. Rev. *34* 459
Lieberman, S., Katzenellenbogen, E. R., Schneider, R., Studer, P. E., Dobriner, K. E. (1953) J. Biol. Chem. *205* 87
Mattox, V. R., Mason, H. L. (1956) J. Biol. Chem. *223* 215
Meyer, A. S. (1953) J. Biol. Chem. *203* 469
Mulrow, P. J., Cohn, G. L. (1959) Proc. Soc. Exp. Biol. *101* 731
Nicolis, G. L., Ulick, S. (1965) Endocrinology *76* 514
Pasqualini, J. R. (1964) Nature *201* 501
R aman, P. B., Ertel, R. J., Ungar, F. (1964) Endocrinology *74* 865
Sandor, T., Lanthier, A. (1963a) Acta Endocrinol. *42* 355
Sandor, T., Lamoureaux, J., Lanthier, A. (1963b) Endocrinology *73* 629
Sandor, T., Lamoureaux, J., Lanthier, A. (1964) Steroids *4* 213
Sharma, D. C., Raman, P. B., Dorfman, R. I. (1966) Intern. Cong. Hormonal Steroids, Milan, 1966. Excerpta Med. International Congress Series No. *111*, p. 138.
Smith, J. (1960) Chromatographic and Electrophoretic techniques. *1* p. 433, W. Heinemann Ltd. London.
Travis, R. H., Farrell, G. L. (1958) Endocrinology *63* 882
Ulick, S., Vetter, K. K. (1962) Abstract J. Clin. Invest. *41* 1406
Zaffaroni, A. (1953) Recent Progress in Hormone Research, Academic Press, New York, *8* p. 51

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 379—388 (1966)

Chromatographic Behaviour of Pancreatic Amylase

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(Received August 15, 1966)

Amylase can be separated chromatographically into 2 or 4 active fractions according to the different ways of recrystallization (presence or absence of urea) and to the different conditions of chromatography (presence or absence of mercaptoethanol). These different fractions appear to be intercovertible, representing different structural forms of the same protein molecule. Chromatographic heterogeneity of amylase is discussed with respect to possible conformational changes in the enzyme molecule.

Investigations on pancreatic proteins are of great interest because of the specific morphological structure and biological function of the pancreas. The structure of the proteins of pancreas has been thoroughly studied for many years in several laboratories and has been completely elucidated in the case of some enzymes, e.g. ribonuclease, chymotrypsinogen.

Fewer data are available concerning pancreatic amylase. The biosynthesis of this enzyme as well as its transport within the cell have been extensively studied (Hokin 1955; Garzó, T. Szabó, Straub, 1957; T. Szabó, Garzó, 1957; Weiss et al., 1958; Siekevitz, Palade, 1958a, 1958b, 1960; Redman et al., 1966), but less is known about its chromatographic separation and structural characteristics.

No amylase peak can be obtained upon chromatographic fractionation of a beef pancreas extract (Keller et al., 1958, 1961), probably because of the very low quantity of amylase as compared to that of other proteins in the extract. Amylase is obtained as a separate protein fraction by chromatography of extracts of pig or dog pancreas (Marchis-Mouren, 1959; Desnuelles, 1961). Amylase of cell fractions of the parotis gland, a tissue which contains this enzyme in a relatively large amount, can also be separated by chromatographic techniques from other proteins (Schramm, Danon, 1961).

In the present paper chromatography of amylase from pig pancreas on a DEAE-Sephadex column is described. It was found that amylase is not a homogeneous protein, as it can be separated by column chromatography into two or four enzymatically active fractions according to the experimental conditions.

Materials and Methods

Preparation of crystalline amylase. Amylase was isolated from pig pancreas according to the method of Fischer and Bernfeld (1947, 1948) as modified by Hatfaludy et al. (1966). The enzyme was recrystallized by either of the following two methods, as indicated in the text.

1. *Recrystallization from ammonia solution.* The crystals were dissolved in an ammonia solution of pH 9 to 10, containing 5×10^{-3} M $CaCl_2$. The solution was concentrated in vacuo over cc. sulfuric acid. Recrystallization was repeated until a specific activity of 4000 to 5000 Smith – Roe units per mg protein (Smith, Roe, 1949) was obtained. This value corresponds to the activity of pure crystalline amylase.

2. *Recrystallization in the presence of urea.* Crystals were dissolved in a 4 to 5 M urea solution containing 10^{-2} M $CaCl_2$. The solution was immediately diluted about twofold to obtain an appropriate protein concentration and a urea concentration of 2 to 2.5 M. Dialysis in the cold against 3×5 l of 5×10^{-3} M $CaCl_2$ solution of pH 7 followed. Amylase crystals appeared in the solution soon upon dialysis. 3 or 4 recrystallizations yielded amylase preparations with specific activities of 4000 to 5000 units per mg protein.

Chromatography of amylase was made on DEAE-Sephadex A–50 (medium) columns. The ion exchanger was equilibrated either with a buffer solution of pH 7.5 containing 10^{-2} M TRIS-HCl, 10^{-4} M mercaptoethanol, 5×10^{-3} M Ca^{++} ions and 2×10^{-2} M Cl^- ions, or with a buffer solution of similar composition except for the absence of mercaptoethanol. The flow rate was 30 to 40 ml/h. Recovery was calculated on the basis of amylase activity.

Throughout this work chromatography was performed in the presence of Ca^{++} ions, and during all manipulations Ca^{++} ions in a concentration of 5×10^{-3} M were present in the protein solution. Ca^{++} ions are indispensable for the activity of the enzyme and at this relatively high concentration alterations of amylase activity caused by Ca deficiency could be prevented.

For elution the column was washed with the same buffer it had been equilibrated with, followed by a continuous chloride gradient. Chromatography was performed at room temperature (18–23°C), under which conditions amylase is stable in the presence of Ca^{++} and Cl-ions. In the different chromatographic runs 4 to 10 ml fractions were collected. Before applying to the column, the protein solution was dialyzed against the equilibrating buffer until its Cl^- concentration was lowered to the same level as that of the equilibrating buffer, i.e. 2×10^{-2} M. Before rechromatography, the protein was lyophilized, dissolved and dialyzed against the equilibrating buffer.

Enzyme activity of amylase was assayed by the method of Smith and Roe (1949). *Protein content* of the chromatographic fractions was determined on the basis of absorbancy at 280 mμ, using the data of Hsiu et al. (1964), according to which $E_{1\%}^{280} = 24.00$ at pH 8. In the course of recrystallizations of amylase, the

method of Lowry (1951) was applied for protein determination. After 3 to 4 recrystallizations the pure protein solution gave identical results when determined by both methods.

Results

Chromatography of amylase recrystallized from ammonia or urea solutions in the absence of mercaptoethanol

If amylase crystallized from ammonia solution is applied to a column which had been equilibrated with the buffer containing no mercaptoethanol, the protein binds strongly to the ion exchanger. It cannot be eluted with mercaptoethanol, a simultaneous elevation of the Cl^- concentration is needed for partial elution.

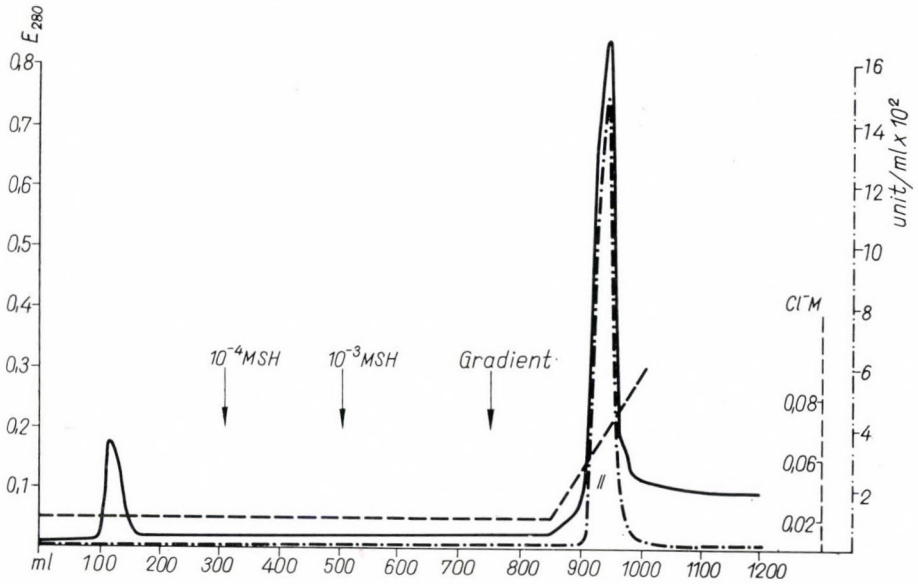


Fig. 1. Chromatography of amylase recrystallized from ammonia solution. Mercaptoethanol absent. Specific activity of amylase: 4,200 U/mg. 2.2 × 30 cm column. 5 ml fractions collected. Protein sample: 18.6 mg in 2 ml. Recovery 58 per cent. E_{280} ———; amylase activity: unit/ml —.—.—; conc. Cl^-: — — —

Amylase crystallized from urea solution, when applied to the column under the same conditions, can be eluted with 90 per cent recovery with the equilibrating buffer, without addition of mercaptoethanol or elevation of Cl^- concentration. Only 10 per cent is bound more strongly to the column. The protein eluted at low Cl^- concentration, however, is separated into two peaks.

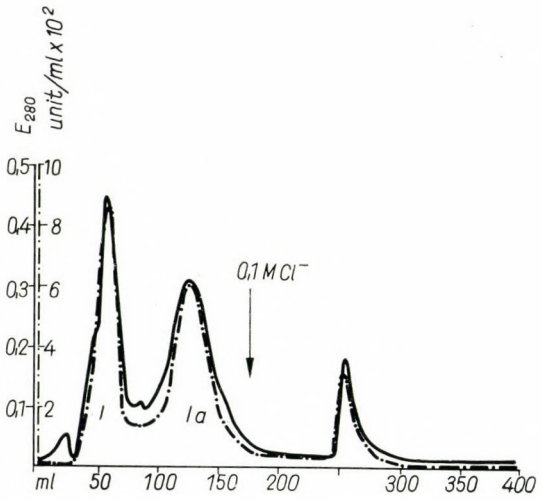


Fig. 2. Chromatography of amylase recrystallized from urea solution. Mercaptoethanol absent. Stepwise elution. Specific activity of amylase: 4,200 U/mg. 1.4×25 cm column. 5 ml fractions collected. Protein sample: 9.32 mg in 2 ml. Recovery: 100 per cent, E_{280} ———; amylase activity: unit/ml —.—.—.

Chromatography of amylase crystallized from ammonia or urea solutions, in the presence of mercaptoethanol

If chromatography of amylase crystallized from ammonia solution is performed in the presence of mercaptoethanol, the protein is eluted in two peaks. The first amylase peak (peak I) appears immediately after the breakthrough peak, the second (peak II) after increasing the Cl^- concentration. These two distinct peaks can be observed irrespective of variations in protein concentration or in the volume of the samples applied.

The first peak was rechromatographed after lyophilization and dialysis. A peak in the same position was obtained as in the first chromatographic run, the protein could be eluted completely without increasing the Cl^- concentration. Rechromatography of the second peak gave different results depending upon the presence of NaCl in the protein solution. If pooled fractions of the second active peak were immediately dialyzed against the equilibrating buffer, containing Cl^- ions in a concentration of 2×10^{-2} M and applied to the column after equilibration with this solution, amylase could be eluted only at a higher Cl^- concentration. The amylase peak thus appeared in the same position as in the first chromatographic run (Fig. 4b). However, if the pooled fractions of peak II were lyophilized without removing the NaCl present in the eluate, and dialysis was performed after lyophilization, upon rechromatography amylase was almost

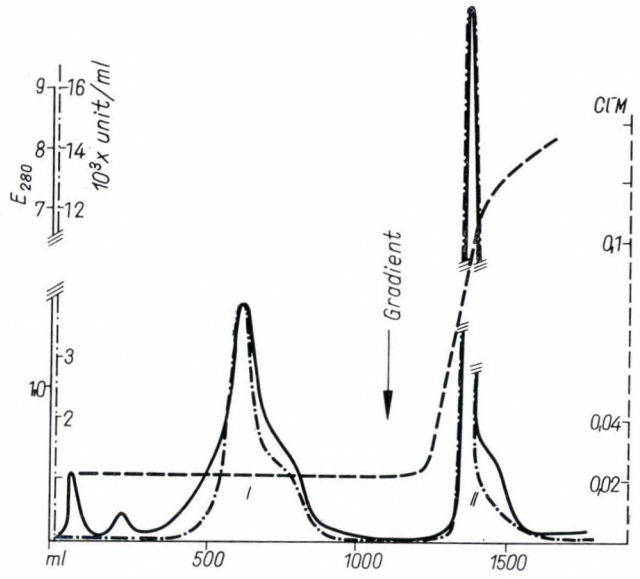


Fig. 3. Chromatography of amylase recrystallized from ammonia solution. Mercaptoethanol present. Specific activity: 4,500 U/mg. 3×32 cm column. 10 ml fractions collected. Protein sample: 435 mg in 10 ml. Recovery 100 per cent. E_{280} ———; amylase activity: unit/ml —.—.—; conc. Cl^-: — — —

completely eluted with the equilibrating buffer (Fig. 4c). Under such conditions peak II appears in the position of peak I; the presence of NaCl thus changes the chromatographic behaviour of amylase.

The effect of high concentrations of NaCl is shown also in the following experiments. Amylase was incubated in 0.1 M NaCl at pH 7.5 at 0°C for 36 h. After incubation the solution was dialyzed against the equilibrating buffer until its Cl^- concentration was decreased to 2×10^{-2} M. Upon chromatography this amylase preparation could be completely eluted with the equilibrating buffer. The same result was obtained if instead of incubation with NaCl, amylase was lyophilized in the presence of NaCl and applied to the column after this treatment (Figs. 5a, 5b).

Chromatography of amylase crystallized from urea solution yielded in the presence of mercaptoethanol the same main peaks I and II as were obtained with amylase recrystallized from ammonia solution, with the difference, however, that after each main peak a minor peak (Ia and IIa, respectively) also appeared. The specific activities as determined at the maxima of the four chromatographic fractions were practically identical.

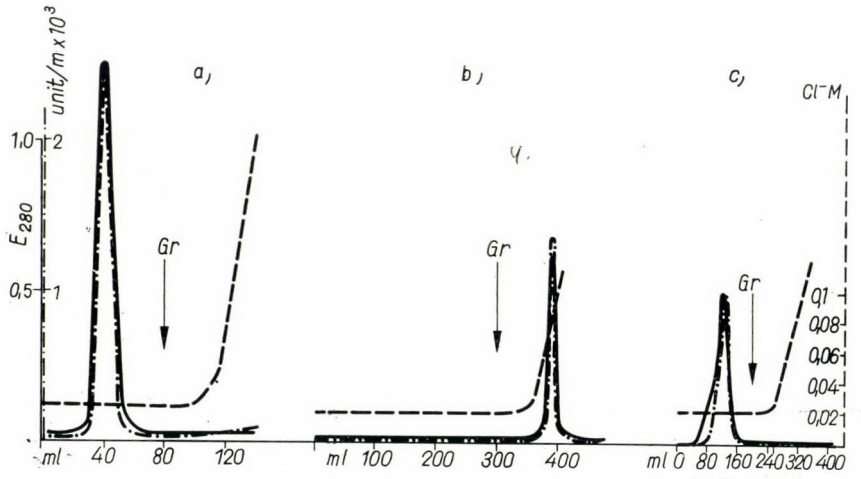


Fig. 4. Rechromatography of peak I and peak II in the presence of mercaptoethanol. *a)* Rechromatography of the pooled fractions of peak I seen in Fig. 3. The solution was dialyzed after lyophilization. Specific activity of amylase: 4,000 U/mg. 0.8×30 cm column. 4 ml fractions collected. Protein sample: 7.4 mg in 2 ml. Recovery 80 per cent. E_{280} ———; amylase activity: unit/ml —.—.—; conc. Cl^-: — — —. *b)* Recromatography of the pooled fractions of peak II from another run not presented here. The pooled fractions were immediately dialyzed. Specific activity of amylase: 4,600U/mg. 0.8×32 cm column. 5 ml fractions collected. Protein sample: 4 mg in 4 ml. Recovery 54 per cent. E_{280} ———; amlyase activity: unit/ml —.—.; conc. Cl^-: — — —. *c)* Rechromatography of the pooled fractions of peak II seen in Fig. 3. The pooled fractions were lyophilized in the presence of NaCl and dialyzed afterwards. Specific activity of amylase: 4,900 U/mg. 0.8×42 cm column. 4 ml fractions collected. Protein sample: 8 mg in 2 ml. Recovery 88 per cent. E_{280} ———; amylase activity: unit/ml —.—.—; conc. Cl^- — — —

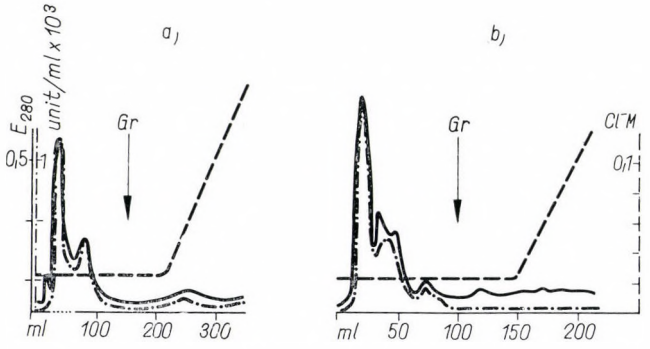


Fig. 5. Chromatography of amylase treated with NaCl. Mercaptoethanol present. *a)* Amylase incubated with NaCl in the cold. Specific activity: 4,100 U/mg. 0.8×30 cm column. 5 ml fractions collected. Protein sample: 8.6 mg in 2 ml. Recovery: 93 per cent. E_{280} ———; amylase activity: unit/ml —.—.—; conc. Cl^-: — — — *b)* Amylase lyophilized in the presence of NaCl. Specific activity: 3,850 U/mg. 0.8×29 cm column. 5 ml fractions collected. Protein sample: 7.6 mg in 2 ml. Recovery: 95 per cent. E_{280} ———; amylase activity: unit/ml —.—.—; conc. Cl^- — — —

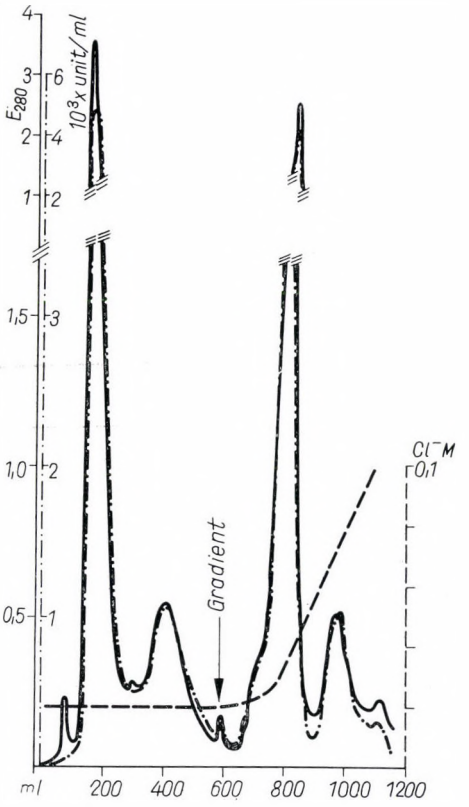


Fig. 6. Chromatography of amylase recrystallized from urea solution. Mercaptoethanol present. Specific activity: 4,500 U/mg. 2.2×30 cm column. 5 ml fractions collected. Protein sample: 105 mg in 10 ml. Recovery: 88 per cent. E_{280} ———; amylase activity: unit/ml —.—.—; conc. Cl^- — — —

Discussion

Chromatography on DEAE-Sephadex reveals heterogeneity of amylase. The chromatographic profile strongly varies with variations in the experimental conditions.

1. In the absence of mercaptoethanol amylase crystallized from ammonia solution is more strongly bound to the ion exchanger than is amylase crystallized from urea solution (Figs. 1 and 2).

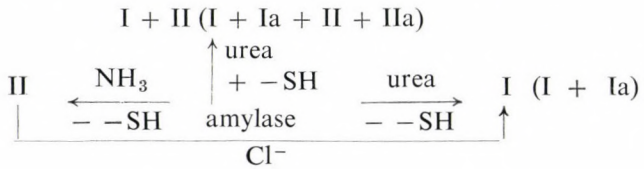
2. In the presence of mercaptoethanol several active peaks are obtained, indicating the presence of several different active forms of amylase. Under such conditions amylase crystallized from ammonia solution exists in two active forms,

whereas amylase as recrystallized from urea solution is present in the form of four different active proteins (Figs. 3 and 6).

3. One of the active forms of amylase can be converted into the other. This is suggested by the following observations. Peak II is shifted to the position of peak I upon rechromatography when during lyophilization NaCl had been present (Fig. 4c). No second peak is obtained from amylase preparations treated with NaCl before chromatography (Figs. 5a and 5b). The chromatographic behaviour of amylase crystallized from ammonia solution is different when mercaptoethanol is absent or present during chromatography. In the former case, no second peak is obtained, in the latter, about half of the amylase is eluted in the position of peak II (Figs. 1 and 3). A conversion in the opposite direction (peak I into peak II) could not be accomplished as yet.

As can be seen in the figures, in different chromatographic runs peak II is eluted at different chloride concentrations. These variations can also be explained by the effect on amylase of various NaCl concentrations.

Chromatographic heterogeneity and interconversion of the different active forms of amylase under mild conditions can be explained by $-SH-SS-$ interchanges in the protein molecules. Amylase contains disulfide bridges as well as blocked sulfhydryl groups. These latter groups become unmasked under various conditions, e.g. in the presence of urea. It is suggested that in the presence of mercaptoethanol interchange of disulfide bridges may occur, in the course of which new intra- and intermolecular linkages are formed. According to the experimental conditions one or more of the different conformations become stabilized. It is interesting to note that these conformational changes are not accompanied by changes in enzyme activity. On the other hand, distribution of positively and negatively charged groups on the surface of the molecule will be different if the same protein is present in different conformations. Amylase may thus undergo reversible structural changes which result in differently charged but enzymatically equally active molecules. Different conditions applied at chromatography of amylase favour conversion in one or the other direction, thereby leading to stabilization of one or other of the different conformations of amylase.



Whether disulfide interchange concerns intermolecular disulfide bridges, i.e. whether the appearance of different active forms of amylase is due to formation and dissociation of dimers, could not be decided as yet. Detection of amylase molecules of different sizes was attempted by applying Andrews' method of

molecular sieving on a Sephadex G 100 column (Andrews, 1964, 1965). Only one peak was obtained. It came off later than expected for a fraction of a molecular weight of 50,000. The peak was broad and the enzyme activity was present in a large volume of the eluate even when only 100 μg of protein were applied to a 50 ml column. This result, however, can be ascribed to the similarity of the structure of Sephadex gel to that of the substrate, which may cause retardation in the appearance of the enzyme in the eluate (Andrews, 1964).

We therefore cannot decide whether a monomer-dimer conversion, or some other types of conformational changes take place in amylase under our experimental conditions. It can be concluded, however, that amylase of pig pancreas may exist in different structural forms which are all enzymatically active and which can be separated by chromatography on DEAE-Sephadex column.

References

Andrews, P. (1964) Biochem. J. *91* 222
Andrews, P. (1965) Biochem. J. *96* 595
Desnuelles, P. (1961) Adv. Enzymol. *23* 129
Fischer, E., Bernfeld, P. (1947) Helv. Chim. Acta *30* 64
Fischer, E., Bernfeld, P. (1948) Helv. Chim. Acta *31* 1831
Garzó, T., T. Szabó, M., Straub, F. B. (1957) Acta Physiol. Hung. *12* 299
Hatfaludy, F., Strashilov, T., Straub, F. B. (1966) Acta Biochim. Biophys. Hung. *1* 39
Hokin, L. E. (1955) Biochim. Biophys. Acta *18* 379
Hsiu, J., Fischer, E., Stein, E. (1964) Biochemistry *3* 61
Keller, P. J., Cohen, E., Neurath, H. (1958) J. Biol. Chem. *233* 344
Keller, P. J., Cohen, E., Neurath, H. (1961) J. Biol. Chem. *236* 1404
Lowry, O. H. (1951) J. Biol. Chem. *193* 265
Marchis-Mouren, G. (1959) Thesis, University Marseille
Redman, C. M., Siekevitz, P., Palade, G. E. (1966) J. Biol. Chem. *241* 1150
Schramm, M., Danon, D. (1961) Biochim. Biophys. Acta *50* 102
Siekevitz, P., Palade, G. E. (1958a) J. Biophys. Biochem. Cytol. *4* 309
Siekevitz, P., Palade, G. E. (1958b) J. Biophys. Biochem. Cytol. *4* 203
Siekevitz, P., Palade, G. E. (1960) J. Biophys. Biochem. Cytol. *7* 631
Smith, B. W., Roe, J. H. (1949) J. Biol. Chem. *179* 53
T. Szabó, M., Garzó, T. (1957) Acta Physiol. Hung. *12* 303
Weiss, S. B., Acs G., Lippmann, F. (1958) Proc. Natl. Acad. Sci. US. *44* 189

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 389—402 (1966)

Structure of Glyceraldehyde-3-Phosphate Dehydrogenase and its Alteration by Coenzyme Binding

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(Received July 29, 1966)

The optical rotatory dispersion, hydrodynamic constants, and the kinetics of hydrogen-deuterium exchange, as measured by infrared spectrophotometry, have been studied in pig muscle glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating), E. C. 1.2.1.12) in the absence and in the presence of the coenzymes, NAD and NADH.

The parameters calculated from optical rotatory dispersion (λ_c, b_o, a_o, K_c, H_{193}, H_{225}) indicate the existence of an ordered structure amounting to about 34 per cent of the total molecular volume for the coenzyme-free enzyme. Addition of the coenzyme NAD, results in a 9 per cent increase in the ordered structure. The addition of NADH to the NAD-free protein causes a 4 per cent decrease in λ_c and b_o.

The rate of the H—D exchange of the amide hydrogens of peptide bonds was measured by infrared spectrophotometry following the decrease in absorption of the amide II band (1554 cm^{-1}). The relative amount of peptide hydrogens practically inaccessible to water was about 32 per cent. Upon addition of NAD and NADH, their amount increased by 10 and 4 per cent, respectively. About 15 per cent of the peptide hydrogens could be exchanged within a measurable time span, while the remaining 53 per cent was exchanged instantaneously.

The hydrodynamic parameters (sedimentation, diffusion, and intrinsic viscosity) show a slight loosening of the globular molecule after the removal of bound NAD and NADH.

Correlation has been found between the amount of peptide hydrogens inaccessible to water and the amount of ordered structure as calculated on the basis of optical rotatory dispersion. The data indicate the existence of a compact hydrophobic core amounting to about 40 per cent of the total molecular volume. The coenzymes render the structure more compact.

Introduction

GAPD* isolated from pig muscle is a globular protein of a molecular weight of 143,000 to 146,000 (Elődi, 1958; Harris, Perham, 1965), and consists of 4 identical subunits. The enzyme contains no disulphide bridges. Its amino acid

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* GAPD = D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating) E. C. 1.2.1.12

composition (Harris, Perham, 1965), hydrodynamic data (Elias, 1960) and the solvent perturbation and iodination experiments of Libor et al. (1964) suggest the existence of a relatively large hydrophobic core amounting to about half of the total molecular volume.

The enzyme crystallizes with 3.0 – 3.5 moles of bound NAD per mole of protein (Velick, 1958; Friedrich, 1965). The coenzyme cannot be removed by dialysis. The dissociation constants, K_D of the coenzymes are 0.6×10^{-7} and 2.4×10^{-7} for NAD and NADH, respectively (Velick, 1958). The NAD-free enzyme differs considerably from the protein-NAD complex by having a lower heat-stability and higher tryptic digestibility, viscosity, and levorotation (Boyer, Schultz, 1958; Elődi, Szabolcsi, 1959; Furfine, Velick, 1965).

The changes in the optical rotatory dispersion of GAPD upon the addition of coenzymes have been studied by several authors (Listovsky et al., 1965; Havsteen, 1965; Markovich et al., 1966) and it was found that NAD increased the organization of the structure.

In other dehydrogenases as alcohol dehydrogenase (Hvidt, Kägi, 1963) and lactic dehydrogenase (Di Sabato, Ottesen, 1965) the H – D exchange is used for the detection of fine structural changes induced by the binding of coenzymes.

In the present work the structure of GAPD was examined by methods fundamentally independent of one-another. In this frame the ratio of ordered polypeptide portions, the hydrodynamic parameters characteristic of the tertiary structure, the accessibility of peptide hydrogens to D_2O and the coenzyme-induced alterations of the protein structure were studied.

Experimental

Materials

GAPD was isolated from pig muscle according to the method of Elődi and Szörényi (1956). Four times recrystallized preparations were used. The molecular activity of the preparations in case of GAP oxidation and in the presence of phosphate, was 16 – 18,000. Calculations were made from the optical density values obtained in the first 30 seconds.

LDH* was prepared from pig muscle by Jécsai's method (1961) and was purified by three-fold recrystallization.

The experiments were performed in 0.1 M phosphate buffer, pH 7.5, at 20°C. Enzyme solutions were freshly prepared before use and gel-filtered on Sephadex G-25 columns at 4°C.

The protein content of the solutions was determined spectrophotometrically in 0.1 N NaOH, by measuring the absorption at 280 mμ and using the extinction

* LDH-lactic dehydrogenase

coefficients $E_{1\,cm}^{0.1\%}$ 1.0, 1.1 and 1.29, for GAPD – NAD complex, GAPD and LDH, respectively.

Bound NAD was removed by charcoal-treatment and the quantitative removal was controlled by measuring the E_{280}/E_{260} ratio of the protein, which increased from 1.1 to 1.8 after charcoal-treatment.

NAD and NADH were commercial preparations (REANAL). In the optical rotatory dispersion experiments NADH was purified chromatographically on DEAE-cellulose columns. The purity of the preparations was checked both spectrophotometrically and enzymically.

The heavy water contained 99.6 M per cent D_2O.

Sodium dodecyl sulphate (Du Pont-Nemours Co.) was recrystallized from ethanol.

All other chemicals were of reagent grade. Solutions were made up with glass-distilled water.

Enzymic activity was checked after every experiment and only those results were taken into account, where the activity remained above 80 per cent.

Methods

Optical rotatory dispersion. The measurements were made in a Bellingham – Stanley Pepol-60 precision polarimeter in the wave-length region of 350 – 600 mμ in 10 cm cells, and in the 0.5 – 1.0 per cent concentration range. The accuracy of the readings was 0.003°.

The contribution of NADH and NAD to the rotation was taken into account.

The values of the rotation constants λ_c and K_c were calculated from the Drude equation according to the method of Yang and Doty (1957) by plotting $\lambda^2[\alpha]$ versus $[\alpha]$.

The values of a_0 and b_0 were calculated from the Moffit and Yang (1956) equation

$$[M] = \frac{MRW}{100}[\alpha] = \frac{a_0\lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2 - \lambda_0^2)^2}$$

where λ_0 was taken as 212 mμ. The mean residue weight (MRW), on the basis of the amino acid analysis of Harris and Perham (1965), was taken as 109.

The values of b_0 and a_0 can be obtained from the plot $[M](\lambda^2 - \lambda_0^2)$ versus $\frac{1}{(\lambda^2 - \lambda_0^2)}$, $b_0\lambda_0^4$ being the slope of the straight line, and $a_0\lambda_0^2$ the intercept with the ordinate, respectively.

The percentage amount of ordered structure was calculated from the above parameters as well as on the basis of Shechter and Blout's (1964) equation where the wave-length dependence of the molar rotation:

$$[M] = \frac{A_{193}\lambda_{193}^2}{\lambda^2 - \lambda_{193}^2} + \frac{A_{225}\lambda_{225}^2}{\lambda^2 - \lambda_{225}^2}$$

where λ_{193} and λ_{225} correspond to the absorption maxima of the peptide bonds and the ordered structure, respectively. The "helix content" can be calculated from the constants in the following way:

$$H_{193} = \frac{A_{193} + 600}{32{,}6}$$

and

$$H_{225} = -\frac{A_{225}}{13}$$

$$\bar{H} = \frac{1}{2}(H_{193} + H_{225})$$

Viscosity. The viscosity of 0.2 – 1.2 per cent protein solutions was determined in Ostwald viscosimeters at 20°C (outflow with distilled water 120 sec). 0.1 M phosphate buffer, pH 7.4, was used as a solvent.

The intrinsic viscosity was extrapolated on the basis of the following formula

$$[\eta] = \lim_{c \to 0} \frac{\ln \eta_r}{c}$$

where η_r is the relative viscosity.

The *sedimentation constant* was determined in a MOM G-120 (Budapest) type ultracentrifuge, by the use of Philpot – Swenson optics, in the 0.1 – 0.5 per cent concentration range, at 20°C, at 60,000 r. p. m. The results were extrapolated to 20°C, water, and 0 concentration. All values represent the mean of 8 – 10 experiments.

The *diffusion constant* was determined by the aid of a diffusion apparatus equipped with polarization interference optics. The measurements were performed at 20°C with 0.05 per cent solutions.

Hydrogen-deuterium exchange. The H – D exchange of protein solutions in D_2O was examined by the infrared spectrophotometric method developed by Nielsen (1961), Blout et al. (1961) and Hvidt (1963), by following the disappearance of absorption of the amide II band (1554 cm^{-1}). The experiments were carried out at a uniform pD value at 20°C. Due to the nature of the method, our investigations concerned only the $-CO-NH-$ hydrogens of the peptide bond.

The samples were prepared in the following way.

From a 3 per cent protein stock solution (in 0.1 M phosphate buffer, pH 7.5) samples of 150 μl were taken, frozen in liquid air, evacuated, and dried for 2 – 3

hours by condensing water in a trap containing liquid air. Drying was completed at room temperature at 10^{-3} mm mercury pressure in 24 hours and the preparations were stored in the dark at 4°C.

For the experiments a sample was dissolved in 150 μl of D_2O and poured into a calcium fluoride cell. UR-10 (Zeiss) double-beam recording infrared spectrophotometer equipped with sodium chloride prisms was used at slit program number 8, at 20°C. The measurements were performed in a detachable calcium fluoride cell-pair with 110 μ light path. Protein-free solutions were applied as controls. Both cells were placed in a thermoregulated cell house. Readings were taken at a constant wave number, 1554 cm^{-1}. Registration was started 2–3 minutes after dissolving the sample in D_2O. 60 and 300 minutes after dissolving the sample the spectra were taken in the region from 400 to 1800 cm^{-1}, in order to correct the base line and to exclude errors due to the penetration of the vapour content of air.

The amide I/amide II absorption ratio was determined parallel in all experiments for 100 per cent deuterium-exchanged samples. In such cases the protein was incubated in 3 per cent sodium dodecyl sulphate solution for 8 hours at 80°C, then the spectra were taken.

The amide I/amide II absorption ratio of deuterium-free protein was experimentally determined by the dry-layer spectra. In such cases the protein solution in water was dried in vacuo on a calcium fluoride plate. The spectra were taken at a mercury pressure of 10^{-2} mm.

Results

Optical rotatory dispersion

In a previous paper we presented data on the optical rotatory dispersion parameters of GAPD and its coenzyme complexes in glycine buffer, pH 8.5 (Markovich et al., 1966). Since the technique of deuterium exchange studies requires the use of a non-volatile buffer which does not absorb in the infrared region, these experiments were carried out in 0.1 M phosphate buffer, pH 7.4. Due to the effect of phosphate ion modifying the structure of GAPD, it was necessary to repeat the optical rotatory dispersion measurements under the same conditions, as the H–D exchange experiments.

The optical rotatory dispersion of pig muscle GAPD was measured in the 350–600 mμ wave-length range and the rotation parameters were calculated. In this region both the Moffit–Yang and Yang–Doty plots were found to be linear.

Table 1 summarizes the rotation parameters of the NAD-free GAPD, of GAPD–NAD complex, and of the GAPD–NADH complex prepared by the addition of 4 mole equivalents of coenzymes to the NAD-free protein. The

Table 1

The rotation parameters of GAPD and the relative amount of ordered structure calculated from them, in the presence of NAD and NADH, in the coenzyme-free, and urea-denatured protein. Solvent: 0.1 M phosphate buffer, pH 7.4. Temperature: 20°C

Preparation	λ_c	K_c	$-a_0$	$-b_0$	Ordered structure, per cent			
					calculated		Shechter and Blout	Average
					from λ_c	from b_0		
NAD-free GAPD	258	1.16	214	180	34	35	34	34
GAPD–NAD complex	272	0.94	145	250	45	42	43	43
GAPD–NADH complex	254	1.00	140	173	31	28	30	30
GAPD + 6 M urea	222	3.90	780	46	7	7	—	7

measurements were performed with 1 per cent solutions. For comparison, the data of GAPD denaturated in 6 M urea are also presented.

The values for the GAPD–NAD complex (b_0, λ_c) are somewhat higher than those for other dehydrogenases (Urnes, Doty, 1961), but are in good agreement with the results of Listowsky et al. (1965). On removal of firmly bound NAD $-b_0$ decreases from 250 to 180; λ_c from 272 to 258, while the value $-a_0$ increases from 145 to 214, and K_c from 0.94 to 1.16. The changes are reversible, upon the addition of 4 mole equivalents of NAD the original values are regained.

Upon the addition of 4 mole equivalents of NADH to the NAD-free GAPD, b_0 and λ_c slightly decrease with a parallel decrease of the values of a_0 and K_c, the two latter approaching the values characteristic of the GAPD–NAD complex.

The changes in helix content for the above transitions, as calculated by the method of Shechter and Blout (1964), are in agreement both qualitatively and quantitatively, with the results obtained by Moffit and Doty's method.

On the basis of rotatory dispersion parameters 43 per cent of the protein molecule is in the ordered state in case of the GAPD–NAD complex, i.e. in helical conformation. This portion of the molecule is probably hydrophobic, of a low dielectric constant, and is located in the interior of the molecule. The removal of firmly bound NAD decreases the amount of peptide chain portions being in α-helical conformation by 9 per cent, and upon the addition of NADH a further 4 per cent decrease occurs. The constants K_c and a_0, which reflect the interaction of side chains with the environment, change in the same manner whether NAD or NADH is added, i.e. it seems that the structure becomes more compact.

Hydrodynamic parameters of GAPD

The hydrodynamic parameters were measured in 0.1 M phosphate buffer, pH 7.4, at 20°C. In these experiments 20 moles of coenzyme per mole of GAPD were added. The results are summarized in Table 2.

Table 2

Hydrodynamic parameters of the GAPD—NAD and the GAPD—NADH complexes, and of coenzyme-free GAPD in 0.1 M phosphate buffer, pH 7.4

Preparation	S°_{20w} 10^{13} sec	D°_{20w} 10^{7} cm^2 sec	$[\eta]$ dl/g	M_{SD} 10^{-3}	Mw Archibald 10^{-3}
NAD-free GAPD	7.51±0.03	4.46±0.05	0.042	144±5	146±5
GAPD—NAD complex	7.88±0.03	4.71±0.05	0.030	148±5	146±5
GAPD—NADH complex	7.64±0.03	4.53±0.05	0.033	146±5	142±5

The values of sedimentation, diffusion and viscosity uniformly indicate the reversible loosening of structure on the removal of bound NAD and NADH without any appreciable change in the molecular weight. Accordingly, the effect of NADH on the tertiary structure, as to its direction, is the same as that of NAD but somewhat smaller.

H—D exchange

Fig. 1 shows the infrared spectrum of the GAPD—NAD complex in the wave number region from 1400 to 1800 cm^{-1} in different stages of the exchange. Within this range five important bands can be found (cf. curve *A*, Fig. 1). The shoulder at 1410 cm^{-1} can be attributed to the ionized carboxyl groups (Erlich, Sutherland, 1954). During the exchange new bands develop at 1450 cm^{-1} and 1464 cm^{-1}, due to the deuterized peptide groups and to the HDO formed during the course of the exchange, respectively. The shoulder at 1554 cm^{-1} is the so-called amide II band, which is of a coupled $-CN-$ vibrational and $=NH$ deformational origin (Miazawa et al., 1958). This is overlapping with the maximum at 1575 cm^{-1}, which is also due to the ionized carboxylic groups of the side chains. The highest maximum can be found at 1655 cm^{-1}, the so-called amide I band, which corresponds to the $C=O$ stretching frequency of the peptide bond. At 1500 cm^{-1} there is an isosbestic point.

It follows from Fig. 1 that the H—D exchange has no effect on the amplitude and position of the amide I band thus this value together with the isosbestic point at 1500 cm^{-1}, can be used as reference. In the following the amide I and amide II designations are used in the sense of the intensities of the corresponding bands. For the base line of amide I the absorption at 1760 cm^{-1} was taken, while for that of amide II, the absorption at 1554 cm^{-1} of the fully deuterized protein was chosen.

For the exact determination of the position of the amide II maximum, difference spectra were taken in time (Fig. 2 and 3), by plotting the difference in the absorptions of the samples at different stages of the exchange as a function of wave number. The position of the maximum proved to lie at wave number

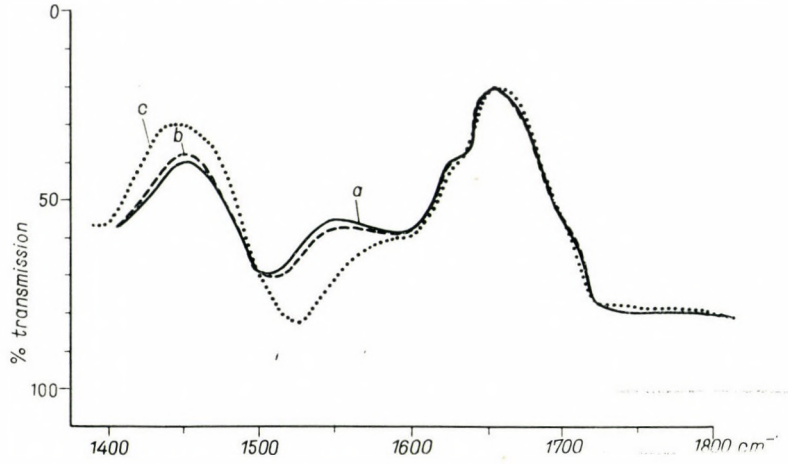

Fig. 1. Infrared spectra of GAPD treated with D_2O at pH 7.5 in 0.1 M phosphate buffer. (UR-10 spectrophotometer, rate of registration 12 cm/min, slit-program 8.) *a)* 1 hour after dissolving in D_2O, *b)* 5 hour after dissolving in D_2O, *c)* sample incubated in 3 per cent sodium dodecyl sulphate (in D_2O) at 85°C for 6 hours.

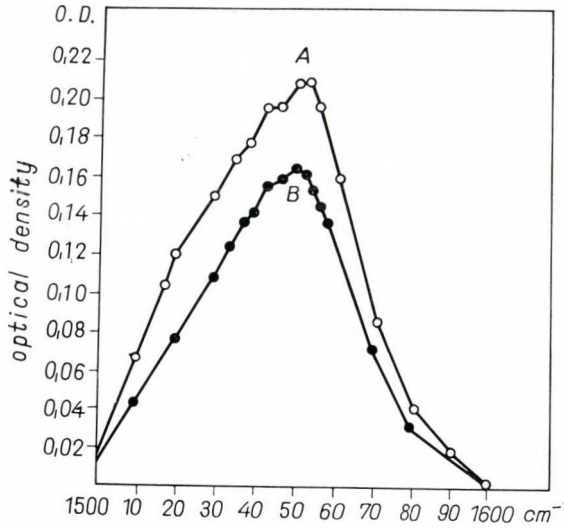

Fig. 2. Infrared difference spectra of the NH-groups of GAPD.
GAPD—NAD complex dissolved in 0.1 M phosphate buffer, pH 7.5
The curves were obtained by calculating the difference between the spectrum of the completely deuterized sample (cf. Fig. 1) and spectra taken 12 minutes and 1 hour after dissolving in D_2O (curves A and B, respectively)

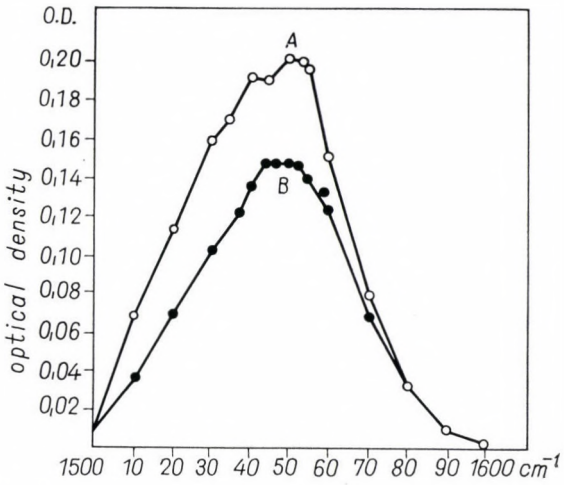

Fig. 3. Infrared difference spectra of the NH-groups of NAD-free GAPD in 0.1 M phosphate buffer, pH 7.5. Curve A and B: as in the legend to Fig. 2.

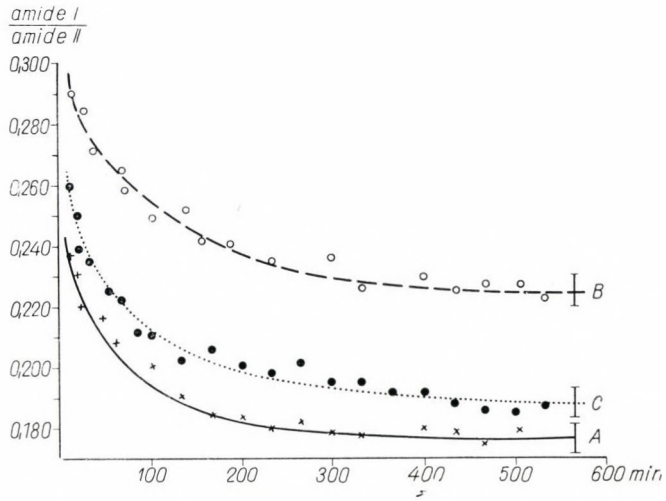

Fig. 4. The rate of H—D exchange in GAPD in 0.1 M phosphate buffer, pH 7.5, at 20°C. A: NAD-free GAPD×, B: GAPD—NAD complex ○, C: GAPD—NADH complex ● The solid lines represent the theoretical curves calculated on the basis of the half-exchange times (cf. Table 3). The points represent the measured values

1554 cm^{-1}. The decrease of the half-width of the difference spectra (from 0.7 to 0.6 cf. Fig. 2 and 3) upon addition of NAD to the NAD-free GAPD also suggests a change towards structural "uniformity". The process of exchange was followed in terms of the change in time of the amide I/amide II ratio (Blout et al., 1961), by registering the changes in transmission at wave number 1554 cm^{-1}.

Fig. 4 shows the kinetics of H – D exchange for the GAPD – NAD, GAPD – NADH complexes and for NAD-free GAPD. The points represent the averages of 4 – 7 experiments. The mean deviation was ±5 per cent. At the beginning of registration (2 – 3 minutes) about half of the peptide hydrogens was already exchanged. In the semilogarithmic plot the composite kinetic curves could be resolved into first order reactions and further three groups of peptide hydrogens could be distinguished. In this way peptide hydrogens could be classified into four groups according to their half-exchange time: I. hydrogens exchanged instantaneously (within 3 minutes), II. and III. hydrogens exchanged within measurable time (from several minutes to several hours) and IV. hydrogens practically unexchangeable, with a half-exchange time of about 60 hours.

The half-exchange time or the rate constant characteristic of the individual groups may be the measure of the accessibility to water.

For the determination of the relative number of hydrogens belonging to the individual groups, the initial value of the amide II/amide I intensity ratio is to be known i.e. the values for the deuterium-free protein. This quotient can be calculated for any protein from the ratio of the molar extinction coefficients of the peptide –NH– and =CO groups, $K_{NH} = 186$ l $mol^{-1}cm^{-1}$ and $K_{CO} = 315$ l $mol^{-1}cm^{-1}$ (Di Sabato, Ottesen, 1965). The value thus obtained is 0.6.

The amide II/amide I value determined by us experimentally for GAPD was found to be 0.53, as calculated from the dry layer infrared spectra. The difference between the calculated and measured values is not significant and can be due to the fact that during the preparation of the dry layer partial denaturation

Table 3

The percentage distribution according to the half-exchange time of the peptide hydrogens of NAD-free GAPD and of the GAPD-coenzyme complexes

Values obtained by the graphical analysis of the exchange kinetic curves

Preparation	I. class per cent $T_{1/2}$ 1'	II. class per cent $T_{1/2}$ 10'	III. class per cent $T_{1/2}$ 60'	IV. class per cent $T_{1/2}$ 60 h
NAD-free GAPD	53	7	8	32
GAPD–NAD complex	44	7	7	42
GAPD–NADH complex	50	7	7	36
LDH	46	8	13	33

could have taken place. This may lead to the modification of the spectrum and consequently of the amide II/amide I ratio as well. For this reason the calculations were based on the value of 0.6.

Table 3 shows the percentage distribution of the four hydrogen groups differing in their kinetic constant i.e. in their half-exchange time.

The removal of firmly bound NAD decreases the number of unexchangeable hydrogens by 10 per cent (Group IV). Groups II and III remain unaffected, while group I increases. This phenomenon can take place in two ways. Either completely buried portions become exposed, or a IV → (II; III) → I transition takes place, and the overall change is reflected in group I.

Discussion

It is known from the investigations of Boyer and Schultz (1958) and Előd i and Szabolcsi (1959) that the binding of NAD by GAPD leads to a decrease in the viscosity of the enzyme while its heat stability, susceptibility to trypsin and levorotation ($-[\alpha]$) are increased. These data have been interpreted in terms of structural changes induced by coenzyme binding.

Recently the effect of coenzyme binding was studied by the optical rotatory dispersion method (Listowsky et al., 1965; Hvidt, 1965; Markovich et al., 1966) and the changes found in the ORD parameters confirm the above interpretation. Listowsky et al. (1965) found, however, that the optical rotatory dispersion curves of the GAPD – NAD complex exhibit a shoulder around 280 mμ. They suggested two alternative explanations for the interpretation of these data. They may reflect either a purely structural alteration of the polypeptide chain, or can be the result of the binding of a chromophore to an asymmetric molecule without any change in the polypeptide conformation.

Optical rotatory dispersion data of proteins are difficult to interpret in the present state of our knowledge. The method, however, provides a very sensitive indicator of conformational changes in general. It is also possible to make a rough estimate of the major changes representing both the alteration in the helical content and the solvation of asymmetry centers, from the changes in b_0 or λ_c and a_0, K_c, respectively. Further details on the nature of the conformational changes, and application of more direct methods for structural analysis are required.

The aim of the present work was to study the steric structure of NAD-free GAPD and GAPD-coenzyme complexes by fundamentally independent methods.

According to our experiments the amount of "hard to exchange" peptide hydrogens in the NAD-free GAPD is about 32 per cent. The exchangeable hydrogens could be classified into three groups on the basis of their half exchange time. This classification of peptide hydrogens reflects the distribution of peptide bonds in the structure of the intact protein molecule.

In the presence of NAD and NADH the number of non-exchangeable peptide hydrogens increases by about 10 and 4 per cent of the total peptide hydrogens, respectively. If in the tetramer we assume the presence of 1300 peptide bonds and 4 moles of bound coenzyme, then one NAD or NADH molecule should overlap about 40 and 13 peptide hydrogens, respectively, which is not probable. In previous work (Linderstrøm-Lang, 1959; Blout et al., 1961; Hvidt, 1963) it has been established that the secondary and tertiary structure of a protein or a polypeptide is an important factor in limiting the exchange of peptide hydrogens. On the basis of these results the decrease in the number of exchangeable peptide hydrogens in the presence of bound coenzymes may be attributed mainly to conformational changes, rather than to the shielding effect of the coenzyme molecules. This assumption is also supported by the slight but definite changes in the hydrodynamic parameters. These results show that the structure of the protein becomes more compact on the binding of the coenzymes.

The optical rotatory dispersion data indicate that NAD-free GAPD contains about 34 per cent ordered structure. These data are in good agreement with the extent of hydrophobic core estimated from H−D exchange experiments. The increase in λ_c and b_0, and the decrease in a_0 and K_c upon the addition of NAD, may be interpreted according to Yang and Doty (1957) and Moffit and Yang (1956), as changes in the ordered structure of the protein. The explanation that the change in optical rotatory dispersion may be caused by an induced extrinsic Cotton effect, as suggested by Listowsky et al. (1965), is not very probable. The extrinsic Cotton effect at 280 and 360 mμ is very weak and insufficient to bring about a 10 per cent change in the parameters.

Upon the addition of NADH to the NAD-free GAPD b_0 and λ_c slightly decrease. At the same time a_0 and K_c decrease parallel approaching the values characteristic of the GAPD−NAD complex. With the exception of λ_c and b_0 calculated for the GAPD−NADH complex, all our results indicate identical changes in the structure of the enzyme upon the binding of any of the coenzymes. However, we should like to stress the point that the contradiction between the changes in λ_c and b_0 values and the changes in the K_c, a_0, H−D exchange and hydrodynamic data may indicate that in the case of NADH binding, overall change to a more compact structure may not be accompanied by increased spiralization. We cannot, however, exclude the possibility of decomposition of NADH during the experiments, though it had been carefully purified and controlled.

On the basis of the above experiments we came to the conclusion that the binding of coenzymes makes the structure of GAPD more compact and closed. This effect in cases of NAD binding is more expressed and goes together with the increase of ordered structure, while the NADH complex formation seems to be accompanied by a slight decrease in spiralization.

It appears very probable that the NAD and NADH molecules alter the whole structure of the protein globule on binding or they cause well-defined

translocations (structural re-organization) in the active center and its wider environment.

As to the interpretation of the mode of action of the coenzyme, the "motile" protein model, suggested by Linderstrøm-Lang and Schellman (1959) seems to be very suitable. This model assumes the permanent fluctuation of the structure of the protein molecule in water solution. In the sense of this model Straub and Szabolcsi (1964) suggested that one of the conformational states of an enzyme molecule is suitable for the binding of substrates and the structural changes induced by substrate binding may be due partly to the stabilization of this particular conformation.

In the light of this model our results can be explained in the following way. In a solution of GAPD at a given temperature and under given conditions, there exists a set of conformations in equilibrium with a constant transition between the individual states. In one conformation the polypeptide chain is more ordered and is in a more compact state, consequently some portions are less accessible to water, while in another state the peptide chain is looser, less ordered, and larger portions are accessible to water. Certain conformations bind the coenzymes more strongly, and the probability of the occurrence of these conformations after coenzyme binding is increased. It seems probable that the optimum conformations for the binding of NAD and NADH are different, and this could be the basis of their different effect.

To sum up, our data indicate that the binding of coenzymes to the protein displaces the structure towards the more ordered and compact state.

In Table 4 the amount of ordered structure calculated on the basis of optical rotatory dispersion is compared with the percentage amount of buried peptide hydrogens of group IV and with the result of Libor et al. (1964) obtained with solvent perturbation and iodine-modification of GAPD, which measure the fractional amount of tyrosyl residues accessible in the native state to the solvent and to iodine, respectively.

Table 4

Comparison of the amount of ordered structure calculated from optical rotatory dispersion, of peptide hydrogens inaccessible to water, and of tyrosyl residues inaccessible to solvent and iodine in case of GAPD and LDH

Preparation	Helical conformation per cent	IV. class per cent	Tyrosyl residues* inaccessible to solvent per cent	Tyrosyl residues* inaccessible to iodine per cent
NAD-free GAPD	34	32	—	—
GAPD—NAD complex	43	42	55	65
GAPD—NADH complex	30	36	—	—
LDH	46	33	45	62

* The data of Libor et al. (1964).

For the sake of comparison we examined the same parameters in LDH, too, and in agreement with Libor et al. (1964) we assume that both enzymes contain a relatively large hydrophobic core of an ordered structure and some loose, motile polypeptide portions.

References

Blout, E. R., De Losé, C., Asadourian (1961) J. Am. Chem. Soc. *83* 1895
Boyer, P. D., Schulz, H. (1958) Sulfur in Proteins, Academic Press, New York, p. 199
Di Sabato, G., Ottesen, M. (1965) Biochemistry *4* 422
Elias, H. G., Grabe, A., Lamprecht, W. (1960) Hoppe-Seyler's Z. für physiol. Chem. *319* 22
Elődi, P., Szörényi, I. T. (1956) Acta Physiol. Acad. Sci. Hung. *9* 339
Elődi, P. (1958) Acta Physiol. Acad. Sci. Hung. *13* 199
Elődi, P., Szabolcsi, G. (1959) Nature *184* 56
Ehrlich, G. and G. Sutherland (1954) J. Am. Chem. Soc. *76* 5268
Friedrich, P. (1965) Biochim. Biophys. Acta. *99* 375
Furfine, C. S., Velick, S. F. (1965) J. Biol. Chem. *240* 1844
Harris, I. J., Perham, R. N. (1965) J. Mol. Biol. *13* 876
Havsteen, B. H. (1965) Acta Chem. Scand. *19* 1643
Hvidt, A. (1963) Compt. Rend. Trav. Lab. Carlsberg *33* 475
Hvidt, A., Kägi, J. H. R. (1963) Compt. Rend. Trav. Lab. Carlsberg *33* 497
Jécsai, Gy. (1961) Acta Physiol. Acad. Sci. Hung. *20* 339
Libor, S., Elődi, P., Nagy Z. (1964) Biochim. Biophys. Acta *110* 484
Linderstrøm-Lang, K. U., Schellman, J. A. (1959) The Enzymes, 2nd ed., vol. 1., chap. 10, p. 443
Listowsky, J., Furfine, C. S., Betheil, I. I., Englard, S. (1965) J. Biol. Chem. *240* 4253
Markovich, D. S., Závodszky, P., Volkenstein, M. V. (1966) Biokhimiya *31*, 837
Miazawa, T., Schimanouchi, T., Mizushima, S. (1958) J. Chem. Phys. *29* 611
Moffit, W., Yang, J. T. (1956) Proc. Natl. Acad. Sci. US. *42* 596
Nielsen (1961) Biochim. Biophys. Acta *37* 146
Shechter, E., Blout, E. R. (1964) Proc. Natl. Acad. Sci. US. *51* 695
Straub, F. B., Szabolcsi, G. (1964) Molecular Biology (in Russian) "Nauka", Moscow
Urnes, P., Doty, P. (1961) Advances in Prot. Chem. vol. 16, p. 401
Velick, S. F., Meyer, I. E., Harting, J. (1953) J. Biol. Chem. *203* 527
Velick, S. F. (1958) J. Biol. Chem. *233* 1455
Yang, J. T., Doty, P. (1957) J. Am. Chem. Soc. *79* 761

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 403—406 (1966)

Separation of Erythrose 4-Phosphate from Glucose 6-Phosphate and Glyceraldehyde 3-Phosphate by Ion Exchange Chromatography

A Preliminary Report

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(Received September 1, 1966)

Two different procedures can be used for the synthesis of D-erythrose 4-phosphate (E-4-P). The first method which is quite laborious involves the phosphorylation of the suitable blocked derivative of D-erythrose (Ballou et al., 1955). The second method far simpler than the first, consists in the oxidation of glucose 6-phosphate by lead tetraacetate. In this latter case the product is contamined by glucose 6-phosphate (G-6-P) and glyceraldehyde 3-phosphate (G-3-P) in addition to inorganic phosphate (P_i) (Ballou, 1963). During our studies on the synthesis of 3-deoxy-D-arabinoheptulosonic acid 7-phosphate by the allosteric enzyme of the aromatic pathway 7-phosphate-2-oxo-3-deoxy-D-arabino-heptonate D-erythrose-4-phosphate-lyase (pyruvate phosphorylating) (EC 4.1.2.15) we observed that the impurities of E-4-P obtained by the oxidation of G-6-P were disturbing the kinetics of catalysis and of allosteric inhibition (Staub et al., 1966). In this paper we describe a simple method for the purification of E-4-P by ion exchange chromatography suitable for kinetic investigations and a thin-layer chromatographic (TLC) method for testing purity.

Thin-layer chromatography. Glass plates (20 × 20 cm) were coated with a slurry of MN cellulose-300 in water using a Desaga applicator to yield a layer 250 μ thick and allowed to dry for 24 h at room temperature before use. The chromatography plates were developed by the ascending method using a solvent system of ethylacetate (110 ml), 90 per cent formic acid (50 ml) and water (15 ml), for 1.5 h, then the plates were removed and dried. The spots of phosphate esters and of P_i were detected by a molybdate spray reagent (Bandursky, Axelrod, 1951). The following R_f values were found: E-4-P: 0.23; G-6-P: 0.43; G-3-P: 0.52; P_i: 0.63.

Ion-exchange chromatography. For the separation of E-4-P obtained by the oxidation of G-6-P, 1 × 12 cm columns of Dowex 2 × 8 (Cl^-) anion-exchange resin (200 – 400 mesh) were prepared and stored at room temperature before use.

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The pH of the mixture of phosphate esters and P_i was adjusted to 8.5 with 0.01 N ammonium hydroxide before chromatography. A sample containing up to 640 μmoles of phosphate esters was applied to the column under gravity flow. This was followed by a wash with two bed volumes of distilled water. The compounds were then eluted with 0.1 M ammonium chloride in 0.005 M $Na_2B_4O_7$. The flow rate was maintained at about 4 ml/min and fractions of 10 ml were collected. The elution of the compounds was followed by determination of P_i and of phos-

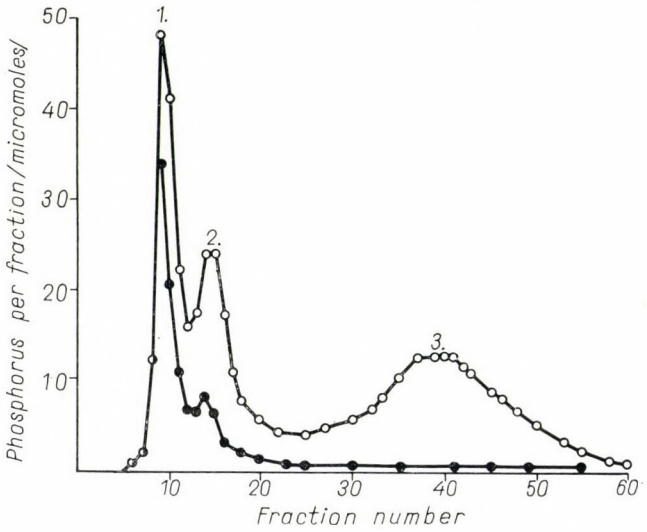

Fig. 1. Chromatogram on Dowex 1 of a mixture of phosphate esters obtained by oxidation of glucose 6-phosphate. Eluting agent: 0.1 M ammonium chloride in 0.005 M M $Na_2B_4O_7$. Phosphate esters, ○—○; inorganic phosphate, ●—●. The peaks: 1: glucose 6-phosphate; 2: glyceraldehyde 3-phosphate; 3: erythrose 4-phosphate

phate esters. For the determination of phosphate esters aliquots of the individual fractions were hydrolyzed with 1.0 N hydrochloric acid at 100°C for 30 minutes and the amount of P_i liberated was assayed (Fiske, Subbarow, 1925). A three-peak elution pattern was obtained as shown in Fig. 1. Fractions representing the peaks were pooled and analyzed for the identification of the phosphate esters by the TLC method. The first peak was identified as G-6-P in addition to P_i, the second one as G-3-P with some P_i impurity, and the third one as E-4-P free of any contaminating compounds.

Although E-4-P can be eluted with the $NH_4Cl - Na_2B_4O_7$ mixture, this procedure was not employed, for it was found that large amounts of borate interfere with the enzymatic reaction. For this reason this elution method was used only for the quantitative determination of E-4-P in the phosphate ester mixture obtained by oxidation of G-6-P. For the preparation of E-4-P for kinetic

studies, after the elution of the first peak with the $NH_4Cl - Na_2B_4O_7$ mixture, E-4-P was eluted with 0.1 M ammonium chloride, as shown in Fig. 2.

With the method described above, analytically pure E-4-P was prepared. The yields, calculated from the amount of E-4-P present in the original mixture, varied from 85 to 95 per cent.

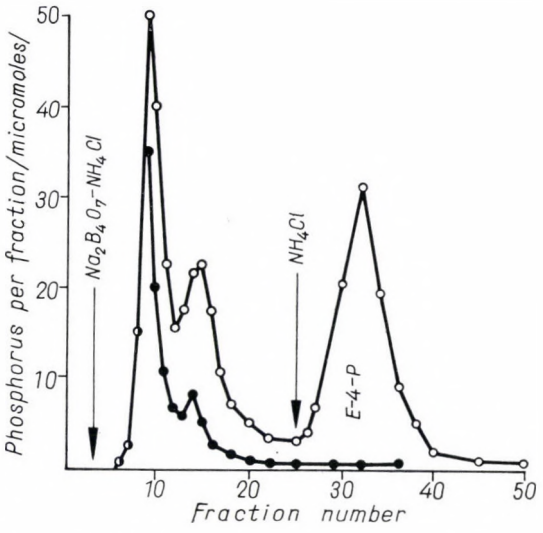

Fig. 2. Separation of erythrose 4-phosphate from phosphate esters and inorganic phosphate by ion exchange chromatography. Phosphate esters, ○—○; inorganic phosphate, ●—●

References

Ballou, C. E., Fischer, H. O. L., MacDonald, D. L. (1955) J. Am. Chem. Soc. *77* 5967
Ballou, C. E., (1963) in Colowick, S. P., Kaplan, N. O. (Editors), Methods in Enzymology, vol. 6. p. 479, Academic Press, New York.
Bandursky, R. S. Axelrod, B. (1951) J. Biol. Chem. *193* 405
Fiske, C. H., Subbarow, Y. (1925) J. Biol. Chem. *66* 375
Staub, M., Łobarzewski, J., Dénes, G. (1966) In preparation.

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 407—412 (1966)

Migration of the Substance of the Anisotropic Band of the Cross Striated Muscle during the Release of Myosin

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(Received January 6, 1966)

A 0.8 per cent Weber—Edsall solution was passed by suction through isolated fibrils prepared from the thorax muscle of Apis mellifica to make the release process slower and the migration of myosin inside the sarcomere was measured quantitatively by means of an interference microscope. About half of the myosin content of the anisotrope band migrated near to the Z-line and formed a thickened Z-line, similar to the contraction line, and was released from here. The other half of the anisotrope band was removed during the experiment. The process of the migration of the substance of the anisotropic band and the release of myosin are well reflected by the densitometric curves. The substance which is able to migrate may play a role in the concentration of the muscle and in the formation of the contraction line.

Introduction

According to the literature the contraction line sometimes appears in the muscle irrespective of contraction. According to some observations the substance of the sarcomere accumulates near the Z-line (Hasselbach, 1953). In our earlier experiments (Tigyi-Sebes, 1964) a similar phenomenon was observed in connection with the release of myosin inside the sarcomere. The protein substance of the A-band migrated from its original place, accumulated beside the Z-line and formed thus a thickened Z-line, similar to the contraction-line. The further investigation of this problem is the aim of the present paper.

Methods

The thorax muscles of Apis mellifica used for the investigations were taken apart with a dissecting needle and the fibrils thus obtained were mounted on a slide in Pringle solution. Preparations made in this way were investigated in a phase contrast microscope. Two time phases were observed during the process of release; a quick phase lasting for 3 to 5 minutes and a slow phase lasting for 25 to 30 minutes. To make the observations more precise isolated fibrils were looked for in the preparations (see the method of Ernst et al. 1956) and the migration of

the substance of the A-band was investigated on such isolated fibrils. The whole process was photographed on a substandard film (16 mm) with a motion picture camera of type AK – 16, which made 64 exposures in a minute. Thus the migration of the protein content of the anisotropic band could be followed step by step in the course of the projection of the film.

To make the release process slower, the Weber – Edsall solution was diluted (0.8 per cent). By using such dilute solutions the first quick phase of release could be prolonged to 20 to 25 minutes. Thus the process could be observed also in an interference microscope of type Baker. Photographs were taken in each stage. The photographs were registered with respect to the phases discussed below by a Kipp automatic photometer.

Results

Fig. 1 shows fibrils investigated and measured in the interference microscope in the phases following each other according to the above discussed method. Fig. 1*a* shows the picture of a freshly prepared fibril. Shortly after the starting of the flow of the fluid the substance of the anisotropic band divides into two; a light band appears and the release of the myosin begins (Fig. 1*b*). The migration of the substance of the anisotropic band towards the Z-line is the following step and in this phase the light line formed in the middle of the anisotropic band increases in size (Fig. 1*c*). Fig. 1*d* which follows shows that the substance of the anisotropic band accumulates closely beside the Z-line. Fig. 1*e* shows the I-line and the Z-line covered with the migrating anisotropic substance and the progress of dissolution. Fig. 1*f* shows the next phase in which the Z-line appears more intensively and seems somewhat thicker than the original Z-line.

Measurements were made in the above phases and the migration of the substance of the anisotropic band could be followed quantitatively. In these cases the anisotropic band, the Z-line and the migrating A-substance were measured. Series were made from such measurements and the averages of the relative values compared to those of the freshly prepared fibril, were calculated. The following results were obtained: Taking the amount of the substance of the A-band as unity, it decreased to its 0.73rd part in the course of the release process, in accordance with Fig. 1*b*, then to its 0.50th part, according to Fig. 1*c* and to its 0.43rd part, according to Fig. 1*d*. In the phase shown in Fig. 1*e* and even more in the phase shown in Fig. 1*f* the decrease approaches zero.

Densitometric curves were also made from the photographs. These curves follow well the release process and the process of migration of the substance. Such curves are represented in Fig. 2. The curve of Fig. 2*a* was made of a freshly prepared fibril, that of Fig. 2*b* reflects the anisotropic band divided into two in the middle. The double peak (A_1, A_2) shows the divided anisotropic band, the minor peaks between them represent the Z-line, and the curve shows the starting

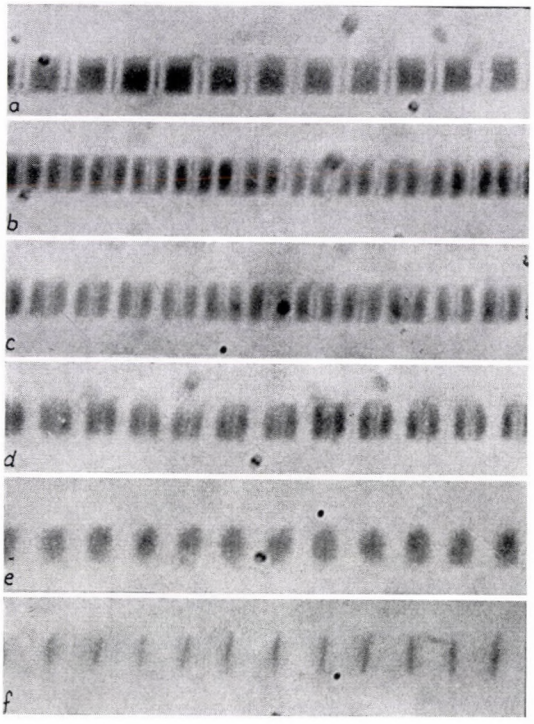

Fig. 1. Fibrils photographed in an interference microscope in consecutive phases during the release of myosin. *a)* Freshly prepared fibril, before treatment, *b)* Fibril, perfused with a 0.8 per cent Weber—Edsall solution, *c)* A light line appears in the middle of the anisotropic band, and the release process begins, *d)* The substance of the anisotropic band accumulates beside the Z-line, *e)* Z-lines are being covered with the substance of the anisotropic band, *f)* Z-lines remain practically in separated state

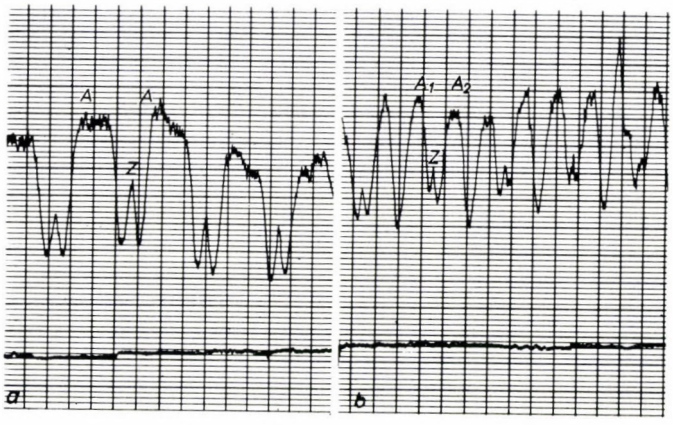

Fig. 2. (See text on p. 410.)

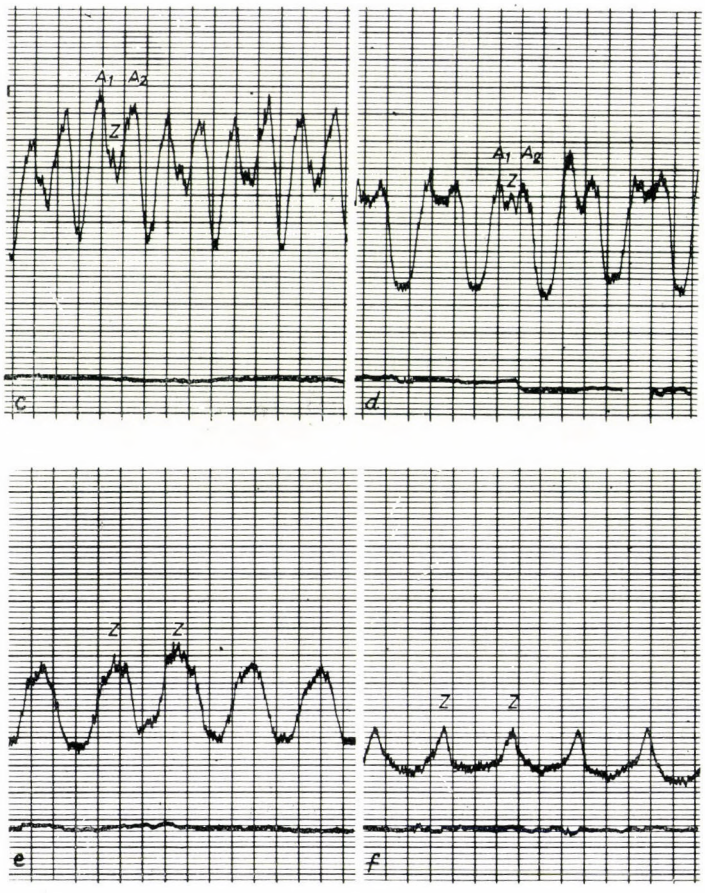

Fig. 2. Densitometric curves taken from the corresponding pictures shown in Figs. 2a, b, c, d, e, f by an automatic photometer of type Kipp. The figures show the process of the migration of the substance, the process of the release of myosin. *a)* Freshly prepared fibril, *b)* The double peak A_1, A_2 designates the anisotropic bands divided into two, the minor peaks show the Z-line, *c)* The accumulation of the substance of the anisotropic band beside the Z-line, *d)* The distribution of the substance beside the Z-line, *e)* The curve of the decreasing anisotropic substance, *f)* Pictures made from the Z-line. The line under the curves is the base line

of the release of myosin as well. In Fig. 2*c* further accumulation of the A-substance beside the Z-line can be seen, Fig. 2*d* designates an increase in thickness beside the Z-line and also a decrease of the substance in the middle of the A-band. In Fig. 2*e* a further decrease in the quantity of the A-substance accumulated beside the Z-line can be seen. The peaks of Fig. 2*f* indicate the Z-lines; A-substance is practically completely released.

Discussion

The above discussed migration of the myosin of the anisotropic band seems to be proved by the measurements performed on the pictures taken, and in the interference microscope. The conclusions drawn from these measurements are the following: One part of the myosin localized in the anisotropic band migrates beside the Z-line during the release process. This accounts for about half of the myosin, as calculated from the mean values of the relative measurements on the anisotropic band. It forms a thickened Z-line there, similar to the contraction line. The other half of myosin is released. During a release lasting for a longer time, the migration of substances comes to an end and only the Z-lines and the substance keeping them together remain. Other authors have also observed the migration of substances (Hodge, 1955; Szent-Györgyi, 1963; Ashley, 1951) from the A-band by different methods, and their results are in good agreement with ours. According to these data the myosin is localized inside the sarcomere in the A-band and is able to migrate and in certain circumstances accumulates beside the Z-line. The observations Szent-Györgyi et al. (1963a) concerning the A-substance in connection with contraction and migration of the myosin can be looked upon as an interesting analogue.

In our previous publication (Tigyi-Sebes, 1962) we have reported the localization of the A-substance and its ash. Our present aim is to investigate the possible co-migration of the A-substance and its ash or rather their localization during migration.

According to our present results this substance of the anisotropic band which is able to migrate may play a role in muscle contraction and also in the formation of the contraction band.

Acknowledgement

Author wishes to express her gratitude to Professor E. Ernst for suggesting the experiments to be performed and for the encouragement given in the course of the work.

References

Ashley, C. A., Porter, K. R., Philpott, D. E., Hass, G. M. (1951) J. Exp. Med. *94* 9.
Ernst, E., Béleczki, L., Nagy, J. (1956) Acta Physiol. Acad. Sci. Hung. *9* 7.
Hasselbach, W. (1953) Z. Naturforsch. *8*.b. 450.
Hodge, A. J. (1955) J. of Biophysical and Biochemical Cytology. *1* 361.
Szent-Györgyi, A. G., Holtzer, H. (1963) Biochim. Biophys. Acta *74* 709.
Szent-Györgyi, A. G., Holtzer, H. (1963a) Biochim. Biophys. Acta *74* 722.
Tigyi-Sebes, A. (1962) Acta Physiol. Acad. Sci. Hung. *22* 243.
Tigyi-Sebes, A. (1964) MBT III. Vándorgyűlés előadáskivonatok, pp. 12.

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 413—418 (1966)

Effect of Visible Light on Stopped Frog's Heart

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(Received June 1, 1966)

Frog's heart stopped by K-free "Ringer" solution were subjected to the effect of very intensive visible light. In the case of an illumination intensity of 60,000 lux, the functioning of hearts could restart, when the K-free "Ringer" solution contained dissolved Na-eosin in a low concentration. In our opinion this effect is connected with the capability of light to excite electrons.

Introduction

The physical and chemical changes caused by illumination are connected with changes produced by the light in the electron states of the illuminated sample (e.g. with the excitation of the electrons or ionization). The biological effects of light are due to the same phenomenon. Some authors have reported experiments in which illumination by visible light altered the function of nerve or muscle (Agnoli and de Barbieri, 1929; Ernst, 1963; Lippay, 1929, 1930, 1930a; Ludkovskaya and Kayushin, 1959; Sandow and Isaacson, 1960; Viale, 1921). Our recent experiments suggest, that the interrupted ability of isolated frog's hearts to produce stimuli can be restored as a result of illumination by visible light, if the K-free "Ringer" solution in the heart contains Na-eosin in a low concentration.

Methods

The hearts for the experiments were prepared from Rana esculenta and they were isolated on Straub's cannulas. Frog's hearts isolated in this way are beating rhythmically for about a week under favourable conditions in the presence of Ringer solution. However, if the Ringer solution is modified so that its KCl- or $CaCl_2$ content is replaced by NaCl, the hearts in the absence of K or Ca stop beating in 1 or 2 days. This is, however, a reversible phenomenon. If the hearts are filled again with a normal Ringer solution, the beating of the hearts restarts and is rhythmical again. The hearts stopped by Ca-free solution do not give any reaction upon mechanical or electrical stimulation, on the other hand, a heart stopped by K-free solution give 1 contraction if stimulated in the above ways.

The light necessary for the illumination was produced by a projection bulb of 500 W, with tungstene filament. The light beam was made parallel by a condenser lens and was passed through a layer (10 cm thick) of a 1 per cent $CuSO_4$ solution in order to filter out heat rays, and finally it was collected by a concave mirror on a 1 cm^2 area. The illumination intensity produced in this way was 60,000 lux at the highest. The light intensity could be decreased by reducing the current of the electric bulb to the extent that the final illumination intensity was 30,000 lux (Fig. 1).

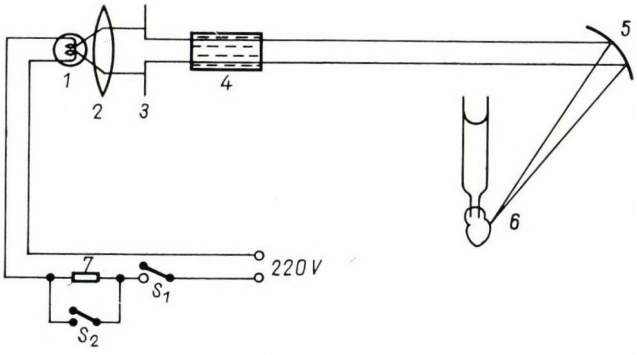

Fig. 1. 1. 500 W electric bulb 2. condenser 3. diaphragm 4. 1 per cent $CuSO_4$ solution 5. concave mirror 6. frog's heart 7. series resistance. The illumination intensity was 60,000 lux when switches S_1 and S_2 were simultaneously turned off and 30,000 lux when only switch S_1 was turned off

The experiments were carried out as follows: The stopped heart was fixey in position so that the spot of light assuring the highest illumination intensitn reached it after switching on the bulb; the sinus venosus was turned in the directios of the light beam. The experiment was continued only when a mechanical stimulu triggered one single contraction (Ernst, 1966).

After this the content of the heart was renewed by a solution of identical composition. It was checked whether or not the change of solution itself caused contraction. Each observation lasted for two minutes. The heart prepared in this manner was illuminated in order to test whether or not contractions took place upon illumination. The illumination intensity was 60,000 lux for two minutes. After these preliminary tests the solution was changed again, but now the heart was refilled with a K-free (or Ca-free) solution, which contained Na-eosin in a concentration of 10^{-5} g/ml. This was followed by an additional observation for two minutes. Occasional contractions were counted. (Sometimes the experiments were somewhat modified. These modifications will be mentioned separately.)

Cases, when the contraction of only some parts (e.g. the atrium) of the heart was observed were also recorded. Illumination intensity was measured by a selenium-cell lux-meter so that the light was transmitted through a grey filter

which diminished the light intensity to its hundredth. (The spectrum of the visible light filtered by a 1 per cent $CuSO_4$ solution differs from that of the white light in the range of 640 – 760 mμ, but both of them have a similar character in the range of 400 – 640 mμ. Although the difference in the spectra introduces some inaccuracy into the results of lux-measurements, the deviation is not important from our point of view. Therefore no corrections were made.)

It was examined whether the very high light intensity in spite of filtering increased the temperature of the heart in the course of the experiment. A pearl thermistor thermometer of small inertia was used for the measurements, the thermistor was connected in a Wheatstone bridge. The deviation of the microammeter of the unbalanced Wheatstone bridge was linearly proportional to the temperature in the range under consideration (15 – 22°C). The thermistor was calibrated by a mercury thermometer. The sensibility of the microammeter of the bridge allowed to record temperature fluctuations with an accuracy of 0.2°C. The measurement was carried out so that the thermistor was put to the illuminated outer wall of the heart; on other occasions the thermistor was made slip into the inside of a heart, or the temperature was measured in the inside of a small part of musculus gastrocnemius of about 1 gm. The temperature rose about 1°C every two minutes when a light intensity of 60,000 lux was applied.

Results

1. 85 frog's hearts stopped by K-free solution were tested. Contractions were found after the renewal of the same solution (in the first phase of the experiments) on three occasions; the hearts restarted beating in the absence of Na-eosin when illuminated by 60,000 lux, on five occasions. Contractions were observed after refilling the hearts with K-free solution containing Na-eosin, without switching on the light-source, in 23 experiments (in these cases the illumination intensity was 5 – 15 lux); but contractions did not occur after refilling the hearts with a solution containing Na-eosin, without illumination in 62 experiments. Contractions took place (or rhythmical beating of the hearts was observed) simultaneously with the illumination of the hearts filled with a solution containing Na-eosin on 39 occasions; 46 hearts, however, did not contract under the effect of illumination.

2. In 16 experiments contractions were observed after filling in the Na-eosin containing solution at once, and the number of contractions rose significantly during illumination. This change might have been caused by the light, however, these cases were not taken into consideration in the evaluation. The results are summarized in Table 1.

3. Some of the experiments reinforce the hypothesis that the ability of the hearts to produce stimuli restarts under the effect of light. In seven experiments an illumination intensity of 30,000 lux was inefficient, but contractions occurred with an illumination intensity of 60,000 lux.

Table 1

Frequencies of the experiments

	After filling in Na-eosin, in darkness	After filling in Na-eosin, illuminated	Sum
Started beating	23	39	62
Did not start beating	62	46	108
Sum of experiments	85	85	170

The hearts restarted beating under the effect of an illumination intensity of 60,000 lux in two experiments, but they stopped, if we switched off the light source, and restarted beating, when the light was turned on again. The phenomenon was repeated two times with one of these hearts, and four times with another one. Although we have only a few experiments of this kind, still the phenomenon is so characteristic that it should not be left out of consideration.

4. Among 44 frog's hearts stopped by Ca-free "Ringer" solution only one started beating during the illumination in the presence of Na-eosin, however, this heart (in contrast to the others) contracted under the effect of a mechanical stimulus, too. The remaining 43 hearts stopped by Ca-free solution, which failed to react to a mechanical stimulus, did not restart even, under the effect of light, but they worked rhythmically if filled with normal Ringer solution.

We may conclude that the frog's hearts stopped by Ca-free solution don't start even under the simultaneous effect of Na-eosin and light. This can be expected, since the musculature of the heart in these cases did not even contract under the effect of a direct stimulus (Belágyi, in the press).

Discussion

The results of the experiments show, that light of high intensity may induce the ability of stopped but excitable frog's hearts to produce stimuli with Na-eosin present. Seemingly this conclusion is inconsistent with experiments in which the presence of Na-eosin caused contractions without illumination. Therefore we controlled by a χ^2-test whether the frequencies of the experiments found with Na-eosin present, but without switching on the light, and the frequencies of the experiments found with Na-eosin present and under simultaneous illumination originate from the same distribution (Table 1).

We got $\chi^2 = 6.49$, which means that the probability of these two frequencies not being different is $P < 0.05$ (in other terms the two frequencies differ at a

probability level of 5%). Consequently we may state with this statistical certainty that frog's hearts stopped by K-free solution may restart beating with Na-eosin present under the simultaneous effect of an illumination intensity of 60,000 lux.

The light energy restarting the beating of the hearts in two minutes was 0.25 cal/cm^2 at an illumination intensity of 60,000 lux; considering, that the mechanical equivalent of 1 lumen light flux is 0.00147 Watt. (This recalculation factor is related to a wave-length of 555 mμ, but it is sufficient to consider this as a mean value for white light, if we want to estimate only the quantity of the absorbed light energy.)

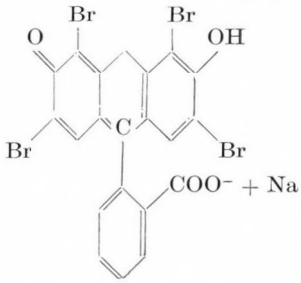

Fig. 2. The structure of Na eosin. The network of π-electrons allows the transport of energy absorbed from the light

This amount of energy would heat a heart of 0.5 g by about 0.5°C; in fact the rise of temperature was as high as 1°C (probably because some infrared rays penetrated through the filter), but this is too little to cause the observed effect.

In our opinion the generating of excitation restarts under the effect of electrons excited by the light energy; the fluorescent Na-eosin absorbs the light energy, which excites electrons and the energy of excitation may migrate over the network of π-electrons to the site of the formation of excitation (Fig. 2). This hypothesis is in accordance with previous reports, which claimed electron excitation and energy migration to be connected with photosynthesis (Riehl, 1940; Szent-Györgyi 1941); soon afterwards the possibility of energy-migration was experimentally supported (Bücher and Kaspers, 1946). Later on an increasing number of reports were being published about semiconductor properties of organic and biological substances (e.g. Eley et al., 1953; cf. Lakatos, 1962). The semiconductor properties of biological substances were also claimed to be connected with the excitation processes (Ernst, 1955–56; Liberman, 1958). These experiments support the hypothesis that the formation of excitation may be connected with some kind of electron process.

Acknowledgement

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

References

Agnoli, R., de Barbieri, A. (1929) Arch. Exp. Path. Pharm. *143* 46.
Belágyi, J. Acta Biochim. Biophys. Hung. (in the press)
Bücher, Th., Kaspers, J. (1946) Naturwiss. *33* 93.
Eley, D. D., Parfitt, G. D., Perry, M. J., Taysum, D. H. (1953) Trans. Farad. Soc. *49* 79.
Ernst, E. (1955—56) Wiss. Z. Humboldt-Univ. Berlin. Math. Naturwiss. Reihe *5* 167.
Ernst, E. (1963) Biophysics of the Striated Muscle Acad. Press, Budapest.
Ernst, E. (1966) Acta Biochim. Biophys. Hung. *1* 61.
Lakatos, T. (1962) Acta Physiol. Acad. Sci. Hung. *22* 297.
Liberman, E. A. (1958) Biofizika *3* 743.
Lippay, F. (1929) Arch. Ges. Physiol. *222* 616.
Lippay, F. (1930) Arch. Ges. Physiol. *224* 584.
Lippay, F. (1930a) Arch. Ges. Physiol. *224* 600.
Ludkovskaya, R. G., Kayushin, L. P. (1959) *4* 104.
Riehl, N. (1940) Naturwiss. *28* 601.
Sandow, A., Isaacson, A. (1960) Biochim. Biophys. Res. Communications. *2* 455.
Szent-Györgyi, A. (1941) Nature, London. *148* 157.
Viale, G. (1921) Arch. di Scienze Biologiche p. 231.

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 1, pp. 419—426 (1966)

Hindered Diffusion along the Membrane and Water Transport in a Two-membrane System

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(Received October 1, 1965)

In a preliminary experiment water transport was observed across a three-compartment system provided with parchment paper and cellophane membrane. The closed central compartment was filled with a colloidal solution, the side chambers with water.

The systems used in further experiments were three-chambered too, but both of the membranes were the same (e.g. parchment paper). The membranes were enclosed between two perforated plexiglass sheets. Water transport took place in the direction of that membrane which was covered by the plexiglass sheet on a larger area. The water transport can be described on the basis of the thermodynamics of irreversible processes and be explained by the flow of dissolved substances in the double slit between the plexiglass sheets. Free diffusion of the dissolved substance is inhibited by the plexiglass sheets. Therefore, concentration gradients are produced at the membranes which induce water transport across the membranes.

Introduction

The membranes of biological objects have in many cases several layers. Therefore the study of transport processes across systems consisting of several membranes seems to be justified. This paper deals mostly with water transport. The simplest membrane series consists of two membranes. Data on such systems can be found in the literature since the second half of the 19th century.

According to a reference by Lepeschkin (1906), Sachs (1865) supposed that the water flows in one direction across some plant cells because the permeability of the cell wall and that of the hialoplasm are different at the site of inflow and outflow, respectively. Pfeffer worked out the classical theory of water transport across a system in the case of a solution enclosed between two different membranes. Lepeschkin (1906, 1908, 1908a, 1909) used this theory for the explanation of certain water transport processes and of bleeding in plants.

Some of the authors (Kedem and Katchalsky, 1958; Katchalsky, 1960; Ginzburg and Katchalsky, 1963) used the thermodynamic theory of irreversible processes for the analysis of the flow across biological membranes. This made the quantitative evaluation of volume flow possible. For example a volume flow (I_v) across a membrane can be described by the following equation.

$$J_v = L_p (\Delta p - \sigma \Delta \pi) \tag{1}$$

where Δp means the difference in hydrostatic pressure and $\Delta \pi$ the difference in osmotic pressure between the two sides of the membrane. L_p is the filtration coefficient and σ is the Staverman reflection coefficient or the so-called selectivity coefficient of the membrane.

Curran and McIntosh (1962) and later Ogilvie et al. (1963) performed volume flow measurements on series system models with two different membranes. They used equation (1) according to the experimental conditions and found it to be correct. According to Dainty and Ginzburg (1964, 1964a) Ginzburg and Katchalsky have also demonstrated that the movement of dissolved substances and water is polarized in a system where the two membranes are arranged in a series and the permeability of the two membranes is different. Dainty and Ginzburg (1964, 1964a) have taken into consideration the above considerations in the explanation of water transport across plant cell membranes.

The above-mentioned authors performed their two-membrane model experiments mostly with sucrose and other crystalloid solutions, and for comparatively short periods. Homola (1962) observed water transport to go on in two-membraned model experiments for more than half a year if the enclosed space between the membranes was filled with a colloidal solution.*

The main subject of this paper is to report a series of experiments in which the water transport is not caused by the difference in permeability between the two membranes, but by the differences in membrane spaces which are covered by impermeable sheets.

Methods

The apparatus used consisted of three compartments were separated from each other by membranes (m_1 and m_2) of the same quality and area. For example both membranes were of commercial mono-layer parchment papers. The area of each membrane was $F = 225$ cm^2. Both membranes were pressed together from both sides by plexi glass sheets which were perforated symmetrically with holes of 3.2 mm in diameter before fitting in the membranes. Fig. 1 shows that the perforations were at the same places on the two plexi glass sheets. Thus the membrane was either left uncovered or was covered on both sides by the plexi glass sheets. There were 1230 perforations beside membrane m_1 while beside membrane m_2 there were only 123. Therefore membrane m_2 was covered on a greater area ($f_2 = 215$ cm^2) than membrane m_1 ($f_1 = 125$ cm^2) by the plexi glass sheets on

* No detailed description of these preliminary experiments will be given in this paper. 15 volume flow experiments were carried out (e.g. a flow of 0.027 ml/day.cm^2 for 215 days). 9 further experiments were performed to determine the hydrostatic pressure difference between the two side chambers on both sides of the system as a result of water transport (e.g. pressure difference of 136 watercm).

both sides. According to the results of later measurements the plexi glass sheets did not press close against the membrane. Thus, there were very narrow slits between the membranes and the plexi glass sheets in which water could flow freely along the membranes. Membrane m_1 had a free area of $F - f_1 = 100$ cm^2 while the free area of membrane m_2 was $F - f_2 = 10$ cm^2. The edges of the membranes were made water-proof and were sealed with liquid paraffin and rubber sheets.*

The other parts of the instrument were made of plexi glass. There were two holes on the top of each compartment. Filling up and de-aeration of the instrument were performed through these holes. The central compartment was filled up

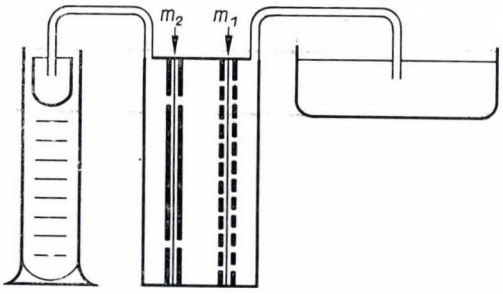

Fig. 1

with 25 per cent gum-arabic or with a 10 or 20 per cent aqueous colloidal dextran solution.** The central compartment was closed throughout the experiment. The two side chambers were filled with distilled water and afterwards only one hole was sealed out of the two holes on the top of each side chamber and a glass tube was placed in the others. When volume flow (J_v') was measured, the glass tube from the side chamber beside m_2, was connected to a small glass vessel, and was filled up with water before starting the experiment. The flow volume is equal with the volume of the solution dripping from the small glass vessel into a measuring cylinder and thus J_v' can be determined. The water content of the side chamber beside m_1 was supplied through a glass tube connected to a broad glass vessel filled with water which was sucked in by the instrument. With this arrangement J_v' could be measured either at a desired difference of water level or at an equal water level. In the experiments described in this paper water levels were equal. The evaporation of the water could be minimized by covering up the equipment.

* The volume of the central compartment was 250 ml. The volume of the side chamber beside m_1 was 60 ml, the volume of the side chamber beside m_2 was 30 ml.

** The ash content of 1.00 g dry substance of the experimental solution was: gum arabic 0.033 g; dextran 0.010 g.

Differences in hydrostatic pressure ($\Delta p'$) can be measured in the two membrane system by adjusting a glass manometer to one of the two holes in each side chamber. In such cases water levels must be kept equal in the two manometers at the beginning of the experiment and the differences between the water levels must be observed (Fig. 2).

The instrument must be taken apart and cleaned after each measurement of either J'_v or $\Delta p'$. New measurements can be made only after replacing the mem-

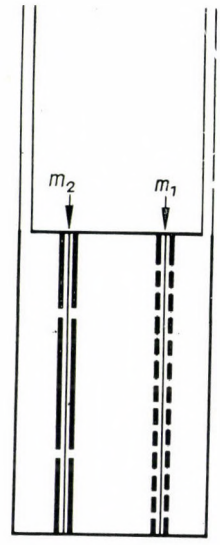

Fig. 2

brane by a fresh one and after filling the instrument up with fresh colloidal solution and distilled water. Thus, substantially each observation is a separate experiment. The experimental conditions can remain the same or can be changed, as preferred. The following changes can be done: the concentration of the colloidal solution, its composition, the temperature of the experimental system, the quality of the membranes and the shape and position of the plexi glass sheets can be changed. After finishing each experiment the concentrations are to be determined in the two side chambers by either refractometry or by methods involving the determination of freezing-point decrease or dry substance determination. Concentration determination is made during the experiment also, in the case of observing J_v' in the sample taken from the small glass vessel.

Results

Volume flow through the experimental system was observed in the direction $m_1 \rightarrow m_2$. In nine experiments volume flow was observed in eight cases. In all the eight cases the central compartment was filled with a 25 per cent gum-arabic solution. The duration of the volume flow was 1 to 32 days. During this time maximum volume was 19.8 ml which flowed through the instrument. After the cessation of the volume flow the concentration of the dissolved substance in the side chamber beside m_1 was found to be always higher (e.g. five times as high) than in the side chamber beside m_2. The membrane built into the instrument was permeable to a certain extent for the dissolved substance ($\sigma < 1$). Despite the concentration difference between the two side chambers the water was flowing in the direction $m_1 \rightarrow m_2$ as long as the water transport effect of the concentration difference did not compensate for the volume flow. The direction of the water transport turned after the compensation because of the increasing concentration difference. When the side chambers were rinsed with distilled water and filled with fresh distilled water at the time of compensation, the volume flow started again in the original direction. Later compensation ensued again. Rinsing could be repeated several times and thus gradual volume flows followed each other. In one of the experiments altogether 74.2 ml flowed through the instrument during 100 days.

The experiments were usually performed at room temperature, with decreasing temperature the volume of the flow also decreased and the compensation ensued sooner. Great decrease in the flow could be induced also by building in cellophane membranes into the instrument instead of parchment paper ones. The permeability of the parchment paper is greater than that of the cellophane paper with respect to the dissolved substance. The decrease of the volume flow will be understandable in the discussion. The permeability is even smaller with dextran. In the experiment already mentioned where no volume flow was observed, two layered cellophane membranes were built into the instrument and the central compartment was filled with a 20 per cent dextran solution.

Volume flow could also be measured in instruments where the covering of the membranes and the shape of the covering sheets were other. Water transport was observed to a lesser extent when no perforated plexi glass sheets were used beside the membranes on the side of the central compartment.

The measurements of the differences in hydrostatic pressure ($\Delta p'$) between the two side chambers were generally in agreement with those of the volume flow experiments under similar experimental conditions. In each of the eight measurements there was a higher hydrostatic pressure in the side chamber beside membrane m_2. The value of $\Delta p'$ varied from 0.4 to 184 watercm. For the formation of $\Delta p'$ 0.5 to 19 days were needed when the described method was used. The value of $\Delta p'$ was greater when using instruments with parchment paper membranes than with cellophane paper. In experiments performed with gum-arabic solutions the value

of $\Delta p'$ was greater than in those performed with dextran solutions. The value of $\Delta p'$ was greater at higher than at lower temperatures. When two layered cellophan paper membranes and a 20 per cent dextran solution were used, the value of $\Delta p'$ was 0.4 watercm only.

Results of the control measurements: There was no water transport if all three chambers were filled with the same colloidal solution or with distilled water. Water of the same volume filtrates across both of the side membranes during the same time if the central compartment is filled up with water under pressure.

Discussion

Curran and McIntosh (1962) and later Ogilvie et al. (1963) have shown experimentally and theoretically that water transport takes place through a two membrane system if the values of σ of the two membranes related to the molecules dissolved in the central compartment are different. In a case like this according to Ogilvie et al.

$$J_v' = \frac{L_{p1} L_{p2}}{L_{p1} + L_{p2}} (\sigma_1 \Delta\pi_1 + \sigma_2 \Delta\pi_2) \tag{2}$$

where $\Delta\pi_1$ and $\Delta\pi_2$ have equal values but different signs. According to the author this equation is applicable to experiments with membranes of parchment paper, cellophan paper and with colloidal solutions, when the two different membranes are built into the same instrument.

For the instrument described in detail in this paper equation (2) is seemingly not applicable because the two membranes are identical, and therefore

$$\sigma_1 = \sigma_2 \tag{3}$$

Considering the concentration differences between the chambers with respect to their sign as well, the differences in osmotic pressure at the beginning of the experiment are

$$\Delta\pi_1 = -\Delta\pi_2 \tag{4}$$

The substitution of equations (3) and (4) into equation (2) gives $J_v' = 0$ and thus the process seemingly cannot be described in this way.

Nevertheless, the water transport seems to be explainable if the concentrations in the immediate vicinity of the two membranes are considered. The dissolved substance slowly flows from the central compartment to the two side chambers across the membranes. The dissolved substance which flows across the free membrane area freely diffuses into the water of the side chamber. There is solution in the slit of both sides beside the covered membrane area. The dissolved substance cannot diffuse freely after flowing across the membrane because the

plexi glass from the side of the side chamber inhibits diffusion. The concentration difference is smaller between the two sides of the covered parts of the membranes than between the two sides of the free parts of the membranes. Let us consider the unit membrane area as consisting of n elemental membrane areas. Let the calculated osmotic pressure difference for the unit membrane area be $\Delta\pi_m$, and the osmotic pressure force difference effecting the elemental membrane area $d\pi_m$. The individual values of $d\pi_m$ differ from each other. Thus, $\sum_1^n d\,\pi_m = \Delta\pi_m$. Membrane m_1 has more uncovered area than membrane m_2. Therefore

$$\Delta\pi_{m_1} > \Delta\pi_{m_2} \tag{5}$$

From this it can be concluded that volume flow takes place in the system in the direction $m_1 \rightarrow m_2$ according to equation (2) also.

The concentration differences responsible for the volume flow are due to the characteristic shape of the cover plates beside the membranes. The cover plate prevents the free diffusion of the dissolved substance which flowed across the membrane on the one hand, and changes the direction of flow of the dissolved substance and the water from perpendicular to the membrane to parallel, on the other.

If we relate the average osmotic pressure difference effecting the membrane to the osmotic pressure difference of the solutions between the side chambers, then for the instrument in operation at a given time

$$\frac{\Delta\pi_{m_1}}{\Delta\pi_1} = q_1 \text{ and } \frac{\Delta\pi_{m_2}}{\Delta\pi_2} = q_2 \tag{6}$$

Because

$$\Delta\pi_{m_1} = q_1\Delta\pi_1 \quad \text{and} \quad \Delta\pi_{m2} = q_2\Delta\pi_2 \tag{7}$$

furthermore

$$L_{p1} = L_{p2} \tag{8}$$

considering equations (3), (4), (7) and (8), equation (2) can be transformed into the following, after disregarding the distinctive indexes of the equal values:

$$J_v' = \frac{L_p L_p}{L_p + L_p}(\sigma q_1 \Delta\pi - \sigma q_2 \Delta\pi) = \frac{L_p^2 \sigma \Delta\pi}{2L_p}(q_1 - q_2) \tag{9}$$

where because of the signs of quotients q the signs of the concentration differences were also taken into consideration.

The experiments reported can be of importance in the understanding of biological water transport processes where the membrane system is multi-layered and where the flow of the dissolved substance is hindered by one of the perforated layers (e.g. a lipoid layer or cells beside membranes).

In biological structures which function according to the operation method of the experimental system, the energy can derive from the concentration differences of the dissolved substances formed during metabolism.

References

Curran, P. F., McIntosh, J. R. (1962) Nature *193* 347.
Dainty, J., Ginzburg, B. Z. (1964) Biochim. Biophys. Acta *79* 102.
Dainty, J., Ginzburg, B. Z. (1964a) Biochim. Biophys. Acta *79* 112.
Ginzburg, B. Z., Katchalsky, A. (1963) Journ. Gen. Physiol. *47* 403.
Homola, L. (1962) Bull. Lect. II. Biophys. Meeting Hung. 36.
Katchalsky, A. (1960) Congress Transport and Metabolism, Prague.
Kedem, O., Katchalsky, A. (1958) Biochim. Biophys. Acta *27* 229.
Lepeschkin, W. W. (1906) Beih. z. Bot. Zentralblatt *19* 409.
Lepeschkin, W. W. (1908) Ber. d. Deutsch. Bot. Ges. *26a* 198.
Lepeschkin, W. W. (1908a) Ber. d. Deutsch. Bot. Ges. *26a* 231.
Lepeschkin, W. W. (1909) Beih. z. Bot. Centralblatt *24* 308.
Ogilvie, J. T., McIntosh, J. R., Curran, P. F. (1963) Biochim. Biophys. Acta *66* 441.

Printed in Hungary

A kiadésért felel az Akadémiai Kiadó igazgatója Műszaki szerkeztő: Farkas Sándor

A kézirat nyomdába érkezett: 1966 X. 3. — Terjedelem: 7,25 (A/5) ív, 24 ábra

66.62959 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

Errata

Vol. 1.

P. 5, line 4: change "protein becomes to bind" into "the protein becomes unable to bind"

P. 74. On the lower myofibril of Fig. 1. the labels A and Z are to be reversed.

P. 76. The two curves of Fig. 4 are to be reversed.

P. 199. Instead of q_l, q_a.

P. 201. In Table 3 the time is 8. In column 4 instead of spec. ac, spec. act$_l$.

P. 401, Table 4, column 5: change "65" into "35" and "62" into "20"

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