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The N-terminal Tryptic Peptide of Rabbit Muscle Aldolase*

(Short Communication)

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(Received November 30, 1970)

According to Udenfriend and Velick (1951) the N-terminal amino acid residue of aldolase is proline. The N-terminal sequence, including three and six residues, has been studied by Lai (1968) and Kochman et al. (1968), respectively. The sequences reported were Pro.His.(His) and Pro.His.Ser.His.Pro.Ala. As lysine or arginine is not encountered among the first six residues, a larger portion of the N-terminal sequence might be revealed through the analysis of the tryptic N-terminal peptide.

Isolation and amino acid composition of the N-terminal peptide

Rabbit muscle aldolase isolated according to Taylor et al. (1949) was carboxymethylated and digested with trypsin as described earlier (Sajgó, 1969). The freeze-dried hydrolysate was gel-filtered on a Sephadex G-25 (fine) column, and the fractions were pooled on the basis of light absorption at 225 nm (Fig. 1).

All fractions were analyzed by two-dimensional high voltage paper electrophoresis (pH 6.5, pyridine–acetic acid–water, 96:4:900, v/v; pH 1.9, formic-acid–acetic acid–water, 20:80:900, v/v; voltage gradient 50 and 75 V/cm, respectively).

The N-terminal peptide was detected with the isatine colour reaction (Monier, Jutisz, 1954). Peptides having proline N-terminal give a characteristic blue colour with isatine on paper. Since during the tryptic hydrolysis of the intact protein the intrachain peptides possessing proline as N-terminal cannot be liberated, owing to the resistance of Lys.Pro and Arg.Pro bonds, the peptide having proline N-terminus in the hydrolysate may only come from the N-terminal end of the protein.

Of all fractions obtained by gel-filtration only fraction 2a contained peptide that gave with isatine the reaction characteristic of proline N-terminus. The

* While preparing this manuscript we were kindly informed of a similar line of work going on in Dr B. L. Horecker's laboratory. (Dr B. L. Horecker, private communication).

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peptide was basic at pH 6.5 and contained histidine as shown by the Pauly-reaction. This peptide was then purified by preparative paper electrophoresis at pH 6.5 and 1.9. Its N-terminus was determined by the dansyl-method (Gray, 1969a), the identification of dansyl amino acids was carried out by means of thin layer electrophoresis (Sajgó, 1970). The N-terminus proved to be purely proline.

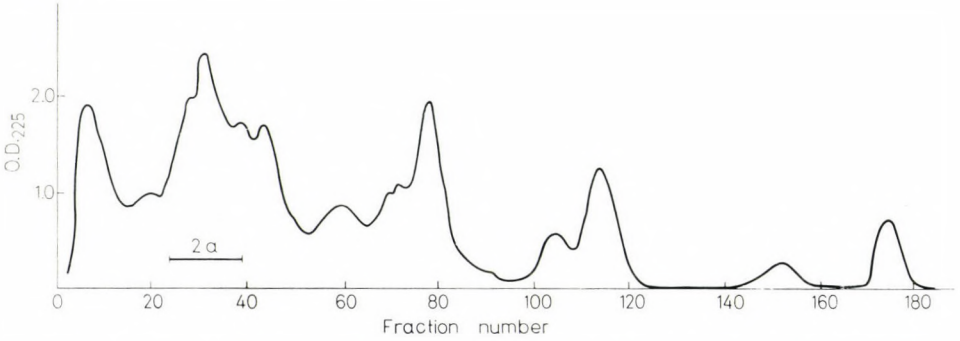

Fig. 1. Fractionation of the tryptic hydrolysate of aldolase on a Sephadex G-25 (fine) column. Size of column: 127 × 2.7 cm; flow-rate: 32 ml/hour; eluent: 0.1 M ammonium bicarbonate containing 1% butanol; V_0: 210 ml; fraction volume: 8 ml

To determine the amino acid composition of the peptide, it was subjected to hydrolysis at 105°C in 6 N hydrochloric acid for 16 hours. Quantitative amino acid analysis was performed according to Dévényi's single column procedure (Dévényi, 1969). The following amino acid sequence was obtained:

$$Pro.(Thr_1Ser_1Glu_2Pro_1Ala_1Leu_1His_{2+}).Lys$$

Degradation of the N-terminus by the dansyl-Edman method

The sequence of the peptide was analysed by the dansyl-Edman method (Gray, 1969b). Histidine was unambiguously shown to be the second residue in the sequence. However, dansyl-Ser and dansyl-His were liberated simultaneously after the second residue. Dansyl-His, dansyl-Pro and dansyl-Ala were identified at the fourth, fifth and sixth positions, respectively, without any contamination. The lack of accompanying contaminations indicated that each cleavage step reached completion.

Analysis of the partial acid hydrolysate and subtilysin hydrolysate of the N-terminal peptide

The peptide was hydrolyzed with concentrated hydrochloric acid at 37°C for 48 hours, and the hydrolysate, after drying, was fractionated by means of preparative paper electrophoresis at pH 6.5.

Table 1

Amino acid sequence of the N-terminal tryptic peptide of aldolase

Dansyl-Edman degradation of the N-terminus	Pro.	His.	Ser. His.	His.	Pro.	Ala					
Peptides isolated from the partial acid hydrolysate of the N-terminus	Pro.	His. His.	His. (Ser$_1$ Ser.	His$_1$ His.	Pro$_1$) Pro.	Ala. Ala.	Leu, Leu.				
								Thr. Thr.	Glu. Glu.	Glu. Gln.	Lys Lys
Peptides isolated from the subtilysin hydrolysate of the N-terminus	Pro.	His.	Ser.				Leu.	Thr.	Glu.	Gln.	Lys
	Pro.	His.	Ser. His.	His.	Pro.	Ala.	Leu.	Thr.	Glu.	Gln Glu	Lys
	1	2	3	4	5	6	7	8	9	10	11

Three out of the peptides produced by hydrolysis with subtilysin (pH 8.5, 0.1% ammonium bicarbonate, 37°C, 2 hours) provided important additional information to the alignment of peptides obtained from partial acid hydrolysis and to the elucidation of heterogeneity at the third position of the sequence.

Table 1 shows the sequences of peptides obtained by partial acid and subtilysin hydrolysis.

The sequence of the N-terminal peptide

The sequence of the N-terminal tryptic peptide, as deduced from dansyl-Edman degradation, partial acid hydrolysis and hydrolysis with subtilysin, is presented in Table 1.

At the third position of the sequence both histidine and serine can be detected. The existence of this duality could also be verified by the direct identification of the phenylthiohidantoin derivatives of serine and histidine originating from the third position of the sequence. Separation of these derivatives was achieved by shaking out in butylacetate or, in case of PTH-His, by extracting in hot absolute ethanol (cf. Bailey, 1967). The final proof of this microheterogeneity is provided by the fact that from the partial acid hydrolysate peptides Pro.His.His and Ser.His.Pro.Ala.Leu, whereas from the subtilysin hydrolysate peptide Pro.His.Ser could be isolated.

Lai (1968) claims that the subunits of aldolase slightly differ from each other. Structural differences supporting this conclusion have been demonstrated

by Lai et al. (1970) in the C-terminal sequence of aldolase. Our results presented here indicate that such structural differences also occur at the third and tenth positions of the N-terminal sequence. The quantitative investigation of this microheterogeneity is in progress.

The devoted technical assistance of Mrs K. Lendvay is gratefully acknowledged. Thanks are due to Mrs J. Báti for the amino acid analyses.

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The Role of the Histidine Side Chains in the Catalytic Activity of B. Cereus Exopenicillinase

(Short Communication)

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(Received August 21, 1970)

The exopenicillinase of B. cereus looses its activity under the effect of iodine in two independent steps (Csányi et al., 1970; Ferencz et al., 1970). Apparently this phenomenon is not the consequence of the existence of two conformations or two isoenzymes with different sensitivities to iodine (Mile et al., 1970a, b) but it can rather be attributed to the properties of the molecules.

The aim of our investigations was to study the role played by the amino acids reacting with elementary iodine in the activity and iodine sensitivity of exopenicillinase. In fact, under appropriate experimental conditions, iodine oxidizes the SH groups and the tryptophan side chains of the proteins, furthermore substitution reactions can occur between iodine and the tyrosine, tryptophan or histidine side chains. The reaction between iodine and the amino acid side chains depends on the pH and on the temperature of the medium as well as on the conformation of the given protein.

In this paper we report some investigations on the role of the histidine side chains in the activity and the iodine sensitivity of the enzyme. A specific reagent, diethyl pyrocarbonate (DEP), was employed in the experiments. This reagent, similarly to iodine, substitutes the proper amino acid side chains, but does not take part in oxidation reactions. According to Mühlrad et al. (1967) and Ovádi et al. (1967), at pH 6 DEP substitutes the imino group of histidine only, forming N-carbethoxy histidine. A similar reaction occurs with histidine residues in proteins provided the former is accessible to the reagent. Thus it is possible to selectively substitute the histidine side chains, and to study their role in enzyme activity in a direct manner. Pradel et al. (1968) employed this method to examine the histidine located in the active center of ATP guanidine phospho-transferase.

We employed B. cereus 569/H exopenicillinase in our experiments. The purification of the enzyme (Csányi et al., 1970) yielded an exopenicillinase preparation the purity of which corresponded to that of a crystalline preparation with a specific activity of 2.12×10^6 E/mg N. DEP ("Baycovin") was a product of Bayer (Levercusen, Germany).

2.8×10^{-5} M penicillinase and 1.7×10^{-4} M DEP were incubated at room temperature. Preliminary experiments showed that 10 minutes were sufficient for the completion of the reaction, i.e. the number of N-carbethoxy histidine

did not change upon further treatment. The protein was dissolved in 0.05 M phosphate buffer at pH 6 while DEP was diluted to the required concentration with ethyl alcohol. The activity of the enzyme was not affected by ethyl alcohol at the concentration (0.5 per cent) employed in the experiment. On the basis of the molar difference extinction coefficient of N-carbethoxy histidine ($\Delta\varepsilon_{240} = 3.2 \times 10^3$; Ovádi et al., 1967) and the protein concentration we calculated the

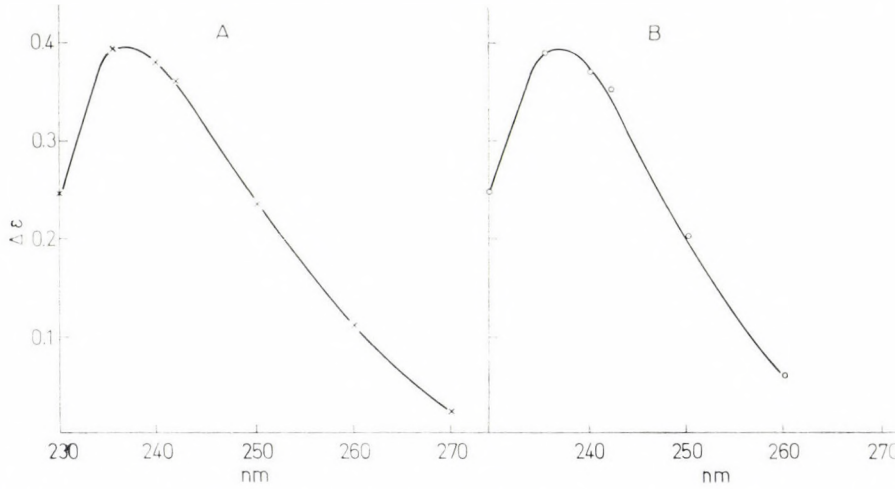

Fig. 1. Difference spectra of penicillinase after treatment with diethyl pyrocarbonate in the absence (x-x-x) and presence (o-o-o) of urea. *A)* 2.8×10^{-5} M penicillinase and 1.7×10^{-4} M diethyl pyrocarbonate were incubated in 0.05 M phosphate buffer, pH 6, for 10 min at room temperature. *B)* 2.8×10^{-5} penicillinase and 1.7×10^{-4} M diethyl pyrocarbonate were incubated in 6.1 M urea at pH 6 for 10 min at room temperature.

amount of histidine residues per molecule, which reacted with DEP. Fig. 1A shows the difference spectrum from 230 to 270 nm of penicillinase treated with an excess of DEP. The difference spectra were taken in the Cary 15 spectrophotometer. According to the experimental data shown in Fig. 1A four N-carbethoxy histidine residues were formed per one penicillinase molecule.

The experiment was repeated in 6.5 M urea in order to determine the total number of histidine residues in the protein (Fig. 1B). The difference spectrum of exopenicillinase treated with DEP in the presence of four N-carbethoxy histidine residues. Thus four histidine side chains per penicillinase molecule reacted with DEP both in the case of the native and denatured protein at pH 6. These results point to the fact that all four histidine residues of penicillinase are located at the surface of the protein and are easily accessible to DEP. The number of histidine residues determined by means of DEP was equal to that obtained by amino acid analysis (Imsande, 1969).

In our experiments the activity of penicillinase upon treatment with DEP was also determined. Enzyme activity was measured according to a previously

reported method (Csányi et al., 1970). The activity of the enzyme containing four N-carbethoxy histidine residues was found to be equal to that of the untreated enzyme. Thus the introduction of DEP in the penicillinase molecule did not cause any change in the activity of the enzyme.

As the protein solution had to be used in a hundred-fold dilution for the determination of enzyme activity by the spectrophotometric method, it could be assumed that the reaction of histidine and DEP was reversible and the enzyme was reactivated by dilution with 0.05 M phosphate buffer, pH 6. Thus we lyophilized the diluted solution and measured the N-carbethoxy histidine content of the concentrated solution prepared after lyophilization. No change was found in the number of histidine residues reacting with DEP. Thus we concluded that the reaction product was stable under the conditions of the enzyme assay.

We investigated the inactivation of the enzyme treated with DEP by iodine in the above described manner employing a previously reported method (Csányi et al., 1970). The sensitivity to iodine of the enzyme was found to be unaltered by treatment with DEP under the given conditions.

Our experimental results seem to exclude the possibility that histidine plays a role in the activity of penicillinase.

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Paper Chromatographic Separation and Determination of Nucleoside Phosphates in Acidic Rat Tissue Extracts

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(Received September 18, 1970)

A method has been developed for the quantitative determination of nucleoside phosphate levels in animal tissues by extraction with perchloric acid, separation of the neutralized extract in a paper chromatographic system and spectrophotometric determination. 1. R_f values of a number of nucleoside phosphates are presented, compared in four solvent systems. 2. The isobutyric acid solvent system, found to be the most suitable for the separation of tissue extracts could be applied to the resolution of a mixture of ATP, ADP, and AMP within an error of $\pm$ 3%. 3. In this system the components of rat tissue extract, ATP, ADP, AMP, NAD, and other nucleoside di- and triphosphates, could be separated and determined. The spots were identified by the aid of added standard compounds and ultraviolet spectra. 4. Normal levels of the above constituents are described for rat skeletal and heart muscle, liver and kidney. Data are presented on the changes that occur upon exhaustion. The findings are checked by measuring the phosphate fractions of different lability, and are compared with the results of other authors.

Introduction

The simple and exact measurement of free nucleoside phosphates is very important for the study of oxidative phosphorylation and energy-requiring processes in animal tissues. Several methods have been applied for this purpose, generally after extraction with perchloric or trichloroacetic acids. Before the advent of modern separation techniques the resolution was based on the different solubilities of heavy metal salts of nucleoside phosphates, followed by chemical analysis (Rapoport, Nelson, 1945; Le Page, 1946; 1946a). Later, sensitivity and selectivity were fundamentally improved by the introduction of enzymic analyses (Rapoport, Nelson, 1945; Kalckar, 1947; Slater, 1953; Glock, McLean, 1955, 1957; Dianzani, 1957) and separation techniques (Hurlbert et al., 1954; Visioli et al., 1964; Fox et al., 1965; Voskoboinikov, 1967). Most of the recent methods use spectrophotometric analysis for the determination of the separated components.

Abbreviations: B_1, B_2, etc. and I_1, I_2, etc: unidentified ultraviolet absorbing compounds on the paper chromatograms (solvent systems No 2 and 4, respectively) in the order of increasing R_f values. P_h = phosphorus after hydrolysis in 1 N HCl at 100°C for 7 minutes, P_t = total phosphorus, P_l = labile phosphorus ($P_h - P_i$), P_s = stable phosphorus ($P_t - P_h$).

In spite of the vigorous work going on in this field, data from different authors show large variation as to the amount of ATP and other nucleoside phosphates in untreated animal tissues. In order to develop a relatively simple and exact method for the measurement of nucleoside phosphate (mainly ATP, ADP and AMP) content of tissue extracts, we studied a number of paper chromatographic systems. The system used in our previous work (Forgách, Rosdy, 1966) for similar purposes with horse muscle extracts rich in ATP, ADP and AMP could not be generally used for other tissue extracts. In this paper the separation characteristics of four different solvent systems are given, together with the obtained Rf values of commercial nucleoside phosphates. A statistical determination of errors is described in conjunction with the most suitable system for the quantitative measurement of a mixture of ATP, ADP and AMP in animal tissue extract. Finally, the amount of acid-soluble nucleoside phosphates in skeletal and heart muscle, liver and kidney of rats is given and compared with the data of other authors.

Materials and methods

Standard materials

Adenine and uracil were purchased from B.D.H. (Poole, England); adenosine from Fluka (Buchs, Switzerland). The following preparations were supplied by Reanal (Budapest): ATP, ADP, 2'(3')-AMP, UTP, UDP, UMP, 2'(3')-UMP, Urd, CTP (Ba-salt), CDP, CMP, 2'(3')-CMP (Ba-salt), Cyd, Cytosine, GTP, GMP, 2'(3')-GMP (Ba-salt), Guo, NAD. NADP was a Boehringer (Mannheim, GFR) preparation. AMP was prepared in our laboratory. The preparations were mainly sodium-salts: the Ba-salts were used in acidic solution. Commercial preparations were used without further purification.

The following paper chromatographic solvent systems were employed:

No 1. Two-phase system. Lower phase: 5% Na_2HPO_4 saturated with freshly distilled isoamylalcohol. Upper phase: freshly distilled isoamylalcohol saturated with 5% Na_2HPO_4 (Cohn, Carter, 1950). Descending technique on Schleicher–Schüll No 2043/b ausgew. paper, duration 9–11 hours.

No 2. 69% (v/v) n-butyric acid, 0.85% (v/v) NaOH in water (Wade, Morgan, 1955). Ascending technique; on Whatman No 3 paper the front migrated 35 cm in 36 hours.

No 3. 2 M acetic acid, 0.05 M citric acid in water, pH 3.0 (adjusted with NaOH). Ascending system. In 2.5 hours the front migrated 17 cm on Whatman DE 81 ion exchange paper.

No 4. Isobutyric acid–cc. NH_4OH–water (66:1:33) (Vardanis, Hochster, 1961). Descending system: on FN-16 (Niederschlag, G.D.R.) or S-S 2043/b ausgew. papers the front migrated 36–40 cm in 17 and 12 hours, respectively.

All chromatographic runs were made at room temperature (23–26°C). The papers were not washed before use. FN-16 and S-S 2043/b ausgew. papers, however, are marketed in washed form.

Detection and quantitative measurement of the compounds

Ultraviolet-absorbing spots were detected in the dark over a light source emitting between 250 and 260 nm ("Germicid" lamp with Schott UG-5 filter).

After the paper chromatographic separation of mixtures containing 0.02–0.2 mg of each component, the spots were cut out together with blank parts of the paper of the same size at the height of each component. The paper squares were cut into small pieces. Elution was performed in test tubes, with 7 ml of 0.1 N HCl, for 3–15 hours (generally overnight), with occasional shaking. The components were quantitatively assayed in the filtrates (in case of FN-16 paper, filtration was not necessary) with a Spectromom 201 type spectrophotometer (MOM, Budapest). Adenosine derivatives were measured at 257 nm, NAD at 260 nm (optical path of cell 1 cm). The readings were corrected for the blanks and the concentrations were calculated on the basis of known molecular extinction coefficients (see below).

Statistical analyses

For statistical evaluation two mixed solutions of ATP, ADP and AMP were separated in solvent system No 4 (0.02 and 0.1 μmole of each component). Four paper sheets were used in each experiment. On each sheet three identical samples of the standard mixture were applied in 10 mm lines with drying by air flow; two blanks could be taken for each component. In this way 12 parallel determinations were made in four groups. A. D. (average deviation) values for each sheet and S. D. (standard deviation) values for the four parallel sheets were calculated.

Preparation of tissue extracts

Female Wistar rats weighing 120–150 g were used in the experiments. Exhaustion was achieved – if indicated – by swimming for 10 minutes. The animals were killed in a rapid chloroform narcosis (to prevent degradation of adenosine phosphates) by decapitation and bleeding. After excision the organs were immediately frozen in acetone-dry ice, and the further steps were made in the cold (cooling mixture, 0–4°C).

Muscles were homogenized with two volumes per weight, liver and kidneys with one and a half volumes per weight of 1 N $HClO_4$ for 3 minutes. (Skeletal muscle in a Waring blendor-type knife-homogenizer, the other organs in a glass Potter-homogenizer.) After centrifugation (15 minutes, 2000 *g* at 1–3°C) the tissue residues were homogenized again with the same volumes of 0.5 N $HClO_4$ for 1.5 minutes. The pooled filtrates were neutralized with 10 N KOH, and the $KClO_4$ precipitate was removed by centrifugation. The extracts were stored at −15 to −20°C.

Nucleoside phosphate analysis of tissue extracts

Each tissue extract was applied on a paper sheet in three parallel lines (150–200 μl each). FN-16 or S-S 2043/b ausgew. paper and solvent system No 4 were used. Descending chromatography was made in a Chopa (VEB Glaswerke, Ilmenau, GDR) glass apparatus, for 26–36 hours. The solvent overrun

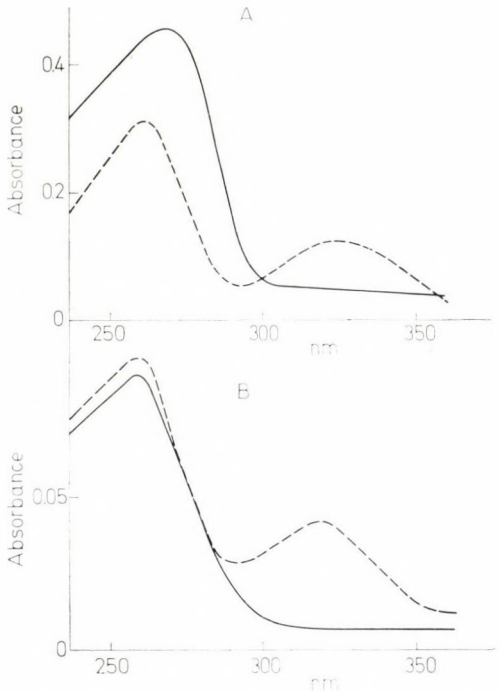

Fig. 1. Ultraviolet spectra of standard NAD preparation and spot "I_5" eluted from isobutyric acid–NH_4OH–water chromatogram of rat liver extract. Dotted line: spectra in the presence of 1 M potassium cyanide. A: standard NAD, B: spot "I_5", identified as NAD

the paper, the lower end of which was cut in zig-zag. After drying in air current the spots were localized, eluted and measured in the way described above. Correction was made for the average of two blank values.

The ultraviolet-absorbing spots were mainly identified by comparing their R_f values with those of standard compounds. These results were checked by adding ATP, ADP and AMP separately to tissue extracts. In case of NAD the spectra of the standard compound and that separated from tissue extracts were also compared in the presence and absence of KCN (Ciotti, Kaplan, 1957) (see Fig. 1).

The nucleoside phosphate content of tissues was calculated according to the following formula:

$$\frac{(E - E_v) \times Ex \times El \times A \times 1000}{\varepsilon \times 10^{-3} \times Fcs} = \mu\text{moles of component per g fresh tissue}$$

where

E, E_v = average optical density values of the eluates of the absorbent spots and paper blanks, respectively
Ex = volume of extracting solvent used for 1 g tissue (ml)
El = volume of solvent used for the elution of each spot (ml)
A = correction factor, under our conditions 1.13; it comes from the extraction losses and the change in volume during neutralization (determined by adding standard compounds to the extracts)
Fcs = volume of neutralized extract introduced onto each line of the paper (μl)
ε = molecular extinction coefficient of the examined compound (pH 1–2)

The values employed were as follows: ATP_{257} 14.7×10^3; ADP_{257} 14.9×10^3; AMP_{257} 15.1×10^3; NAD_{260} 18.0×10^3 (Pfleiderer, 1961; Kornberg, Pricer, 1953).

In cases of unidentified compounds as well as when the total amount of adenosine phosphates or ultraviolet-absorbing compounds was measured, the average value for adenosine phosphates (14.9×10^3; 257 nm) was used for the calculation.

Analysis of phosphorus fractions in the tissue extracts

Inorganic phosphorus was measured by a modified Briggs method (Martland, Robison, 1926). 60% (w/w) perchloric acid was used in the colour reaction instead of 30% (v/v) sulphuric acid (final acid concentration: 0.73 N). When hydrolysis was omitted, the colour reaction was measured 15, instead of 30, minutes after the addition of the last reagent, to avoid the acidic degradation of nucleoside di- and tri-phosphates.

Labile phosphorus fractions were hydrolyzed in 1 N HCl at 100°C for 7 min (P_h). Stable phosphorus compounds were hydrolyzed by heating in concentrated perchloric acid until total hydrolysis (about 2 hours) (P_t). Labile and stable phosphorus fractions are given as differences of $P_h - P_i$ and $P_t - P_h$, respectively.

The amounts of phosphorus fractions in the fresh tissues were calculated from the formula:

$$\frac{P\ (\mu g/\text{ml extract}) \times Ex \times A}{31} = \mu\text{moles of phosphate per g fresh tissue}$$

Results

In order to find a suitable method for the separation of nucleoside phosphates from various mixtures, especially tissue extracts, the separation characteristics of the indicated four solvent systems were studied from several aspects.

Various types of papers were used for the separation of standard ATP, ADP and AMP. Table 1 shows the Rf values of these three compounds in several combinations of experimental conditions.

In cases of solvent systems No 1 and 4, FN-16 and S-S 2043/b ausgew. papers were the most suitable for complete separation. Only Whatman DE 81

Table 1

R_f values of ATP, ADP and AMP in four solvent systems on various papers

Compound	Solvent system*	R_f values				
		FN-16	S-S 2043/b ausgew.	Whatman 1	Whatman 3	Whatman DE-81
ATP	No 1.	—	0.86	0.80	0.88	—
	No 2.	0.30	0.33	—	0.37	0.09
	No 3.	—	—	—	—	0.03
	No 4.	0.25	0.28	0.20	0.35	0.02
ADP	No 1.	—	0.80	0.74	0.82	—
	No 2.	0.48	0.45	—	0.49	0.26
	No 3.	—	—	—	—	0.18
	No 4.	0.32	0.42	0.32	0.48	0.09
AMP	No 1.	—	0.71	0.66	0.75	—
	No 2.	0.63	0.57	—	0.59	0.53
	No 3.	—	—	—	—	0.58
	No 4.	0.45	0.61	0.44	0.66	0.32

* See Materials and methods.

paper can be used with solvent system No 3. The most compact spots, important for quantitative measurements, were obtained on paper FN-16.

In Figure 2 the separation of standard compounds with adenine skeleton can be seen in the four solvent systems. As the figure shows, the main disadvantage of solvent systems No 1 and 2 is their inability to separate AMP and NAD. System No 1 cannot even satisfactorily separate ADP and ATP from each other. Solvent system No 3 cannot separate adenosine from adenine, but it can separate NADP and NAD, an advantage over all other systems (not indicated in Figure 2). This system, however, could not be used at all for the separation of tissue extracts.

Isobutyric acid–ammonia–water solvent system (No 4) was studied in detail, as it was found the most convenient system to separate tissue extract constituents before quantitative measurement. Table 2 shows the R_f values of a number of various standard nucleoside phosphates in this system.

Of the examined solvent systems, No 2 and 4 could be suitably used for tissue extracts. The separation of ultraviolet-absorbing compounds of skeletal muscle and liver extracts is summarized in Table 3.

Because of its ability to separate AMP and NAD, solvent system No 4 was used for routine analyses. As can be seen in Figure 3, this system is able to resolve the four main constituents (ATP, ADP, NAD, AMP) of muscle, liver and kidney extracts. The analyses were not substantially influenced by the pres-

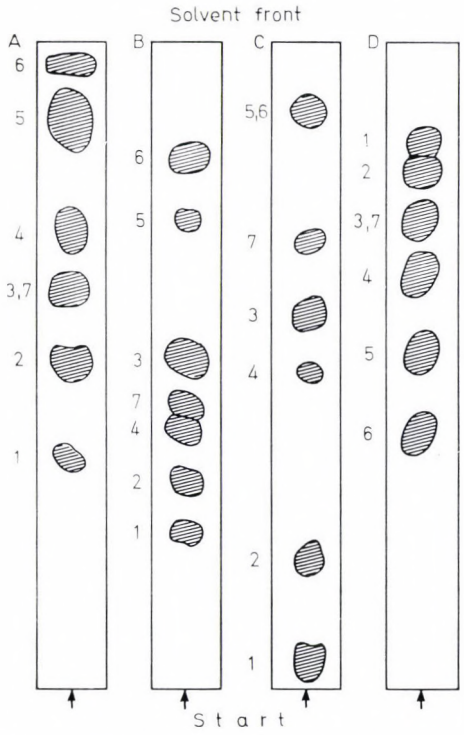

Fig. 2. Tracing of adenine compounds after paper chromatography in different solvent systems. A: Butyric acid 69% (v/v); NaOH 0.85% (w/v); Wh. 3. paper; ascending run (System No 2); B: Isobutyric acid–cc. NH_4OH–water (66:1:33); FN-16 (Niederschlag, GDR) paper; descending run (System No 4); C: Acetic acid 2 M, citric acid 0.05 M, pH 3.0 (with NaOH); Wh. DE 81 paper; ascending run (System No 3); D: Isoamylalcohol-5% sodium phosphate; Schleicher–Schüll 2043/b ausgew. paper; descending run (System No 1). Ultraviolet-absorbing standard compounds: 1: ATP, 2: ADP, 3: AMP, 4: 2'(3')-AMP, 5: adenosine, 6: adenine, 7: NAD

ence of fluorescent spots, found mainly on chromatograms of liver and kidney extracts. These spots could most often be locally separated, and if not, they did not absorb considerably at the used wavelengths anyway.

Using highly purified ATP or ADP in this system, we got homogeneous spots after chromatography; no decomposition could be detected. Thus the system proved to be suitable to resolve different phosphorylated nucleosides in a mixture.

Figure 1 shows the identification of NAD from liver extract. The absorption spectra of the separated compounds and standard preparation were compared

Table 2

R_f values of purine and pyrimidine nucleoside phosphates, nucleosides and bases in isobutyric acid–cc. NH_4OH–water (66:1:33) solvent system (pH 3.7–4.1) on Schleicher–Schüll 2043/b (ausgew.) paper

Compound	ATP	ADP	AMP	2'(3')-AMP	Ado	Adenine
R_f value	0.28	0.42	0.61	0.73	0.90	0.95
Compound	GTP	GDP	GMP	2'(3')-GMP	Guo	
R_f value	0.08	0.15	0.24	0.31	0.48	
Compound	UTP	UDP	UMP	2'(3')-UMP	Urd	Uracil
R_f value	0.09	0.15	0.22	0.27	0.43	0.57
Compound	CTP	CDP	CMP	2'(3')-CMP	Cyd	Cytosine
R_f value	0.14	0.24	0.36	0.41	0.59	0.75

in the presence and absence of 1 M KCN. In addition to the identity of R_f values, the examined compound (I_5) proved to be identical with NAD in this respect too.

The paper chromatographic separation method with solvent system No 4 and quantitative analysis of the separated components were statistically evaluated in a model experiment, in which a mixture of standard ATP, ADP and AMP was used. The results are summarized in Table 4. Average deviations of three values obtained on the same paper may be compared, as this was chosen the routine way of work in tissue analyses. Accurracy is similarly expressed by the

Table 3

R_f values of ultraviolet-absorbing compounds of extracts from rat skeletal muscle and liver in two solvent systems

A: Isobutyric acid–cc. NH_4OH–water (66:1:33), solvent system No 4. Paper: Schleicher–Schüll 2043/b ausgew.

B: Butyric acid 69% (v/v), NaOH 0.85% (w/v) in water, solvent system No 2. Paper: Whatman 3

Solvent system	Organ	UV-absorbing spots						
		I_1	I_2	ATP	ADP	NAD	AMP	I_7
A	Muscle	0.09	0.14	0.27	0.32	0.39	0.45	0.60
	Liver	0.09	0.13	0.28	0.32	0.39	0.44	—
		B_1	B_2	B_3	ATP	ADP	NAD AMP	
B	Muscle	0.18	0.23	0.42	0.46	0.52	0.60	
	Liver	0.24	0.31	0.44	0.48	0.53	0.61	

standard deviation of four separate determinations. In case of 0.1 μmole of each component (or more), the accurracy of the method is rather high, standard deviations between experiments with 3 parallels are within ± 3% of the average values. Even if only 0.02 μmoles are used of each component, these deviations do not exceed ±20%. In the latter case, however, the results and accurracy

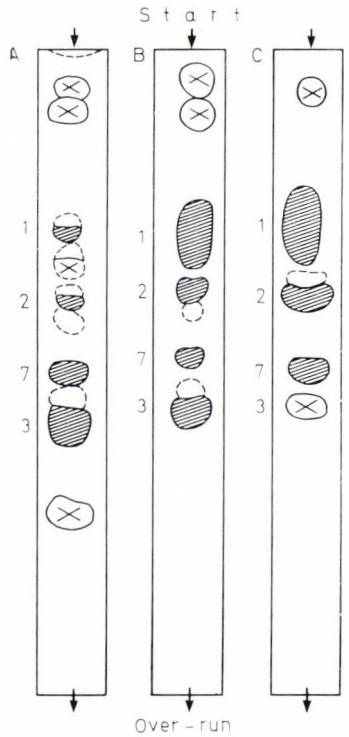

Fig. 3. Separation of ultraviolet-absorbing constituents from acidic rat tissue extracts. System: Isobutyric acid–cc. NH_4OH–water (66:1:33); FN-16 (Niederschlag, GDR) paper; descending run. A: kidney; B: liver; C: skeletal muscle. Numbering of compounds as in Figure 1. Spots indicated by dotted lines are fluorescent in ultraviolet light. X: only slight absorbance in the ultraviolet

are influenced by the degradation of nucleoside phosphates in the very diluted sample.

The method described above was used for the measurement of nucleoside phosphates in perchloric acid extracts of rat skeletal and heart muscle, liver and kidney. Our results are corrected for extraction losses and are given in micromoles per g fresh tissue. In addition to ATP, ADP, and AMP content, the amount of unidentified ultraviolet-absorbing spots as well as that of the main phosphorus fractions is given. The latter values, especially labile phosphorus content, can

Table 4

Statistical evaluation of the quantitative determination of ATP, ADP and AMP after paper chromatographic separation

One figure means the average of three determinations from parallel separations on the same paper sheet. Solvent system: isobutyric acid–cc. NH_4OH–water (66:1:33). Paper: FN-16 (Niederschlag, GRD). As to the details see Materials and methods.

Approximate amount of each component, μmoles	Compound	Recovery μgrams/spot ± A.D.				Average ± S.D.
		I.	II.	III.	IV.	
0.1	ATP	45.8 ± 0.7	43.5 ± 0.8	44.2 ± 3.1	42.8 ± 1.2	44.1 ± 1.2
	ADP	35.1 ± 0.1	36.4 ± 2.2	34.2 ± 1.2	35.5 ± 0.8	35.3 ± 0.9
	AMP	39.2 ± 0.4	40.0 ± 1.2	38.2 ± 0.9	39.8 ± 0.7	39.3 ± 0.8
0.02	ATP	5.1 ± 1.0	3.3 ± 0.2	4.0 ± 0.1	3.7 ± 2.0	4.1 ± 0.8
	ADP	8.4 ± 0.3	10.0 ± 0.5	9.5 ± 0.7	10.1 ± 1.0	9.7 ± 0.8
	AMP	9.0 ± 0.4	8.7 ± 0.0	8.0 ± 0.1	8.7 ± 0.2	8 7 ± 0.4

Table 5

Nucleoside phosphate and phosphorus analyses of rat skeletal and heart muscles

Values are given in μg/g fresh tissue, ± A.D.

Muscle	Skeletal	Skeletal	Skeletal	Skeletal, exhausted	Heart
Number of experiments	7	7	3	3	5
Number of animals in one group	1	3	3	3	4
I_{1-2}	—	0.31 ± 0.07	0.26 ± 0.01	0.22 ± 0.02	—
ATP	5.12 ± 0.37	5.51 ± 0.31	5.47 ± 0.06	5.02 ± 0.18	1.57 ± 0.11
ADP	1.04 ± 0.05	0.96 ± 0.06	0.89 ± 0.03	0.73 ± 0.03	1.31 ± 0.15
NAD	0.52 ± 0.06	0.31 ± 0.08	0.54 ± 0.03	0.51 ± 0.02	0.58 ± 0.14
AMP	0.15	0.15	0.15	0.16 ± 0.01	2.20 ± 0.26
I_7	0.18*	0.11* ± 0.025	0.13 ± 0.037	0.11 ± 0.01	—
Total aden. phosphate	6.26 ± 0.40	6.54 ± 0.36	6.46 ± 0.10	5.91 ± 0.19	5.07 ± 0.32
P_i	19.10* ± 2.70	21.20 ± 1.40	21.30 ± 0.29	21.00 ± 0.20	12.60 ± 0.26
P_l	11.00* ± 0.80	12.00 ± 0.98	14.00 ± 0.44	11.80 ± 0.77	4.74 ± 0.68
P_s	15.00* ± 1.31	17.40 ± 1.04	13.10 ± 0.44	12.90 ± 0.84	14.10 ± 0.78

* Number of experiments lower than indicated.

Table 6

Nucleoside phosphate and phosphorus-analyses of rat liver and kidney

Values are given in μg/g fresh tissue, $\pm$ A.D.

Organ	Liver	Liver	Kidney
Number of experiments	7	7	5
Number of animals in one group	1	3	4
I_{1-2}	0.93* $\pm$ 0.06	1.18 $\pm$ 0.04	0.58 $\pm$ 0.06
ATP	1.53 $\pm$ 0.08	1.67 $\pm$ 0.04	1.19 $\pm$ 0.06
ADP	0.80 $\pm$ 0.08	0.93 $\pm$ 0.09	0.49 $\pm$ 0.05
NAD	0.38 $\pm$ 0.05	0.45 $\pm$ 0.04	0.42 $\pm$ 0.04
AMP	1.72 $\pm$ 0.16	1.55 $\pm$ 0.09	1.89 $\pm$ 0.33
I_7	—	—	0.24 $\pm$ 0.05
Total aden. pho.	4.06 $\pm$ 0.47	4.14 $\pm$ 0.34	3.58 $\pm$ 0.19
P_i	7.49 $\pm$ 0.48	7.69 $\pm$ 0.64	10.90 $\pm$ 0.27
P_l	3.55 $\pm$ 0.40	4.05 $\pm$ 0.63	0.96 $\pm$ 0.43
P_s	15.20 $\pm$ 0.72	15.80 $\pm$ 0.68	16.40 $\pm$ 0.57

* Number of experiments lower than indicated.

be used to follow the changes in the amount of nucleoside phosphates containing easily hydrolyzable phosphorus. Our results about skeletal and heart muscle are shown in Table 5, and those about liver and kidney in Table 6. In skeletal muscle only traces of AMP were found; 82–85% of adenosine phosphates was ATP, in contrast to heart muscle, where AMP predominated. Upon exhaustion the ATP level decreased only 8.2% ($0.1>P>0.05$), that of ADP 18% ($0.05>P>0.02$). Though some increase could be noticed in AMP level, the total amount of adenosine phosphates decreased (8.5%; $0.1>P>0.05$). Alterations in labile phosphorus followed the above pattern. In the acid-soluble fraction of kidney tissue ATP, ADP and I_{1-2} (mainly other nucleoside di- and triphosphates) levels are considerably lower than in liver extracts, in agreement with phosphorus values.

Discussion

Four paper chromatographic systems were studied in detail with regard to their ability to separate mixtures of pure commercial nucleoside phosphates. For the rapid separation of mixtures containing ATP, ADP and AMP only, solvent system No 3 on ion-exchange paper turned out very suitable. However, at pH 3.0 this solvent produces more compact spots than earlier systems (Paolini, Serlupi-Crescenzi, 1964), which is very advantageous for quantitative determina-

tions. Of the systems studied, Nos 2 and 4 could be used to separate the components of rat muscle, liver and kidney extract. Solvent system No 2, however, has the disadvantage of not being able to differentiate AMP from NAD, thus solvent system No 4 has been considered as the best one for routine analyses.

On the basis of these studies a method has been developed for the direct quantitative determination of the main nucleoside phosphates present in acidic tissue extracts, after paper chromatographic separation. This method is much more simple and rapid than ion-exchange techniques, and in the authors' opinion it could be favourably used to follow the changes caused by biologically active substances, in intermediates of oxidative phosphorylation. The accuracy of the method, separation and spectrophotometric measurement included, was found to be within $\pm$ 3%, with 0.1 μmole amounts of the components. Under these conditions highly purified commercial ATP or ADP preparations showed homogeneous spots after chromatography, were not detectably decomposed, thus nucleoside phosphates phosphorylated to different degrees could be measured separately from the same mixture, without cross-contamination.

Data are presented of the nucleoside phosphate composition of rat skeletal and heart muscle, liver and kidneys. The unidentified compound I_{1-2}, the sum of two spots with low R_f values and not completely resolved, is probably a mixture of non-adenosine nucleoside di- and triphosphates. Its amount can give further useful information about the degree of phosphorylation.

Data of nucleoside phosphate composition were followed and checked by the measurement of P_i, P_l and P_s phosphorus fractions. Values of P_l closely followed the differences in nucleoside phosphates containing labile phosphorus-groups.

Some of the methods cited in the introduction were applied to rat tissue extracts. It is evident from Le Page's data (Le Page, 1946) that, at least in the case of skeletal muscle, narcosis is indispensable. Our data concerning skeletal muscle and liver only partly agree with the findings of Le Page, who used extraction with trichloroacetic acid and fractionation with barium ions, followed by colour reaction. ATP level in skeletal muscle is somewhat lower according to our measurements; Le Page also found a considerable amount of AMP, in contrast to our results. This might be due to the contamination of barium fraction containing AMP. For similar reasons, we found higher ATP and AMP levels, and lower ADP level in liver. Rapoport and Nelson (1945) already used more specific methods for precipitates obtained with mercury acetate. Their values for ATP are in good agreement with ours, but ADP and AMP values are strongly shifted in favour of ADP, possibly because of the unspecific nature of labile phosphorus measurements.

Voskoboinikov (1967) reported data on rat heart muscle, using electrophoretic separation and spectrophotometry. The data agreed well with those of Fox et al. (1965) obtained with dog heart muscle by means of anion exchange chromatography. We could not reproduce the relatively high ATP (4.2 μmoles/g) and low AMP (1.1 μmoles/g) values obtained by these authors, but in turn our data

closely agree with those of Parker (1954) who used specific enzymatic methods. Electrophoretic separation gives high ATP and low AMP values for liver, too, due to its lower ability to separate compounds similarly phosphorylated and to the appearance of hardly separable side fractions.

The effects of exhaustion on nucleoside phosphate composition of skeletal muscle could be well followed by the method described. We found considerable decreases in ATP and ADP levels and less increase in AMP level, whereas the fraction of mixed nucleoside di- and triphosphates was somewhat decreased. Visioli et al. (1964) found similar changes in rat heart muscle, without significant increase in nucleoside monophosphates.

In conclusion, we suggest that the method described above is an improvement on previous methods and is suitable for the rapid and selective separation and direct measurement of NAD in addition to ATP, ADP and AMP in acidic tissue extracts. The reliability of the method is supported by the fact that our results satisfactorily agree with those of other authors (Rapoport, Nelson, 1945; Parker, 1954; Glock, McLean, 1955, 1957; Threlfall, Stoner, 1957; Sacks et al., 1966) who used modern separation techniques and specific tests.

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Further Characterization and Cellular Localization of an ATP: Triacetic Acid Lactone Lyase in Pigeon Liver

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The occurrence of triacetic acid lactone (TAL) in nature is already a well-established fact (Harris et al., 1966; Bentley, Zwitkowits, 1967a, b). Enzymes capable of synthetizing TAL from acetate and malonate have been purified from several sources (Yalpani et al., 1969; Nixon et al., 1968; Dimroth et al., 1970). Bentley and Zwitkowits (1967a) have reported that in a Penicillium Stipitatum colony also ergosterol is produced from TAL, even though TAL is no intermediate in the biosynthesis of ergosterol. Thus, in this case, TAL has to be decomposed into acetyl-CoA to render the synthesis of ergosterol possible; however, as to the mode of this decomposition the above mentioned authors have given but speculations. As to the decomposition of TAL we have data only about its hydrolysis. Meister (1949) as well as Witter and Stotz (1948) have purified an enzyme from rat liver and other organs which hydrolyses the lactone into triacetic acid. It has recently been shown in our laboratory that a non-hydrolytic decomposition of TAL may take place (Szabó et al., 1970). We have demonstrated that a fraction of the pigeon liver, between 40 and 70 per cent saturation of ammonium sulfate, produces acetyl-CoA from TAL in the presence of ATP and CoA. In the present paper we describe a more precise and simpler method to measure the activity of this TAL-splitting enzyme. In addition, its intracellular localization in terms of subcellular fractions will be described: the enzyme can be found in the mitochondrial and cytosol fractions, and its specific activity is considerably higher in both fractions of the liver of fasting than of normally fed animals.

Materials and methods

Chemicals

CoA was obtained from Nutritional Biochemical Corporation (Cleveland, Ohio); 4-nitroaniline was purchased from Hoechst, Frankfurt, Germany; ATP, EDTA, Na-acetate, and the organic solvent from Reanal (Budapest) and mercaptoethanol form Merck (Darmstadt). Alumina Cγ-gel was prepared according to the procedure of Wilstätter and Kraut (1923); the $Al(OH)_3$ content was 11 mg/ml. TAL was prepared by Collie's method (1891).

Acetyl-CoA was prepared according to the acetic acid anhydride method (Colowick, Kaplan, 1957). The yield was 92% as measured by the hydroxamic acid method (Lipmann, Tuttle, 1945) and 43% on the basis of the enzymic test to be described below.

Enzyme assay

The activity of the TAL-splitting enzyme was measured according to the method of Tabor et al. (1953); TAL served as acetyl-donor and, in addition to the acetyltransferase enzyme, our own enzyme preparations were used. Specific activity was defined as follows:

$$\text{Spec. act.} = \frac{E_{405} \times 10^3}{\text{mg protein} \times \text{hour}}$$

Purification of arylamine-acetyl-transferase

The acetyl-transferase was prepared as described by Tabor et al. (1953) but the alumina Cγ-gel step was omitted, for the enzyme was sufficiently pure also without gel-adsorption. This preparation did not give any reaction in the course of the enzyme assay with acetate and TAL, and reacted only after the addition of acetyl-CoA. Thus it proved a suitable auxiliary enzyme (protein concentration = 12 mg/ml).

Cell fractionation

The pigeon was decapitated, its liver pressed through a liver-press. A 20% w/v suspension was made in 0.25 M sucrose and then the suspension was homogenized in a Potter-type apparatus (six times up and down). This was followed by centrifugation at 2500 *g* for 15 min. The residue was resuspended and rehomogenized in 0.25 M sucrose and centrifuged again. The supernatant was discarded and the residue suspended in 10 ml of sucrose (nuclear fraction). The first supernatant was then centrifuged at 18 000 *g* for 15 min, the residue washed just as in the case of the nuclear fraction, and then suspended in 5 ml of sucrose (mitochondrial fraction). The second supernatant was centrifuged at 100 000 *g* for one hour, washed and dissolved in 5 ml sucrose solution (microsomal fraction).

Protein determination

The protein content of solutions was measured with the biuret method (Ditterbrandt, 1948).

Experimental animals

Pigeons of the same breed were used; when fasted, no food was given for 3 days before killing.

Results and discussion

Cell fractionation was made for two reasons. First, we wanted to decide in which fraction was the TAL-splitting activity localized. Secondly, we hoped to find, in one of the fractions, a starting material, better than acetone powder, for further purification of the enzyme. The study of subcellular fractions resulted in three important observations:

1. Well-washed nuclear and microsomal fractions do not exhibit any measurable activity, except if obtained from fasted animals where also the microsomal fraction is active. The enzyme is localized in the mitochondrial and cytosol fractions, with a higher specific activity in the latter.

2. Both the mitochondrial and cytosol fractions of the liver of fasted animals exhibit a specific activity higher than that of fed animals, a feature mainly due to the decreased protein content of these fractions in fasted animals as compared with normal ones.

3. We have succeeded in obtaining fractions that display considerable TAL-splitting activity without any acetate-activating effect.

This last finding is especially important as to the mechanism of the reaction. In a previous paper (Szabó et al., 1970) we have demonstrated that the acetylating effect of TAL is not based on hydrolysis into acetate. Thus, we have now a

Table 1

Intracellular localization of the TAL-splitting activity

The TAL-splitting activity was measured as follows: 16 μmoles of TAL, 10 μmoles of ATP, 10 μmoles of $MgCl_2$, 100 μmoles of CoA, 10 μmoles of Na_2-EDTA, 10 μmoles of mercaptoethanol, 10 μmoles of KOH, 1.3 ml of 0.2 M phosphate buffer, pH 6.8, and 0.1 ml of the fraction to be investigated were put into a test tube. The mixture was pre-incubated at 37° for one hour. The final volume was 2.12 ml. The mixture was then centrifuged at 16 000 rpm for 20 min. After pouring the clear supernatant into a cuvette and adding 0.1 ml of acetyl-transferase and 0.1 ml of 0.2 M 4-nitroaniline and the absorbance at 405 nm was measured at 10 min intervals. The blank contained all of the above constituents, but only 0.1 μmoles of 4-nitroaniline and no TAL. Specific activity was calculated as described in Materials and methods, from the decrease of absorbance in the first hour. If no activity was detected we checked the functioning of the transferase system by adding acetyl-CoA

	Normal, fed animals		Fasted animals	
	TAL-splitting spec. act.	Protein concentration mg/ml	TAL-splitting spec. act.	Protein concentration mg/ml
Nuclei	0	26.6	—	—
Mitochondria	19.0	16.3	67.0	3.6
Microsomes	2.0	30.4	52.0	17.6
Cytosol	38.0	12.0	82.0	9.0

Table 2

Comparison of the acetylating ability of TAL and acetate

The specific activity was measured as desribed in Table 1 except that TAL or acetate was used at the same concentration

	Specific activity (normally fed animals)		Specific activity (fasted animals)	
	TAL	Acetate	TAL	Acetate
Nuclei	0	63.0	—	—
Mitochondria	19.0	18.0	67.0	86.0
Microsomes	0	0	52.0	0
Cytosol	38.0	0	82.0	12.0

new argument against the hydrolytic mechanism, for the mentioned fractions do not contain any acetate-activating system. Table 1 shows the TAL-splitting activity of cell fractions from normally fed and fasted animals, while Table 2 gives a comparison of the acetylating ability of acetate and TAL.

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New Chromatographic Method for the Separation of Single-Stranded DNA, Double-Stranded DNA and RNA

(Short Communication)

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Several chromatographic procedures are known for the separation of single-stranded denatured DNA from the native double-stranded species, using nitrocellulose (Armstrong et al., 1965), MAK* (Mandell et al., 1960) or hydroxylapatite (Miyazawa et al., 1965). Here we report a new chromatographic method on benzoylated DEAE-cellulose, which proved more satisfactory in our laboratory in respect of convenience and reproducibility. BD-cellulose has already been successfully applied for the fractionation of tRNA (Gillam, et al., 1968), but the elution of other nucleic acid species from BD-cellulose required extreme pH and high concentration of urea (Sedat, et al., 1969). The pretreatment of BD-cellulose described in this paper ensures good recovery of all bacterial DNA and RNA species. The separation is probably based on differences in secondary structure rather than in molecular weight or base composition.

BD-cellulose was prepared according to Gillam et al. (1967). The resin was thoroughly washed with 96% ethanol, then with 2 M NaCl containing 25% ethanol and finally with 0.3 M NaCl until the OD_{260} of the washing solution became negligibly small (less than 0.04). The resin was then suspended again and washed several times in 96% ethanol. After this washing followed the crucial step: suspending the resin in 96% ethanol that contained 1% Na-deoxycholate. This mixture was slowly stirred for 20 hours at room temperature. The resin was ready for use after washing with at least 100 volumes of 0.3 M NaCl.

Fig. 1 shows the chromatography of rRNA, native DNA and denatured DNA on the pretreated BD-cellulose column. ^{32}P-labelled DNA and ^{3}H-labelled rRNA had been prepared from Salmonella typhinurium by conventional methods as described earlier (Venetianer, 1969). DNA was denatured with alkali. The sample was applied to a 2×10 cm column in 0.3 M NaCl and eluted by a linear NaCl-acetone gradient (from 0.3 M NaCl – 0 % acetone to 1.6 M NaCl–14% acetone; volume of mixing chamber: 250 ml). The elution sequence was:

* Abbreviations: MAK = methyl-albumin-kieselguhr, DNA = deoxyribonucleic acid, RNA = ribonucleic acid, tRNA = transfer ribonucleic acid, rRNA ribosomal ribonucleic aicd, DEAE-cellulose = diethyl-aminoethyl-cellulose, BD-cellulose = benzoylated-diethyl-aminoethyl-cellulose.

rRNA, native DNA, denatured DNA. Recoveries: RNA 80%, native DNA 80–90%, denatured DNA 70–80%. tRNA and pulse-labelled RNA eluted together with rRNA under the conditions described here.

Reduction of the molecular weight of the DNA by means of sonication did not change the elution profile. Renatured DNA (obtained by incubation of the denatured DNA at 67°C in 0.3 M NaCl for 3 hours and subsequent slow

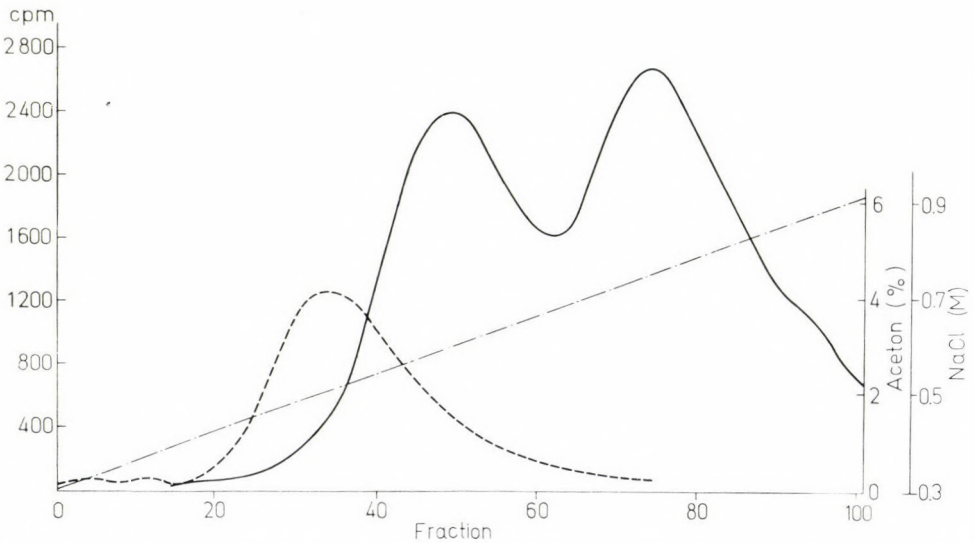

Fig. 1. Separation of rRNA, native DNA and denatured DNA on deoxycholate-treated BD-cellulose. The mixture of approx. 0.4 mg ^{3}H-rRNA, 2 mg native ^{32}P-DNA and 2 mg alkali-denatured ^{32}P-DNA was put on a 2×10 cm BD-cellulose column which was pretreated with deoxycholate as described in the text. Elution was carried out at room temperature with a linear NaCl (0.3 M–1.6 M) + acetone (0%–14%) gradient. Mixing chamber: 250 ml. Radioactivities of the effluent fractions were determined in a Beckman scintillation counter. Tritium counts were corrected for the "spillover" from ^{32}P. — — —: ^{3}H counts per minute. ——: ^{32}P counts per minute. ·—·—: concentration gradient

cooling) behaved on the column as the denatured species. However, if the DNA was sonicated prior to annealing its position on the elution diagram shifted towards that of the native DNA. After 5 minutes sonication with a 60 W MSE ultrasonic disintegrator the renatured DNA behaved on the column exactly as the native DNA.

The chromatographic procedure outlined here has been successfully employed for the separation of RNA–DNA hybrids from denatured DNA (to be published elsewhere), and offers many other possible applications by variation of the elution conditions.

The mechanism of separation still remains to be elucidated. We assume that the deoxycholate "coating" of the resin particles weakens the otherwise

too strong interaction between nucleic acids and BD-cellulose thus resulting in improved chromatographic properties.

We wish to thank Professor F. B. Straub for his support, and one of us (A.U.) for his invitation which made this research possible.

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Investigation on the Relationship between Dose and Energy Response of the Photon Radiation in Film Dosimetry

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The importance of assessing photon energy in personnel monitoring and its adaptation to photographic dosimetry is stressed. The factors correcting for the changes in film sensitivity were studied, as a function of photon energy, in the 12 to 128 keV range according to Dresel's system. The ratio of blackenings measured at the film under empty field and under lead filter, respectively, was used for the assessment of energies from 130 to 1225 keV. The dose dependence of the relationship was investigated. Suggestions were advanced for personnel monitoring.

Introduction

The actual value of the response recorded by the personnel dosemeter is represented by those informations that are decisive for the interpretation of the biological effects of ionizing radiations. For external radiations these are mainly: the dose level and distribution, radiation energy and the region of the body where the dosemeter is worn.

The site of the measurement is known and the dose is directly recorded by the personnel dosemeter. However, the determination of radiation energy and its distribution presents a more complex problem, among others, that of energy-dependent or independent measurement. Several and often different conceptions are prevailing in this respect as well as on the assessment of exposure as related to the critical organs, a fact that may lead to some uncertainty in the field (Attix, 1967, 1968).

The measurement principles of personnel dosimetry should be co-ordinated with the Recommendations of the I.C.R.P. (1959). According to these Recommendations, the maximum permissible dose levels represent the doses absorbed in the critical organs.

In addition to exposure, the determination of the absorbed dose requires the knowledge of radiation energy, too.

The response recorded by the personnel dosemeter generally expresses only the exposure. Accordingly, the absorbed dose should be determined from this value. Considering the problem of energy-dependent or independent measurement from this aspect, though the energy-dependence of the measuring method

cannot affect the exposure measured, the importance of the components of radiation energy should still be emphasized. Evidently, exposure to the staff in any X-ray working site is due to heterogeneous radiation. In the biological assessment of exposure, e.g. the soft energy component, playing a less important part in absorption, should be assessed, whenever possible, separately from the components above 30 keV. The difference in absorption is even more striking if e.g. one considers the dose absorbed in the bone marrow as a function of energy

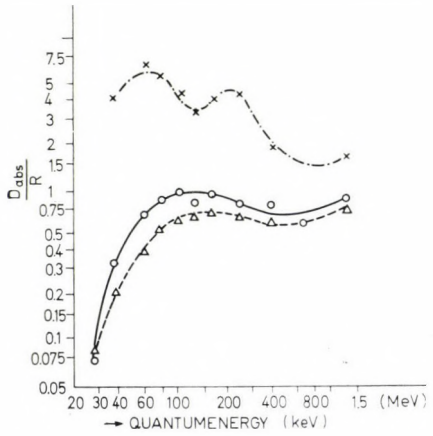

Fig. 1. Dose actually absorbed by the bone marrow (pro R recorded by the film dosemeter) during frontal, rotating and posterior irradiations with various quantum energies (Jones, 1964). –·–·– irradiation from the posterior direction, ——— isotrope, – – – – irradiation from the frontal direction

and for entry doses from various directions (Jones, 1964) (Fig. 1). All these factors show that whatever method is used to mesaure exposure, it should provide information also on the energy of the entry dose, necessary for the determination of the absorbed dose.

Next, it should be reviewed how this requirement is realized by the measuring methods used in personnel dosimetry. The criterium for the energy-independent dosemeter is that the energy absorption by the dosemeter should vary to the same extent as the absorption by the human tissues (Piesch, 1967, 1968). When applying an energy-dependent dosemeter (as e.g. film), to obtain the correct value of exposure, the energy dependence has to be corrected for. However, the energy dependence of the response may be made of use in the determination of the components of radiation energy (Langmead, 1965; Landauer, 1968). Knowing the energy components, the exposure measured may be converted into the absorbed dose by the *R*/rad conversion factors (Jones, 1966).

The experiments reported were designed to show those relationships that would permit to assess the components of the energy of the photon radiation

in the personnel monitoring photographic dosimetry. By making use of the conversion factor, the absorbed dose can be approximated from the exposure measured.

Experimental arrangement

The measurements were performed in the energy range of 0.012 to 1.2 MeV. Exposure data are summarized in Table 1. In the 12 to 23 keV interval the effective energy of radiation was determined by H.V.L. measurements. For the range of 34 to 170 keV the effective energy values were used according to other authors (Miyanaga, Yamamoto, 1963; Kraus, 1964). In the range of soft X-rays the homogeneous radiation of the required effective energy was produced by Siemens Dermopan, above 34 keV by THX-250 X-ray apparatus. Siemens dosemeter and Victoreen Minometer were used for dose measurements. To

Table 1

Radiation sources and effective energies used for the irradiation of the films

X-ray			
Exciting potential (kV_p)	Current (mA)	Filters (mm)	E_{eff} (keV)
29	25	1.0 Be + 0.568 Al	12
29	25	1.0 Be + 1.02 Al	15
29	25	1.0 Be + 3.58 Al	20
29	25	1.0 Be + 10.63 Al	23
43	25	1.0 Be + 5.01 Al	30
60	10	10.0 Al	34
70	10	1.0 Cu	46.5
75	10	1.0 Cu	49
80	10	1.0 Cu	53
100	10	2.0 Cu	65
120	10	3.0 Cu	80
140	10	4.0 Cu	98
160	10	5.0 Cu	113
180	10	7.0 Cu	128
180	10	22.23 Cu	170

γ-ray	E (keV)
^{51}Cr	320
^{137}Cs	661
^{60}Co	1225

identify the doses delivered in the X-ray and γ-ray energy ranges, respectively, the two dosemeters were calibrated to each other by homogeneous radiation of 170 keV effective energy. The maximum difference obtained for the Victoreen Minometer ran to -7 per cent.

ORWO films (emulsion Nos. 643 – 107, 642 – 105, 543 – 103 RD 3 and RD 4) were used. The exposed films were developed in AGFA 30 developer at 18 ± 0.5°C, for 6 minutes. Densitometry was performed by Baldwin's photometer.

The films to be exposed were put into two different film badges. The badge designed to measure exposure in the 12 to 23 keV interval was equipped with the following filters: 0.53 mm Al; 1.06 mm Al; 1.5 mm Al and was of open window (unfiltered film) during exposure. This film badge was developed especially for the measurement of low energy X-ray doses (Bojtor, Dósay, 1970). Above 30 keV, the blackening on the film was measured either under 142.8 mg/cm^2 polystyrol (empty field), or under 0.05 mm Cu; 0.5 mm Cu and 1.1 mm Cu filters, respectively.

Evaluation method and results

In the 12 to 128 keV energy range the films were evaluated according to Dresel's system (1960). In the range of soft X-rays Dresel's method was adopted for Al filters; the optimum thickness of the filter was determined by experiments. For the evaluation of the films, the factors correcting for the sensitivity of the emulsion (Haerte-Faktor) were calculated as a function of the apparent dose-ratios at every energy used. These factors are known to be energy-dependent. The apparent dose-ratios are directly read off when evaluating the films (these values are, in fact, the dose-ratios of the blackenings measured on the empty field and under the various filters, respectively, read off from the calibration curve. The calibration curve represents the peak of the energy diagram for the emulsion blackening. Accordingly, to obtain the actual dose, the apparent dose should be corrected for by the sensitivity correction factors). Radiation energy was determined as follows.

The apparent doses (D^*) under the various filters are energy-dependent, as it appears from the following equation:

$$S_F(E) = S_0(E)e^{-\mu(E)x},$$

where

$S_F(E)$ represents the blackening on the film under filter of thickness x, at an exposure of energy E,

$S_0(E)$ represents the blackening on the unfiltered film at energy E, and

$\mu(E)$ is the absorption coefficient of the filter at energy E.

Accordingly, the diagram for the relations between the apparent dose ratios and the effective energy, as shown in Table 1, has been plotted. The diagram for the dose range of 12 to 23 keV is shown in Fig. 2. The calculation was

made for the blackening of $S = 1$, for the straight part of the dose-response curve.

On this basis radiation energy may be given for three components. The relation expressed by curve 2 proved to be suitable for the determination of the respective component only up to 20 keV.

Fig. 3 shows the relation between the apparent dose and the effective energy for the interval from 30 to 128 keV at blackening $S = 1$.

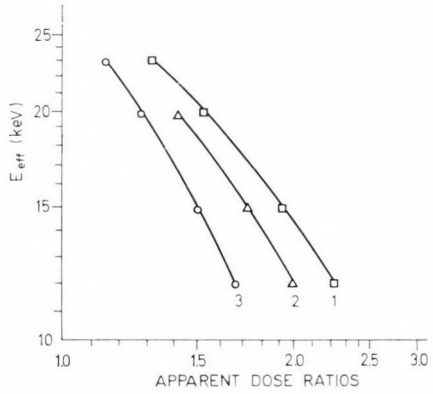

Fig. 2. Relations between the apparent dose ratios and energy in the 12 to 23 keV energy range, when using ORWO RD 4 film. Curve 1 taken up at the ratio: $\frac{D^*_{\text{unfiltered film}}}{D^*_{0,53\ \text{Al}}}$; Curve 2 taken up at the ratio: $\frac{D^*_{0,53\ \text{Al}}}{D^*_{1,0\ \text{Al}}}$; Curve 3 taken up at the ratio: $\frac{D^*_{1,0\ \text{Al}}}{D^*_{1,5\ \text{Al}}}$. Blackening: $S = 1$ in all the three cases

Those evaluation methods in film dosimetry that are based on the correction for the energy-dependence of the film cannot, as a rule, be used for energies higher than the conventional X-ray range. Their upper limit varies according to the filter combination used and the emulsion of the film. Experience has shown that, above 120 to 150 keV, the decreasing energy-dependence of the film may be compensated for by metal filters of high atomic number (Storm, Shlaer, 1965). So, the dose will be obtained independently of energy.

In contrast with the method reported, it is the relation between the dose response and photon energy that is required for the determination of energy above 120 to 150 keV. One of the possible methods is the following:

The response curve obtained for the empty field at various energies is normalized to the curve obtained for a lead filter (practically parallel to the X axis). The relation so obtained is demonstrated by Fig. 4. Provided the radiation is homogeneous, the energy value may simply be read off. At radiation of mixed photon energies (0.113 to 1.2 MeV), according to our experiments with various doses, the relation expresses the weighted mean value of the energy.

Both the apparent dose ratios and the ratio $\frac{S_{142,8\ \mathrm{mg/cm^2\ polystyrol}}}{S_{1,0\ \mathrm{Pb}}}$ show a certain dependence on the dose and on the blackening, respectively. Fig. 5 represents this dependence in the range of 12 to 128 keV at various blackenings. As related to the value calculated at $S = 1$, the maximum change of

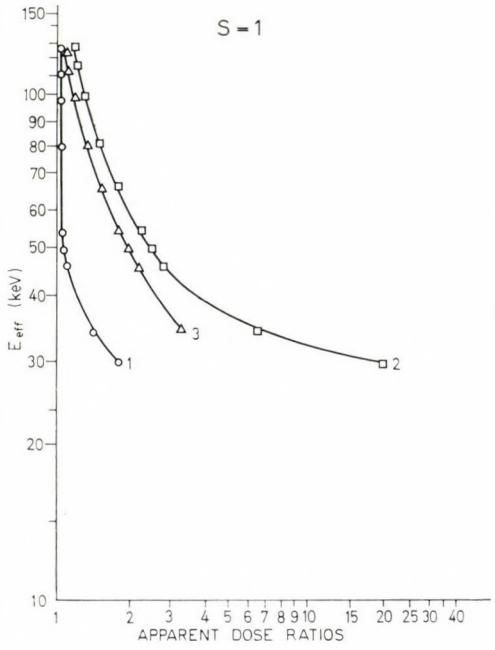

Fig. 3. Relations between the apparent dose ratios and energy in the 30 to 128 keV energy range, when using ORWO RD 4 film. Blackening, $S = 1$. Curve 1 $\frac{D^*_{142,8\ \mathrm{mg/cm^2\ polystyrol}}}{D^*_{0,05\ \mathrm{Cu}}}$, Curve 2 $\frac{D^*_{0,05\ \mathrm{Cu}}}{D^*_{0,5\ \mathrm{Cu}}}$; Curve 3 $\frac{D^*_{1,1\ \mathrm{Cu}}}{D^*_{0,5\ \mathrm{Cu}}}$

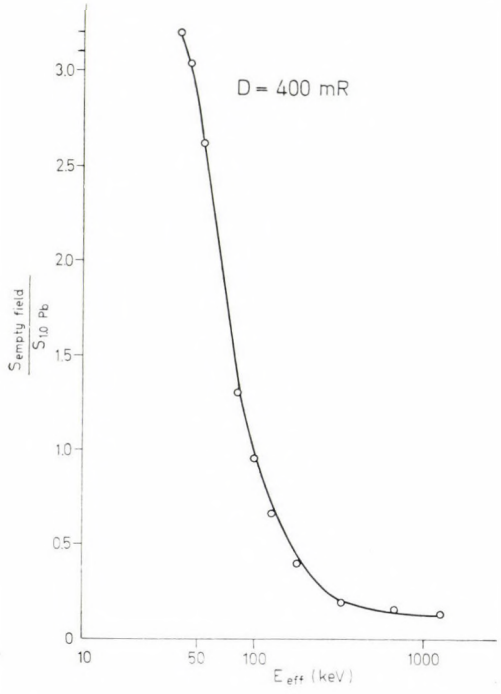

Fig. 4. Variation, with photon energy, of the $\frac{S_{142,8\ \mathrm{mg/cm^2\ polystyrol}}}{S_{1,0\ \mathrm{mm\ Pb}}}$ blackening ratio. ORWO RD 3 film exposed to 400 mR

$D^*_1 = \frac{D^*_{\mathrm{unfiltered\ film}}}{D^*_{0.53\ \mathrm{Al}}}$ was found at 12 keV: +2.65 per cent −10.06 per cent; it decreases at higher energies. In the range of medium energies, the maximum change of $D^*_1 = \frac{D^*_{142,8\ \mathrm{mg/cm^2\ polystyrol}}}{D^*_{0.05\ \mathrm{Cu}}}$ was found at 34 keV, above which the ratio is practically constant. On the other hand, as expected, the dependence decreases at $S > 1$ values of the straight part of the dose-response curve.

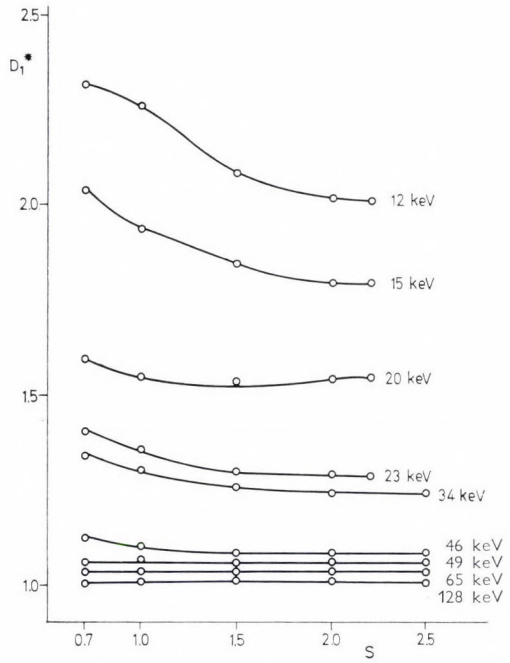

Fig. 5. Variation, with blackening, of the apparent dose ratios (D_1^*). In the 12 to 23 keV interval: $D_1^* = \frac{D^*_{\text{unfiltered film}}}{D^*_{0{,}53\ \text{Al}}}$. In the 34 to 128 keV interval: $D_1^* = \frac{D^*_{142.8\ \text{mg/cm}^2\ \text{polystyrol}}}{D^*_{0{,}05\ \text{Cu}}}$

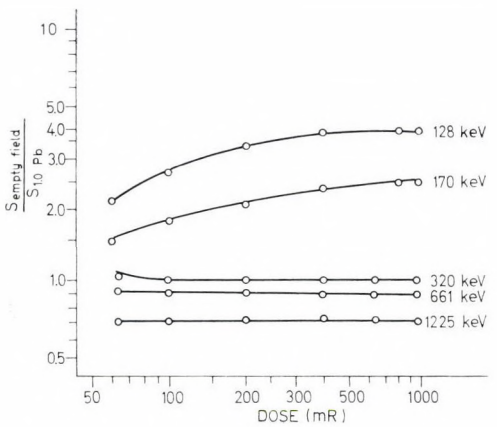

Fig. 6. The $\frac{S_{142.8\ \text{mg/cm}^2\ \text{polystyrol}}}{S_{1.0\ \text{Pb}}}$ ratio versus dose determined at various exposures. Energy range: 128 to 1225 keV

Fig. 6 demonstrates the changes of the $\frac{S_{142,8\ \mathrm{mg/cm^2\ polystyrol}}}{S_{1,0\ \mathrm{Pb}}}$ ratio at various doses. The tendency of dose dependence is similar to that shown in Fig. 4, the maximum dependence being found at 128 keV, in the range from 60 to 400 mR. As related to the mean value of the curve (120 mR), the limits of error are +35.5 per cent and −25.6 per cent, resp. Minimum dose dependence was found above 400 mR at all the energies.

Discussion

The energy-dependence of the film is either corrected for by the filters themselves – in this case the film response will be energy independent – or, the response is corrected for by given correction factors. In the second alternative calibration relates to the surface exposure; the determination of the absorbed dose requires the knowledge of radiation energy. According to our experiments with ORWO dosemeter films, the relations obtained by Dresel's method in the range of conventional X-rays, and with a filter combination different from the usual one, permit the assessment of the energy components of soft X-rays. Above 128 keV the relation $\frac{S_{142,8\ \mathrm{mg/cm^2\ polystyrol}}}{S_{1,0\ \mathrm{Pb}}}$ could be used for the same purpose.

The dose dependence of the above relations should, however, be emphasized. The results reported confirm the importance of energy-information – as stressed by several authors.

The interpretation of the relevant data in literature and that of the results of our own experiments suggest that

1. the values measured on account of professional exposure should be assessed from the aspect of the absorbed dose, as recommended by the I.C.R.P., and

2. the calibration conditions, as well as the number and site of the personnel dosemeters worn should be chosen in agreement with para 1.

The author thanks Miss Éva Bobok for her valuable assistance in the experimental work.

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Examination of Laser-Inactivation on T7 Phages

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The inactivating effect of $\lambda = 694.3$ nm laser light was investigated on a T7 phage—E. coli system. It was found that light energy of 30×6 joule exerts no effect on phages suspended in M9. Making use of the photosensitizing effect of an $0.4 \times \times 10^{-6}$ mole methylene blue solution, the curve of kinetics of the inactivation was determined. It was established that the curve is of multi-hit character and the effect of light on the host cells is an irreversible one. The finding is considered as an injury of the protein coat caused by the effect of the one-photon absorption of light.

Introduction

In the last few years laser light has begun to spread partly as damaging agent but also as therapeutic means both in biology and medicine (Smith, 1969; Mester, 1969). In connection with the biological-medical application of coherent light the problem arises whether any difference from the non-coherent light could be demonstrated as to the biological efficiency and mechanism of action and, what specific effects of laser light can be observed as a consequence of the interaction of light and living systems (Smith, 1969; Tomberg, 1966).

In our present investigations the effect of the light of a ruby-laser ($\lambda = 694.3$ nm) was studied on a simple biological system, the T7 phage—E. coli system well known by us. Our aim was to study the change of the plaque-forming activity characterizing the biological activity of the system and, in connection with this, to draw conclusions as to the molecular mechanism of the action.

Methods

Throughout the experiments the irradiation was performed with a ruby pulsed-laser equipment with a pulse duration of 1.2 ms; the whole output energy of a single pulse was usually 1 joule which corresponds to an average power of 830 W when also the pulse duration is taken into account. In one part of the experiments the output energy was focused, and pulses of a maximum power

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of 4.9 KW were applied. In the other part of the experiments the energy was decreased by means of a Zeiss-filter to 1/20 th of the original (0.05 joule, 41 W). This means that during one pulse 1.7×10^{17} photons fall to the sample to be irradiated.

Throughout the experiments the biological effect under investigation was the plaque-forming activity of T7 phages; an E. coli B/r strain was used as host cells; in some of the experiments the host-cells were treated with 4 mg/ml of caffeine in order to avoid host-cell reactivation (Sauerbier, 1964; Rontó et al., 1967). The irradiation of the phages was performed partly after suspending them in M9 buffer and partly in a methylene blue solution of 0.4×10^{-6} mole in order to sensitize to light effects (McLaren, Shugar, 1964; Yamamoto, 1958). In the case of such a concentration an average of 10^5 dye molecules fall to one phage particle.

Results and discussion

1. In the case of an unstained phage suspension suspended in M9 the plaque-forming activity was determined by irradiating it with a maximum incident light energy of 30×4.9 KW. On the basis of the result obtained in $12-12$ measurements we can establish that even such a high energy fails to bring about any essential change in the plaque-forming ability of the irradiated phage population; the difference between the treated and untreated samples is not significant, $P \gg 0.5$. In our opinion this experience corresponds to the fact that the nucleic acid and the protein coat of the phage do not absorb at all at the wave length of the light emitted by the laser, and so the linear absorption (Smith, 1969), as one of the possible mechanisms of action is quite out of question. The appearance of the two-photon absorption as well as the formation of mechanical waves (Hamrick, Cleary, 1968) is also considered impossible in consequence of the low laser energy available.

2. In further experiments a methylene blue solution was used as a sensitizer. The absorption of the solution can be seen in Fig. 1. We note here that the concentration of the measured solution was 10 times lower than the concentration used for sensitization. In the Figure the line drawn at $\lambda = 694$ nm corresponds to the wave length of the irradiating laser-light. It can be seen that this wave length falls within the absorption range of methylene blue.

Since the unpaired T phages lose their plaque-forming ability in the presence of methylene blue even at average room illumination (Yamamoto, 1958), but the dye has no effect on phages kept in perfect darkness, all samples were kept as light-proof as possible during the laser irradiation quite up to the plate-pouring. The irradiation was stopped after each pulse, and the remaining plaque-forming activity was determined (N_L/N_O). At the time of the irradiation we took a sample from a non-irradiated suspension which served as control (N_V/N_O).

Fig 2 shows the kinetic curve of the inactivation. The abscissa shows the light-energy fallen to the phage suspension and the ordinate the natural logarithm of a quotient, which indicates how many times is the 0.4×10^{-6} mole methylene blue more effective in the presence than in the absence of laser light. The denoted

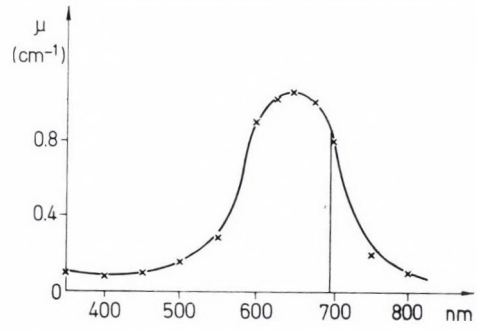

Fig. 1. Absorption spectrum of 0.4×10^{-7} mole methylene-blue solution in the range of visible light

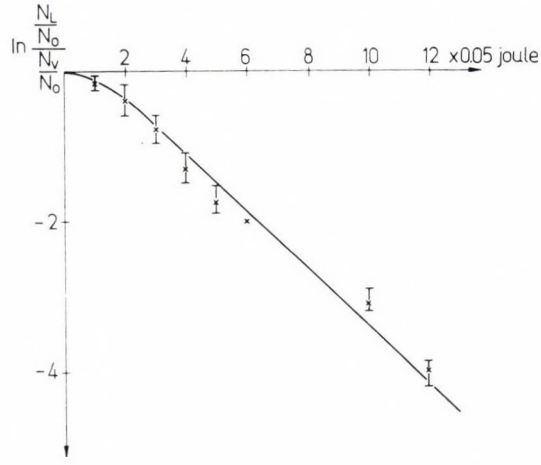

Fig. 2. Kinetic curve of phage inactivation. The horizontal axis shows the dosage, and the vertical one shows the natural logarithm of a quotient indicating how many times is the survival rate (N_L/N_O) lower under the effect of laser light than without it (N_V/N_O)

points of measurement represent the averages of 20 measurements each. The standard deviations of the data are also indicated in the Figure. Considering the character of the curve we can conclude to a multi-hit kinetics.

3. In connection with Fig. 2 we remark that, if the curve of kinetics was determined on host cells containing caffeine, the measured points fully agreed

with the points of the presented curve. This result indicates that the damage caused by laser in host cells is an irreversible one, i.e. the exclusive (selective) damage of DNA cannot be considered in any way as the cause of the laser-induced inactivation of the phage (Rupert, Harm, 1966).

On the basis of the above results we consider the following statements to be essential in connection with the mechanism of action of laser. *a)* The kinetics of phage inactivation is indicative of the multi-hit nature of the damage. *b)* There is no recovery from the damage of the host cells. Collation of these statements with Yamamoto's (1958) opinion, according to which it is the specific receptor site of the unpaired T phages which binds the sensitizing dye, we suggest the following scheme of reaction for the laser damage of T7 phages. The photon of the irradiating light is absorbed by the dye molecule attached to the protein coat of the phage, and the dissipation of the absorbed energy happens in the protein coat. The release of energy causes a damage on such a part of the protein, of which more (2, perhaps 3) can be found in one phage. If each place of the same function in one phage undergoes a damage, the phage-development fails, and this manifests itself in the inactivation of the phage. The mechanisms repairing the damage of the phage-DNA do not influence inactivation.

On the basis of our results we can draw also the conclusion that, by means of a combined treatment with methylene blue and laser, we can selectively damage the protein coat of the phage.

The quantitative research on some details of the mechanism of action of the inactivation is in progress.

Thanks are due to Mrs G. Jolán Tota for the technical assistance in connection with the irradiation as well as Mrs Várszegi for her help in carrying out the experiments.

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A Kinetic Model for Calculations of the Number of tRNA Molecules

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A simple kinetic model has been constructed which may be useful for figuring out the number of tRNA and aminoacyl-tRNA-synthetase molecules belonging to one kind of amino acid per ribosome, on the basis of measurements on an in vitro protein synthetizing system. The first part of the model may serve as a method to make a fast estimation of the number of tRNA molecules in a cell after partial denaturation caused e.g. by ionizing radiation or any other noxious physical agent.

There is no need to suppose a particular mechanism referring to the activity of template or aminoacyl-tRNA-synthetase which would limit the applicability of the model. Well-known data and conceptions of the ribosomal protein synthesis serve as basis of the calculations.

Introduction

There are a lot of data and theories available on the mechanism of bacterial protein synthesis. Some biochemical and biophysical methods known up to now set limit to the quantitative evaluation, or rather to the direct measurement, of certain data. Several attempts were recently made on the basis of other theoretical considerations to device such a model of bacterial biopolymer synthesis, which is compatible with our present biological knowledge also from a quantitative point of view (Pollard, 1961, 1963; Maniloff, 1969).

We tried to develop a simple kinetic model serving as basis to start experiments which would enable calculations to be performed in order to obtain important parameteres of the ribosomal protein synthesis.

As it is known, specific tRNA-s and aminoacyl-tRNA-synthetases (E.C. 3.5.1.14.) belong to each one of the twenty ordinary amino acids. In the course of our kinetic obsevations we obtained a relationship, from which we can determine the number of tRNA and of aminoacyl-tRNA-synthetase molecules specific to a given amino acid and belonging to one ribosome.

The kinetic model

We consider the following enzyme reaction:

$$E + AA + ATP \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [E - AA - AMP] + PP \qquad (1)$$

$$[\text{E - AA - AMP}] + \text{tRNA} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} [\text{E - AA - AMP - tRNA}] \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \text{E} + [\text{AA - tRNA}] + \text{AMP} \tag{2}$$

where E and AA denote the i-typed aminoacyl-tRNA-synthetase enzyme and the amino acid, resp. Expressions in brackets indicate transitory complexes.

Because of the conditions, a simplified scheme may be used instead of Eqs (1) and (2):

$$[\text{E - AA - AMP}] + \text{tRNA} \overset{K}{\rightarrow} \text{E} + [\text{AA - tRNA}] + \text{AMP} \tag{3}$$

This reaction may be considered quasi-irreversible because of its high energy requirement. It also follows from the conditions that, at high amino acid excess, the concentration of E–AA–AMP complex approximately equals the concentration of the enzyme. The acylating enzyme very likely operates on the linear section of its kinetic curve since, because of the dilution, it is not saturated with tRNA.

Let us take a given ribosomal protein synthetizing system which does not contain endogen mRNA. Let us examine the operation of the system if at $t = 0$ time we dilute it with a buffer only containing triplets coding in excess mRNA a certain *i*-typed amino acid and the suitable labelled amino acid. In a short time the system reaches a stationary equilibrium. If the dilution is high enough the time difference between the incorporation of an amino acid and the acylation of a $tRNA_i$ may be neglected in comparison with the average time an $AA–tRNA_i$ gets to the ribosome from the enzyme with the help of diffusion. The operating ability of the system after the dilution is rendered probable by the fact that the K_m for the tRNA referring to the aminoacyl-tRNA-synthetase is 10^{-7} M/l, and the in vivo enzyme/tRNA ratio, e.g. in the case of tyrosyl-RNA-synthetase is equal to nearly one (Calender, Berg, 1966).

Thus it may be supposed that a collision of one $tRNA_i$-synthetase molecule must be effective to all probability if the system has *i*-typed amino acids and a source of energy. If we suppose the difference between the diffusion constant of the AA-tRNA and the tRNA to be negligible, we can state that every $tRNA_i$ – which is specific to the *i*-th amino acid – stays with equal probability in charged and uncharged state.

Introducing some symbols which are necessary further on: The total number of *i*-typed tRNA for one ribosome: n_i; the number of *i*-typed AA-tRNA for one ribosome at a *t* time: ${}_*n_i'(t)$. Thus the number of *i*-typed uncharged tRNA for one ribosome will be $n_i'(t) = n_i - {}_*n_i'(t)$ at a *t* time. ${}_*c_i'(r)$ indicates the AA-tRNA concentration, $c_i'(r)$ the uncharged tRNA concentration, m_i the number of *i*-typed acylating enzyme for one ribosome and C_i indicates its concentration. Because of the statistical distribution, C_i is independent of the distance measured

from the centre of ribosome. $\Sigma_i n_i = N$ and $\Sigma_i m_i = M$ are the total number of tRNA-s and aminoacyl-tRNA-synthetases belonging to one ribosome, respectively, while Θ denotes the number of ribosomes in V_0 volume. Further on, if we consider only one ribosome and the $\frac{V_0}{\Theta} = V'$ volume, it is conceivable that both ${}_*c_i'(r)$ and $c_i'(r)$ depend only on the distance measured from the centre of the ribosome. We must know that the AA–tRNA keeps forming in the V' because of Eq. (3); but it decreases on the surface of the ribosome or rather turns into tRNA. Therefore, because of the steady state assumption, the originating and decreasing of AA–tRNA and tRNA are in equilibrium in V':

$$\frac{1}{2} n_i = n_i'(t) = {}_*n_i'(t) \tag{4}$$

This statement holds true independently of time and further dilution. Let ω mark the number of amino acids incorporated during a given period of time by one ribosome, and τ the number of tRNA charged during a given period of time in a volume of one ribosome (V') in the total incubation mixture.

Having met the above-mentioned conditions, after reaching the stationary state:

$$\omega = \tau \tag{5}$$

comes true independently of time.

The speed of formation of AA–tRNA on the basis of Eq. (3):

$$\frac{d\,{}_* c_i'(r)}{dt} = K C_i c_i'(r) \tag{6}$$

We denote the concentration of AA–tRNA in r distance from the centre of the ribosome:

$${}_*c_i'(r) = \frac{d_* \xi_i'(r)}{d V} \tag{7}$$

where the number of AA–tRNA, to be found in the dV infinitesimal volume, is $d_*\xi_i'(r)$.

On the basis of this, Eq. (6) can be written also in the following way:

$$\frac{d}{dt}\left[\frac{d}{dV}\left({}_*\xi_i'(r)\right)\right] = K C_i c_i'(r)$$

As the operators $\frac{d}{dt}$ and $\frac{d}{dV}$ are interchangeable:

$$\frac{d}{dV}\left[\frac{d_* \xi_i'(r)}{dt}\right] = K C_i c_i'(r) \tag{8}$$

Let us take $\varphi_i(r) = \dfrac{d_* \xi_i'(r)}{dt}$. Separating the variables:

$$d\varphi_i(r) = K C_i c_i'(r)\, dV \tag{9}$$

where $d\varphi_i(r)$ denotes the number of AA–tRNA produced during a given time in a dV infinitesimal volume which is r distance off the centre of the ribosome. The $c_i'(r)$ concentration may be considered constant in the dV volume.

Integrating both sides of Eq (9):

$$\int_0^{\tau} d\varphi_i(r) = K C_i \int_v^{V'} c_i'(r)\, dV \tag{10}$$

where v denotes the volume of the ribosome.

If the radius of the ribosome is ρ, and R denotes the radius of V' which is supposed to be spherical, then on the right side of the Eq. (10) we may write $dV = 4\pi r^2 dr$ variable instead of dV:

$$\int_0^{\tau} d\varphi_i(r) = 4\pi K C_i \int_{\rho}^{R} r^2 c_i'(r)\, dr$$

Integrating the left side:

$$\tau = 4\pi K C_i \int_{\rho}^{R} r^2 c_i'(r)\, dr$$

With the help of Eq. (5):

$$\omega = 4\pi K C_i \int_{\rho}^{R} r^2 c_i'(r)\, dr \tag{11}$$

In connection with $c_i'(r)$, we have a context analogouos with Eq. (7)

$$c_i'(r) = \frac{d\xi_i'(r)}{dV} \tag{12}$$

where $d\xi_i'(r)$ denotes the number of uncharged tRNA being present in dV.

Converting Eq. (12) we get:

$$d\xi_i'(r) = c_i'(r)\, dV \tag{13}$$

Writing $dV = 4\pi r^2 dr$ expression into Eq. (13) we get:

$$d\xi_i'(r) = 4\pi r^2 c_i'(r)\, dr$$

Integrating this Eq. in $\rho \leq r \leq R$ interval

$$n_i' = 4\pi \int_{\rho}^{R} r^2 c_i'(r)\, dr$$

Using Eq. (4):

$$n_i' = 8\pi \int_{\rho}^{R} r^2 c_i'(r)\, dr \tag{14}$$

From Eqs (14) and (11)

$$\omega = \frac{K C_i n_i}{2} \tag{15}$$

According to the definition of C_i:

$$C_i = \frac{m_i}{V' - v}$$

where $V' - v$ is approximately equal to V', because of the relatively small volume of the ribosome. Therefore:

$$C_i = \frac{m_i}{V'}$$

and:

$$V' = \frac{V_0}{\Theta}$$

so:

$$C_i = \frac{\Theta m_i}{V_0}$$

and writing this into Eq. (15)

$$\omega = \frac{K \Theta m_i n_i}{2 V_0} \tag{16}$$

Eq. (16) refers to one ribosome, thus doubting the applicability of our considerations. But if we take all the ribosomes into consideration, then Eq. (16) is reliable, as it is easy to manage statistically.

If we carry out the measurement for t time, the number of amino acids incorporated into proteins per volume V_0 can be determined:

$$\Omega = \omega \Theta t$$

that is:

$$\Omega = \frac{K\,\Theta^2\,t}{2}\,n_i\,m_i\,\frac{1}{V_0} \tag{17}$$

It can be seen from Eq. (17) that measuring Ω in different dilutions and plotting it versus $\frac{1}{V_0}$, the linear section will be in the region where the applied conditions are realized. From the slope of this linear section, the $n_i \cdot m_i$ product, can be calculated if t-time is the same in every measurement, and the Θ and K are known. The Θ and K can be determined in separate experiments.

If the linear part of the expression $\Omega = \Omega\left(\frac{1}{V_0}\right)$ is at α angle to the $\frac{1}{V_0}$ axis, then according to Eq. (17):

$$\operatorname{tg}\alpha = \frac{K\,\Theta^2\,t}{2}\,m_i\,n_i = (\Omega_2 - \Omega_1)\frac{V_{01}\cdot V_{02}}{V_{01} - V_{02}}$$

V_{01} and V_{02} are different volumes of two dilution degrees of the system. That is:

$$n_i\,m_i = (\Omega_2 - \Omega_1)\frac{V_{01}\cdot V_{02}}{V_{01} - V_2}\cdot\frac{2}{K\,\Theta_2\,t} \tag{18}$$

Regarding the fact that we want to make use of the results obtained for in vivo conclusions, we have to reckon with decreases in the number of active tRNA, aminoacyl-tRNA-synthetase of ribosomes in the course of preparations necessary for measurements. The γ, δ and ε denote correction factors:

$$\gamma = \frac{N^0 \text{ active tRNA after the preparation}}{n_i}$$

$$\delta = \frac{N^0 \text{ active acylating enzyme after the preparation}}{m_i}$$

$$\varepsilon = \frac{N^0 \text{ active ribosome after the preparation}}{\Theta}$$

then the modified form of Eq. (18) is

$$n_i\,m_i = (\Omega_2 - \Omega_1)\frac{V_{01}\cdot V_{02}}{V_{01} - V_{02}}\cdot\frac{2}{K\,\Theta^2\,t}\cdot\frac{1}{\gamma\,\delta\,\varepsilon^2} \tag{18a}$$

We may determine γ, δ and ε experimentally with the use of the before-mentioned components.

For further investigations we introduce the following expression:

$$n_i m_i = \alpha_i \tag{19}$$

With the help of isotope technics we may determine the quantity of:

$$\frac{n_i}{n_k} = \beta_{ik} \tag{20}$$

in a relatively simple way.

According to the way proposed in the Appendix, we obtain the following relationships from Eqs (18), (19), and (20):

$$NM = \Sigma_i \Sigma_j \alpha_j \beta_{ij}$$

Calculating N from separate experiments the M, n_k and m_k can be determined:

$$M = \frac{1}{N} \sum_i \sum_j \alpha_j \beta_{ij}$$

$$n_k = \frac{N}{\sum_i \beta_{ik}}$$

and

$$m_k = \frac{M}{\sum_j \frac{\alpha_i}{\alpha_k} \beta_{kj}}$$

Discussion

Having the studied kinetic model and accomplishing the measurements permitted by our present-day knowledge, we can determine M, m_k and n_k with the help of N, K and Θ after measuring α_i and β_{ij}.

Experiments based on this model may approximate *in vivo* conditions fairly well, if the ribosomes are separated from the endogenous mRNA after homogenization of the cells, and placed back to the system in a buffer containing in suitable excess one labelled amino acid and the mRNA coding this and only this sole amino acid.

Further intervention, such as making the system diffusion dependent, i.e. its dilution, unlike to any other procedure, causes a relatively slight damage to

the physiological conditions. The realisation in principle of this system is granted by the low K_m of tRNA referring to the aminoacyl-tRNA-synthetase. According to Calender and Berg (1966) the ratio of enzyme/tRNA is nearly 1. Therefore on the basis of Eq. (18) without knowing the total N^0 of tRNA referring to one ribosome (N), we can obtain the approximate values for both n_i and m_i – i. e. the N^0 of tRNA and aminoacyl-tRNAi-synthetase molecules belonging to one kind of amino acid and referring also to one ribosome – because in this case $n_i \cdot m_i \approx n_i^2 \approx m_i^2$. The γ, δ and ε correctional factors may be neglected if we carry out the experiments cautiously. Since ε is raised to the second power in the last formula it may cause a greater possibility of making a mistake; therefore it is advisable to determine it in separate experiments.

We find our system especially advantageous in the case of comparing data when the changes of the number of tRNA-s or acylating enzymes are examined as fundamental question after *in vivo* interventions, such as irradiation, amino acid fasting, genetic defects, etc.

Appendix

In connection with the β_{ik} quantity in Eq. (20) we have two obvious identities:

$$\beta_{ii} = 1 \tag{A1}$$

$$\beta_{ij} = \frac{1}{\beta_{ji}} \tag{A2}$$

Writing Eq. (19) for the j and k typed components, then dividing the equations with one another we obtain:

$$\frac{n_j m_j}{n_k m_k} = \frac{\alpha_j}{\alpha_k}$$

With the help of Eq. (20) and (A2) we obtain:

$$m_j = \frac{\alpha_j}{\alpha_k} \beta_{kj} m_k \tag{A3}$$

From the equation $\Sigma_i n_i = N$, using Eq. (20):

$$N = n_k \sum_i \beta_{ik} \tag{A4}$$

In a similar way:

$$M = m_k \sum_j \frac{\alpha_j}{\alpha_k} \beta_{kj} \tag{A5}$$

Multiplying (A4) with (A5) we get:

$$NM = n_k m_k \sum_i \sum_j \frac{\alpha_j}{\alpha_k} \beta_{kj} \beta_{ik}$$

This expressions is simplified with the substitution of Eq. (19):

$$NM = \sum_i \sum_j \alpha_j \beta_{kj} \beta_{ik}$$

Because $\beta_{kj}\beta_{ik} = \beta_{ij}$ so:

$$NM = \sum_i \sum_j \alpha_j \beta_{ij} \tag{A6}$$

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Biology and Mathematics*

II. Probability and Biology**

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Due to his knowledge of statistical mathematics and physics Mendel succeeded in formulating the Mendelian laws according to the data of probability theory. In contrast, the validity of probability is denied for the field of biology by some scientists arguing that life is very improbable. But single events must not be considered from the point of view of probability, on the one hand, and the problem of life can be treated from the point of view how it was stabilized, on the other. This leads to genetics; genetics can not do without information theory, and the latter not without the theory of probability. Furthermore many other mathematical data and experimental results (phyllotaxis, one-stranded helix of protein molecules) were used for the double helix of DNA.

1. It is common knowledge that several biophysical questions, e.g. muscular activity, are in the focus of international attention; as another example biocybernetics can be mentioned, the importance of which is also indicated by the fact that one of the so-called Main Directions of biological research chosen by the Hungarian Academy of Sciences for 1971–1985 is "Control Mechanisms of the Life-Process". In connection with this task we should – according to my preliminary study – realize that "*It is now a shortcoming of exact biology that the basic knowledge of some biologists of mathematics and of the exact sciences falls short of the required standards.*"

It is a commonplace today to say that without mathematics there is no natural science: physics, chemistry, physico-chemistry, colloidics; on the other hand it is only these on which *exact biology*, as a program of our century, can be built. This, of course, requires biologists who are in possession of proper exact knowledge. Moreover to follow this line of thought, even if we had enough biologists of this type, we could not succeed without the cooperation of *specialists* in the above-mentioned sciences in the effort to make biology an exact science. This is also beginning to be accepted, viz. not only in principle, so that specialists

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in the exact sciences interested in biology, and even working in the field of biology are fortunately increasing in numbers.

2. Shall I give an example of what a biologist's adequate grounding in natural sciences means? Fig. 1 is a half-schematic presentation of the results of one of Mendel's series of experiments. It shows that the ratio of 1/4 : 3/4 applies to the numerical distribution of the offsprings of green + yellow peas. Passing by the particulars of the experiments I only stress the fortunate recognition of the characteristic numeral results, since Mendel's ratios agree with the numerical data of probability.

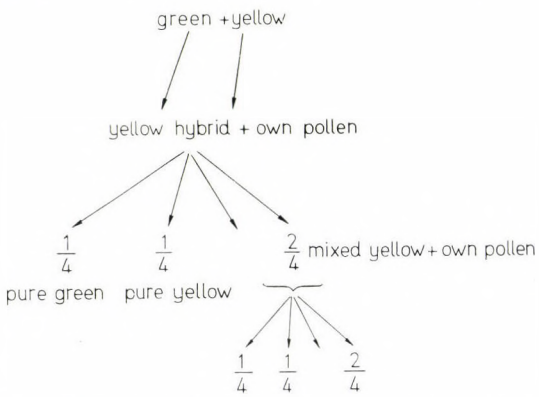

Fig. 1. Numerical distribution of pea-hybrids (Mendel)

Let us imagine that equally large numbers of white and black balls in a basket are thoroughly mixed, and two balls are taken out at random; the question about the probability of the colours of the balls is asked. They may be

white + white
white + black
black + white
black + black

1st question: What is the probability of both balls being white (or black)? — Answer: 1/4.

2nd question: What is the probability that a certain colour will be represented by at least one of the balls? — Answer: 3/4.

Similar results were obtained by Mendel in the pea-hybrids; in 150 thousand experiments in 1909 Darbishire found 75.1 per cent yellow and 24.9 per cent green ones in the offsprings of yellow hybrids; genetics became an exact science. Mendel's great efficiency is explained by the facts that he had carried out a great deal of statistical calculations in connection with his meteorological observations

and, furthermore, that for a long time he had taught physics in the s.e. higher middle school for science in Brünn.

3. In contrast, let us now see what Schrödinger (1944), the theoretical physicist and Nobel prize winner, thinks of the role played by probability in biology. According to him biological events are such (p. 79) . . . "whose regular and lawful unfolding is guided by a 'mechanism' entirely different from the 'probability mechanism' of physics". Right here I should like to add that it is in the same book that Schrödinger explains that (p. 86) in the cell 'the single cog is not of coarse human make, but is the finest masterpiece ever achieved along the lines of the Lord's quantum mechanics." Therefore it might appear that the literature did not attach great importance to this excellent theoretical physicist's opinion about biology. A similar inference seems to be contained in Bernal's (1949) work entitled "The Physical Basis of Life" claiming (p. 65): ". . . mystical interpretations . . . serve simply to conceal ignorance." After this one would hardly have expected that Schrödinger's opinion that biology and the exact sciences are principally opposed would be repeated by scientists.

However, this is not the case. As an example let me quote what was pronounced from the president's chair at a symposium on bioenergetics: (Arnold, 1960) "I overheard a comment, made by one of the physicists at this Symposium, that shocked me to my core. He said, 'I don't believe that the mechanism is very probable'. Any statement about 'probable' might be all right in Havana, where you could make money on it; but, this is a meeting on bioenergetics, and when it comes to biology, this is simply an extraneous consideration of no importance." A similar attitude is expressed, perhaps in an even more definite manner than this by Szent-Györgyi (1960) (p. 3): "Biology is the science of the improbable . . ." This shows how deeply the view that probability is an aspect foreign to biology has penetrated into biology.

I need hardly say that – on the contrary – the aspect of probability is at least as important and indispensable to biology as to any other exact science, if not even more important. It is enough to refer to the data of biometry, which enable the significance of experimental results to be established on the required level of probability. But even apart from biometry, the aspect of probability in biology has an importance which cannot be overlooked, as I expounded in an article (Ernst, 1962) two years after the statements by the latter two authors. In spite of that, at a symposium held five years later, Mendelssohn (1969), a physicist of Oxford, states (p. 416): "Physicists, dealing with a world of random processes connect complexity by statistical treatment to improbability. According to this, life is extremely improbable." That is to say in a meeting of physicists, chemists and biologists, among them 8 Nobel prize winners, the statement about the extreme improbability of life was repeated.

4. In contrast to this standpoint I mention Rényi's work (1954) entitled "Probability Calculation". "The Calculus of Probability only deals with stochastic mass events" and "no probability can be attributed to a single event; probability can only be spoken of in connection with categories of events."

The mathematician Rényi's wording at the same time exposes the basis of the erroneous stand taken by the above mentioned physicists and biologists who apply the calculus of probability, which is only valid for mass phenomena, to the single event of life. This erroneous attitude of theirs would expell from the field of biology the calculus of probability, which, however, is one of the most fundamental aspects of all exact sciences and, by doing so, would raise a barrier of principle between biology and the exact sciences.

This conceptual difficulty is easy to overcome also on the basis of a simple consideration; I personally recall a memory from my schooldays when in the spring of 1910 opinions were published "from the end of the world", that the earth would be in collision with Halley's comet on 18th May. This comet was discovered by Halley in 1682, and the comet, as well as his discoverer, may be regarded as single events, therefore there is no scientific sense in speaking about the probability of either. If, in spite of this, such a question is raised, then let us ask further: how much more improbable is it that in the XVIIth century 10^{28} atoms were joined together to form an astronomer Halley than the event that another group of atoms formed a comet of the same name. And what sense would there be in speaking about the probability of the event that in 1682 one group of atoms observed the other?!

5. A quotation from my mentioned article follows this line of thought: "Life has appeared in the history of the Earth. There is no sense in determining the probability of such an event or that of an event after its having taken place. Why, if people are at all gambling on lottery, one number must win; to investigate afterwards the probability of the winning of the number already drawn is nonsensical. But, if the same number were repeatedly drawn, i.e. if this event were 'stabilized', an intervention could reasonably be suspected. Hence the real problem lies not in the field of probability or improbability but in the situation in which a phenomenon the supervention of which though 'improbable' has once taken place, has been stabilized."

It is hardly necessary to emphasize that the question of stabilizing the phenomena of life leads to genetics; the basis of today's exact genetics is information theory, the mathematical backbone of which is the calculus of probability. Moreover, one of the sectors of genetics which deals with how nucleic acids code the sequence of amino acids in proteins also directly uses the calculation of variation from the theory of probability. Viz. if the molecular weight of a nucleic acid is 10^6, then it contains about 3000 bases, so that the possibilities of the order of arrangement of the four bases in the molecule are (r = repetition)

$$V_4^{3000r} = 4^{3000} \sim 10^{1800}.$$

This is such a great number that it would be more than enough for coding, even though every star of the universe were inhabited by living beings, similarly to the Earth. It is known that the much simpler way was chosen when the question

was raised how 4 bases can code the 20 amino acids. The answer was obvious:

$$V_4^{3r} = 4^3 = 64,$$

i.e. third class repeating variations of the four bases serve the coding.

6. This latter formulation is in close connection with the Watson—Crick model, the mathematical foundations of which go even much more deeper than the theory of probabilty. To demonstrate this we go far back into ancient times

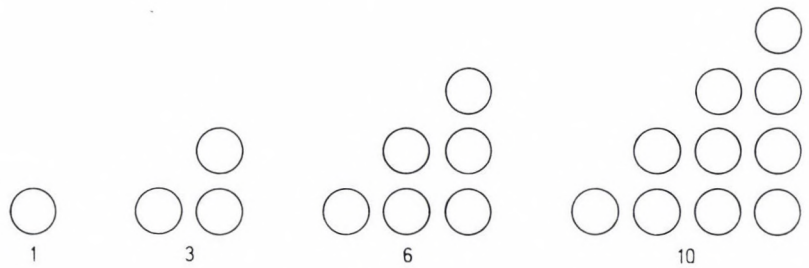

Fig. 2. The s.c. triangular numbers

when the brilliance of mathematics was, from time to time, outshone by mathematical mysticism. As a product of the latter (see Hogben, 1949) may be regarded the so-called triangular numbers (Fig. 2) as well as the series of the s.c. magic numbers in Table 1. In this any number of a series can be calculated by adding

Table 1

The s.c. magic numbers

	0	1	2	3	4	5	6	7
0	1	1	1	1	1	1	1	1
1	1	2	3	4	5	6	7	8
2	1	3	6	10	15	21	28	36
3	1	4	10	20	35	56	84	120
4	1	5	15	35	70	126	210	330
5	1	6	21	56	126	252	462	792
6	1	7	28	84	210	462	924	1716
7	1	8	36	120	330	792	1716	3432

together the number preceding it and that over it. At that time this was rather a play on numbers, but later human intelligence was able to profit from it. Who does not recognize e.g. this sequence: 1, 3, 6, 10, 15, . . .: second class combinations of the elements n = 2, 3, 4, 5, 6, . . . Furthermore, if e.g. the 5^{th} row and the 5^{th} column are viewed diagonally: 1, 5, 10, 10, 5, 1 are coefficients of the binomial thesis.

Around 1200 Leonardo Fibonacci also produced the s.c. Fibonacci series:

$$0,\ 1,\ 1,\ 2,\ 3,\ 5,\ 8,\ 13,\ 21,\ 34,\ 55,\ 89,\ \ldots,$$

in which

$$u_n = u_{n-1} + u_{n-2},$$

viz. any number is equal to the sum of the two preceding numbers. 700 years later Lucas, the French mathematician, created the Lucas series

$$\frac{1}{2},\ \frac{1}{3},\ \frac{2}{5},\ \frac{3}{8},\ \frac{5}{13},\ \frac{8}{21},\ \frac{13}{34}\ldots,$$

each number of which occurs in Fibonacci's series, furthermore, from the third term onwards the numerators and the denominators are the sums of the numerators and denominators of the two preceding terms, respectively:

$$\frac{u_n}{v_n} = \frac{u_n - 1 + u_n - 2}{v_n - 1 + v_n - 2}.$$

7. In this Lucas series the numerical regularity of the so-called leaf arrangement (phyllotaxis) was recognized (Fig. 3). Let the perpendicular in Fig. 3*a* be the stem axis of a plant, on which the nodes are; these are the points of insertion of the leaves. Concerning this, abundant experience has accumulated, and the numerical data on phyllotaxis are on the whole in agreement with this series. For better elucidation let us insert Fig. 4; on the circle in Fig 4*a* let 3 "leaves" be placed at equal distances, at 120° from each other. If the circle is pulled apart to form a helix, even then the three "leaves" will be at $\frac{2}{3}\pi = 120$ degrees from each other (Fig. 4*b*). In view of this, the calculation will be understandable that if the helix is drawn on a cylinder jacket assumed round the stem axis, then it is e.g. after two complete turns that a leaf can be seen in superposition namely 5 leaves between the two nearest superpositions. This arrangement of the leaves can be characterized by the fraction $\frac{2}{5}$ (numerator: the number of turns; denominator: the number of leaves) or, thinking of the circle pulled apart, by the angle between two neighbouring leaves: $\frac{2.360^\circ}{5} = 144^\circ$; (both values may be called a *divergence)*.

8. Let us take another step: 1951; in their work on the behaviour of the protein molecule Pauling and Corey elaborated the question of the helical structure of the protein molecule; by this is to be meant what was said above about phyllotaxis, however, the Lucas series could not be applied to it. On the other hand, another series could be written, which also returns to the Fibonacci series, namely

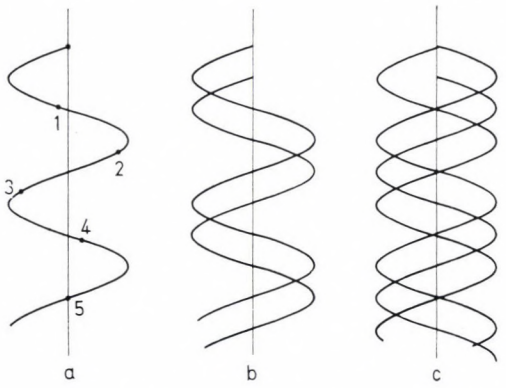

Fig. 3. *a:* single-, *b:* double-, *c:* three-stranded helix

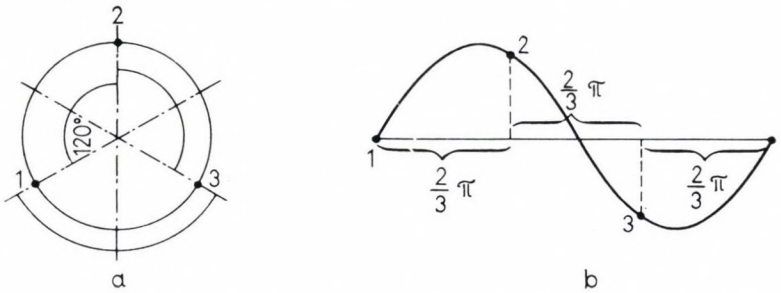

Fig. 4. Helix as a circle drawn apart; *a:* circle with 3 "leaves", *b:* the helix

$$\frac{1}{3}, \frac{1}{4}, \frac{2}{7}, \frac{3}{11}, \frac{5}{18}, \frac{8}{29}, \frac{13}{47}, \ldots,$$

which can be formed similarly to the foregoing from the third term onwards. This can also be called a Fibonacci series. For the first terms of the series no actual protein has been found, but the further ones already correspond to known polypeptides. Thus it is after 5, 8 or 13 complete turns that there is an amino acid in superposition; X-ray investigation has shown the distance between the two superpositions nearest to each other to be 27, 43, and 74 Å; X-rays also demonstrate that the distance between two neighbouring amino acids is 1.5 Å. Thus, dividing the distances between two neighbouring superpositions of 27 Å

by 1.5 gives the number 18, for the 43 Å distance 29, for the 74 Å distance 47, i.e. the number of amino acids between two superpositions. Also in these cases the numerators indicate the numbers of the complete turns in the helix, at which there is an amino acid in superposition, and the denominators give the total number of amino acids between two neighbouring superpositions (poly-alanin and poly-γ-methyl-glutamate; with the latter the 8/29).

After two years Pauling and Corey's view of protein was continued in the theory of the double helix. If the "spiral" in Fig. 3*a* is regarded as a one-stranded helix, then the second strand of the double-stranded helix (3*b*) can be drawn with a phase difference of 90°*. On the other hand it can be imagined that the two-stranded helix results from originally double-stranded circular turns drawing apart.

To this corresponds Watson–Crick's double "spiral" model, which – as has now been seen – is an ingenious and valuable result of thoughts and experiments thousands of years old. This model has proved very useful in scientific research, on the one hand, but leaves fundamental problems unanswered, on the other. Frey-Wyssling's (1955) attempt at explanation, according to which: "eine möglichst dicke Packung auf der Schraubenlinie angestrebt wird" (= . . . on the helical line packing as close as possible is striven for) can hardly be regarded as satisfactory. Yet, for the time being, let us accept it as a partial explanation, in particular, for the Å distances on the one-stranded protein helix, mentioning that the attraction and repulsion of the differently charged residues can be an important factor in the mechanism of development of the helix.** Again, the same explanation can hardly be applied to the macroscopic distances in the case of phyllotaxis.

9. Anyway, we have obtained a definite scientific picture, to which result the use of the calculus of probability contributed. In connection with this picture it was not the mathematically unfounded question of probability or improbability of the phenomenon of life, or of the DNA molecule either, that played a role but the much more promising problem of how DNA was implicated in stabilization and how life on the Earth was stabilized.

We have mentioned before the astronomer Halley and the comet observed by him; and now that biology has been related to astronomy, let us continue the flight of thought about this comparison. Not speaking about ancient times, natural science has hardly ever been faced with a question like the probability of formation of the solar system, for the solar system was taken for granted, and some of its data were computed even for milleniums in advance, as e.g. the so-called sun-table on the basis of the Ptolomaic system. It was regarded as quite natural that this system was stable, furthermore, this view could only be based on the conviction that the mechanism of this stability was completely automatic.

* Fig. 3*c* shows the three-stranded helix, collagen, schematically.

** This view may be brought into interesting relationship with the mechanism of transformation of fibrous and globular forms into each other (Staudinger, 1947).

Our present talk proceeds along similar lines, attributing the stabilization of the phenomenon of life on Earth also to an automatism. And as every kind of view outside the natural sciences has fallen concerning the solar system into disrepute, similarly any teleological explanation about the phenomena of life must be eliminated once for all. On the other hand, *the thesis of the automatism of life on the Earth* attains fundamental importance.

A firm basis for this view is furnished by cybernetics, the biological sector of which – biocybernetics – can only arrive at the exact statements of the biological automatism by means of this new branch of science. Similarly, every basic biological thesis itself can only attain the degree of exactness through its close relation with the exact sciences. On the other hand, without adequately developed natural sciences even the most beautiful biological theory is built on sand, no matter to what great detail it is elaborated.

Let us conclude our discourse like this: throughout the milleniums of human culture time and again the misconception has prevailed that statements by somebody who has once said something correct in a problem or created something valuable, are in every respect and always right. At all times it is the contents of the statements that should be considered, not the author's person.

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On the Effect of Tension on Myosin

(Short Communication)

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(Received October 7, 1970)

In a recent paper (Garamvölgyi et al., 1971) we suggested the two kinds of myofilaments to represent regular structural constituents of vertebrate smooth muscle, too. In the electron microscope we observed the presence of thick filaments consisting presumably of myosin even in muscles relaxed by atropine, provided that the muscle was fixed at a constant length higher than its excised length. We interpreted our observations so that the well known lability of smooth muscle myosin prevents the thick filaments from surviving the electron microscopic handling except if they become more resistive by mechanical tension, presumably by a *crystallization* of myosin (Ernst, 1963). Since the muscle used by us (longitudinal layer of guinea pig ileum) is continuously subject to a certain degree of tone in its physiological state, we considered the existence of thick filaments in vertebrate smooth muscles to be a physiological event. Lowy et al. (1970) using the method of X-ray diffraction obtained essentially similar results.

On the other hand, in previous works on the flight muscle of the bee (i.e., on a certain kind of striated muscle) we glycerinized samples of this muscle in a highly stretched state for several weeks (Garamvölgyi, 1969). The thick myosin-containing filaments of the A-band were intact, as shown in the electron micrographs taken in the course of this work (Figs 1–5 of Garamvölgyi, 1969). Moreover, if muscles like these were released and homogenized, phase contrast micrographs showed the myofibrils to be able to return to a sarcomere length corresponding to their resting length (Fig. 7*a–e* of Garamvölgyi, 1969).

If muscles were glycerinized, released (by cutting) and immediately fixed in osmium tetroxide, most fibrils exhibited a total lack of myosin filaments (Figs 1 and 2). They resemble myosin-extracted fibrils of this muscle (e.g. Garamvölgyi, Kerner, 1966) though, of course, no deliberate myosin-extraction was used in the present case. This is obviously due to either fixation or embedding, thus representing an *artifact*, a consequence of electron microscopic preparation. The I-segments consisting of the Z-lines and of bilateral sets of actin filaments (Huxley, 1963) survived and even traces of the M-material originating in the destroyed M-lines are clearly visible. This indicates a virtually selective removal (or destruction) of myosin.

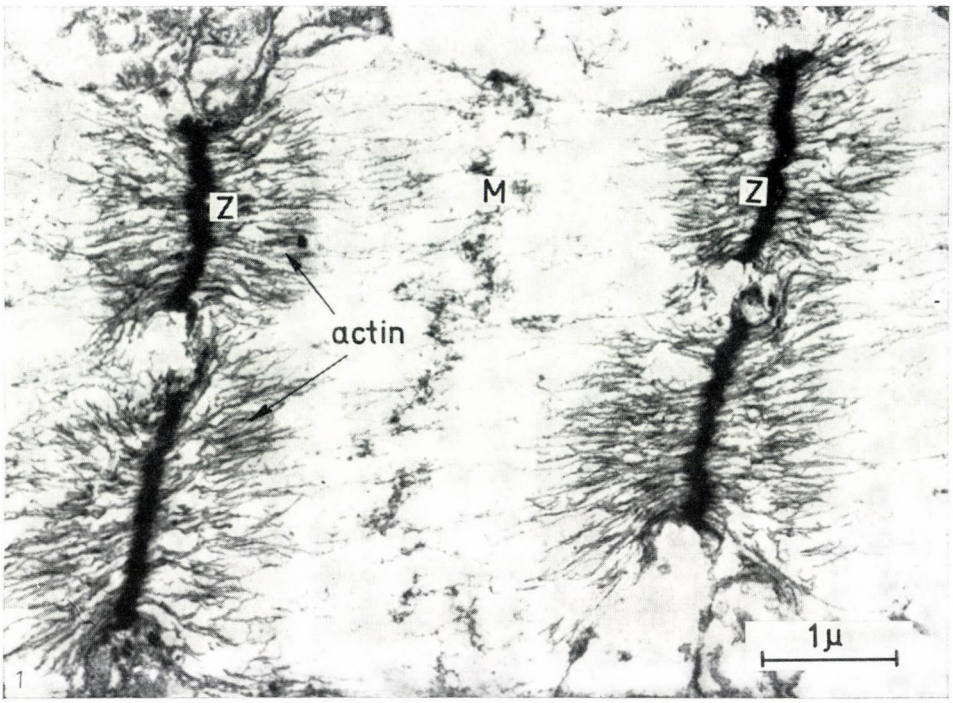

Fig. 1. Myofibrils from the indirect flight muscle of the bee glycerinized for two months, released and immediately fixed in osmium tetroxide. For further details of preparation see Garamvölgyi (1969). The absence of myosin in this case is obviously the consequence of the lack of mechanical tension and represents, thus, an artifact

The origin of this artifact can be explained as follows: during long-lasting glycerinization the substance of the myosin filaments "recrystallized" under the influence of a long-lasting extreme stretch. In this way the state of myosin changed and when released, it became less resistive to uncontrollable effects occurring in the course of specimen preparation. This resulted in a virtually total disappearance of myosin filaments. When fixed under mechanical tension (Garamvölgyi, 1969) this was not the case. It is very probable that glycerol-extraction also introduced changes in the association of myosin molecules (Guba et al., 1968).

It is true, however, that by using another kind of fixation the myosin filaments might have been probably preserved. Our observation serves only to demonstrate that *it may happen* even in a striated muscle that myosin filaments are totally missing as a consequence of preparation. The same artifact may occur in vertebrate smooth muscles, too (see also Kelly, Rice, 1969). This confirms our assumption that the lack of thick filaments may be an artifact also in smooth muscle. If our assumption is correct, the contractile mechanism of smooth muscle

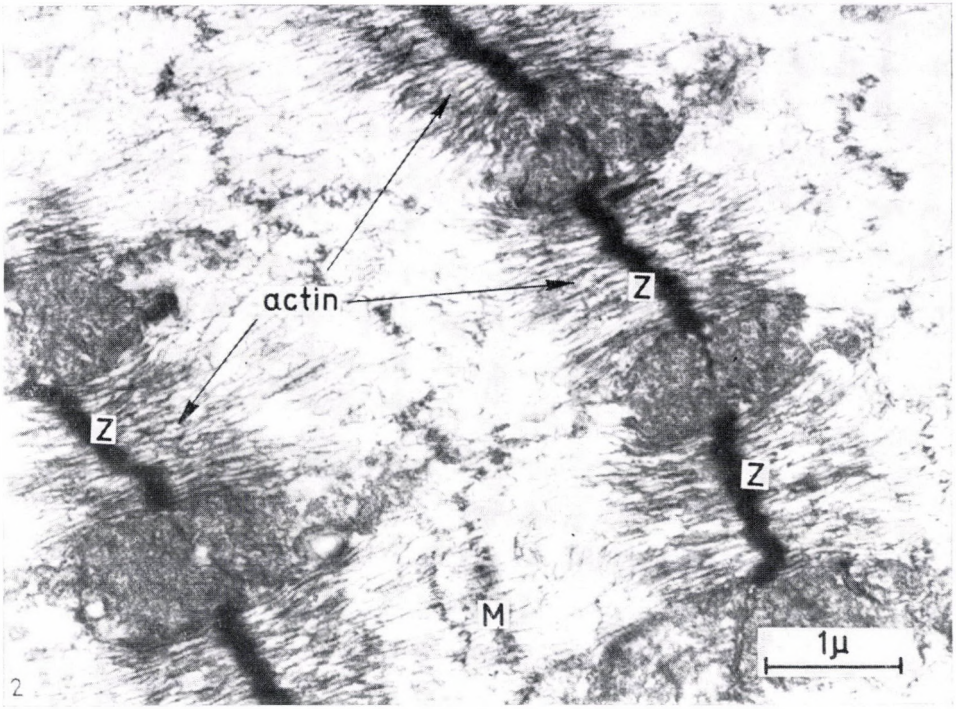

Fig. 2. The same as Fig. 1

does not differ considerably from that of striated muscle (Garamvölgyi et al., 1971). On the other hand, we emphasize the importance of the effect of mechanical tension on the state of myosin (Ernst, 1963).

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Book Reviews

Radiation Protection and Sensitization. Proceedings of the Second International Symposium on Radiosensitizing and Radioprotective Drugs, Rome, 6—8 May 1969. Edited by H. L. Moroson and M. Quintiliani. Taylor and Francis Ltd., London, 1970, 524 pages.

At the Second International Symposium on Radiosensitizing and Radioprotective Drugs organized by the European Biochemical and Pharmacological Society and the Italian Nuclear Energy Commission 25 countries were represented. The Proceedings of the Symposium contains the full text of five reviews and 68 papers complete with tables, diagrams and photographs. The reviewer has no easy task since the papers cover a variety of subjects, but due to excellent editing it is possible to give some ideas about the material of this rich and versatile congress. The consistent way in which the papers are edited, the brief summaries and the Subject and Authors indices help the reader gain the sought for information. The volume has the further advantage of presenting a list of references at the end of each paper by means of which more thorough insight can be gained into the discussed subject.

The first chapter contains the five reviews the first of which is an excellent survey by G. E. Adams on the present status of research concerning the molecular mechanism of action of radiosensitizing and radioprotective drugs. The author stresses in an up-to-date manner the importance of the fixation and repair processes. The second report discusses the radiodamage of the DNA system. P. Alexander and his team have subjected to thorough investigation the radiosensitivity of DNA, the possibility of a break in the double and single strand of DNA with special reference to the role of oxygen. They have investigated the reversible (repair) and irreversible (lethal) process of DNA damage due to radiation and the conditions to prevent damage. The next review by H. S. Kaplan is on the radiosensitizing mechanism of action of halogenated pyrimidine analogues including clinical trials with these drugs. A. Pihl and T. Sanner presented a paper on the radioprotective effect of substances with sulphur content, in which they summed up the possibilities of improving radioresistance and repair on a molecular basis in the case of cells which had suffered ionizing radiation. The mechanism of action of radioprotective drugs containing sulphur was the subject of L. F. Semenov's paper too, primarily from the aspect of hypoxia.

In the second chapter we find ten papers on radiosensitization and radioprotection on a molecular level. In this chapter the papers dealing with the problems of electron and energy transfer are particularly interesting (Greenstock et al., Fielden, Lillicrap, Lohmann).

The main subject of the 27 papers in the third chapter is the radioprotection and radiosensitization of single cells. The experimental objects include E. coli, Saccharomyces cerevisiae, various mammary and tumour cells. The investigations were carried out partly *in vivo* and partly *in vitro* on tissue cultures.

The 17 papers in the fourth chapter deal with the radiosensitization and radioprotection of tissues and organs, thus of multicellular systems. Interest is focussed here on the effect of AET and other radio-

protective drugs on various tissues (embryonal, medullar) of various animal origin (mouse, dog, sheep, etc.).

The fifth chapter contains 10 papers on the study of the biochemical and pharmacological effect of radiosensitizing and radioprotective drugs. Several interesting papers were delivered on the biochemical mechanism of action of aminothiols (Romantzev et al.), on changes in the redox potential of rat and mouse blood under the effect of cystamine (Duychaerts, Liebecq) and on the cardiovascular, biochemical and haematological effect of AET (Dienstbier et al.).

In the sixth chapter four papers report on the clinical applications of radiosensitizing and radioprotective drugs and put into a new light the role and application of these materials.

These few randomly chosen examples are intended to illustrate the excellent survey of the latest experimental results in the study of radioprotective and radiosensitizing substances presented in these Proceedings. The typography and whole presentation of the volume are admirable.

I. ÁRKY

Strukturelle Grundlagen der biologischen Funktion der Proteine (Structural basis of the biological function of proteins). T. DÉVÉNYI, P. ELŐDI, T. KELETI, G. SZABOLCSI (edited by P. Elődi) Akadémiai Kiadó, Budapest, 1969.

In this extensive monograph the authors — whose name is familiar to fellow specialists all over the world — have set themselves the task of presenting a survey of the results achieved in the search for correlations between the structure and function of proteins. Selection of the material and the entire conception of the book reflect the "functional" attitude of the authors. They have presented a fundamental work which may for a long time to come serve as a reference book for those seaking information on proteins in general and on the functional and structural unity of biological phenomena.

The book has five chapters. The first chapter by T. Dévényi deals with the primary structure of proteins. He discusses in detail the most up-to-date methods and goes on to the description of the more important results achieved in the field of primary structure research (insulin, ribonuclease, cytochrome C, lysozyme, haemoglobins, etc.). A separate chapter deals with the influence of the primary structure on the steric configuration of proteins.

The second chapter written by P. Elődi has the steric configuration of globular proteins as its object. It deals in detail with those proteins whose steric configuration and the orientation of their polypeptide chains are already well-known (haemoglobin, myoglobin, ribonuclease, lysozyme, etc.). Methods applicable to the investigation of conformation are described.

The third chapter by T. Keleti deals with enzyme activity, including the active centers of enzymes and the theoretical and experimental aspects of reaction kinetics. This chapter is centered around our present knowledge about NAD dehydrogenases.

The fourth chapter (P. Elődi) sums up our present knowledge on embryonal and abnormal proteins, including the evolution of haemoglobin, myoglobin, cytochrome C and certain other enzymes.

In the last, fifth chapter G. Szabolcsi discusses the correlation between protein structure and enzyme activity with special references to the effect of the structural modifications of proteins on enzyme activity.

Protein research is a relatively young science, but due to the rapid development of test methods progress has been tremendous. For this reason it is extremely difficult to write an up-to-date book in this field. This book has a permanent value not only because it sums up the most important results up to the time of its publication, but will serve as a rich source of information for many years to come. Protein chemistry has by now stepped out from the framework of biochemistry in the narrower sense of the word, so that the book will be of interest to biologists and physicians just as much as to experts in pharmacology or agriculture. The book is an important and representative witness to the internationally recognized standard of protein research in Hungary. The pleasing print and typography are the result of the usual high standard work of the Publishing House of the Hungarian Academy of Sciences.

J. GERGELY

Biochemical and Clinical Aspects of Alcohol Metabolism. Edited by V. M. SARDESAL. Charles C. Thomas Publisher, Springfield, Illinois 1969.

A symposium on the basic and clinical research of alcohol metabolism was held in Detroit in 1968. The papers presented at the Symposium by established and well-known investigators as well as by young scientists were collected in a volume of more than 300 pages, edited by V. M. Sardesai and published by Ch. C. Thomas Publisher in 1969.

The first part of the book deals with analytical methods for the determination of alcohol in blood and other biological fluids.

The second part includes papers on alcohol dehydrogenase with special emphasis on the zinc content of the enzyme and the complexes formed with the coenzyme.

The third part contains experimental data on the metabolic effects of alcohol, followed by the fourth part on the effect of alcohol on tissues and organs.

The last part deals with the aspects of alcohol metabolism, including differences between alcoholics and nonalcoholics.

The book contains discussions after each paper and a very good and useful subject index and author index. The book is well prepared and carefully compiled.

This collection of papers should be warmly welcomed by all those who are actively engaged in basic or especially in applied research in this area.

T. KELETI

Neurobiology of Invertebrates. Proceedings of a Symposium held at the Biological Research Institute of the Hungarian Academy of Sciences, Tihany, September 4–7, 1967

Edited by J. Salánki. Akadémiai Kiadó, Budapest, 1968. pp. 501.

Invertebrate neurobiology is the main field of interest of the group of research workers in the well-known Tihany Institute at the picturesque Lake Balaton. The team under the direction of János Salánki has taken the initiative to organize an international Symposium on this complex problem. According to the account of the participants – among them the rapporteur of the present review – the conference has been highly successful. The volume reflects accurately the proceedings of the four days meeting dealing with the actual research on the nervous system of annelids, molluscs, crustaceans, and insects.

The first part of the volume includes nine papers on the submicroscopical as well as on the histochemical background of neural regulation. The eight papers of the second part deal with physiological events on cellular level. Most of these contributions are devoted to the problems of chemical specificity and physical properties of neurones. The third part of the book comprises six papers on enzymes, active and transport substances of the nervous system, and the heart of invertebrates. Finally the fourth part contains ten interesting contributions on the problems of integration. The studies on the mechanisms of learning and behaviour must be accentuated in special.

The volume is introduced by the opening address of János Salánki, and by some important remarks of János Szentágothai on the technical difficulties in studying the morphology of neuronal networks. The volume includes all the discussions, a rich bibliography, the list of participants, etc.

The thirty-four papers of this excellent book represent an important contribution to contemporary elementary neurobiology, considering that most major problems of neuronal biology have been solved on invertebrate preparations, thus the importance of the topic grows year by year. The volume will be of great value to neurophysiologists, neurochemists, psychologists, neuromorphologists, and other specialists interested in the principal problems of neuronal functions.

G. ÁDÁM

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The Influence of Effectors Modifying Phosphorylase-*a* in the Phosphorylase Phosphatase Reaction

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(Received July 23, 1970)

The role of conformational changes of phosphorylase-*a* in its inactivation reaction catalyzed by phosphorylase phosphatase has been investigated.

The phosphatase reaction was inhibited by AMP, the extent of inhibition increased with the concentration of AMP, it diminished with increasing phosphorylase-*a* concentration and was unaffected by the amount of phosphatase.

The phosphatase did not transform the phosphorylase-*a* complex containing AMP and prepared by compensation dialysis, whereas it transformed the native enzyme.

The inhibition of the phosphatase reaction by glucose-1-P was decreased by increasing concentrations of phosphorylase-*a*, just as in the case of the inhibition caused by AMP.

The extent of inhibition of the phosphatase reaction increased in a sigmoid manner with increasing AMP and glucose-1-P concentration.

The inactivation of phosphorylase-*a* was stimulated by glucose-6-P, and this effect decreased with increasing amounts of phosphorylase-*a*.

Glucose-6-P could moderate or cancel the inhibition of phosphatase reaction caused by AMP. The rate of phosphatase reaction depended on the ratio of glucose-6-P and AMP.

Several intermediates of glycogenolysis inhibited the phosphatase reaction, but only glucose-6-P was able to enhance the reaction or to ward off the inhibition. The strong inhibition caused by UDPG could not be suppressed by glucose-6-P.

Phosphatase could only transform the dimeric form, but not the tetrameric one, of phosphorylase-*a* to phosphorylase-*b* as shown by experiments carried out at 18 °C.

The experimental results suggest that the ligands employed in the investigations affected the phosphatase reaction by changing the conformation of phosphorylase-*a*.

It is well known that the conversion of active phosphorylase-*a* into inactive phosphorylase-*b* plays an important role in the regulation of glycogenolysis of the muscle. The formation of phosphorylase-*a* is catalyzed by the enzyme phos-

Abbreviations used: P_i, inorganic phosphate; glucose-1-P, glucose-1-phosphate; fructose-1-P, fructose-1-phosphate; mannose-1-P, mannose-1-phosphate; glucose-6-P, glucose-6-phosphate; ribose-5-P, ribose-5-phosphate; fructose-di-P, fructose-1,6-diphosphate; UDPG, uridin diphosphate glucose; 2-P-glyc. acid, 2-phosphoglyceric acid; 3-P-glyc. acid, 3-phosphoglyceric acid; AMP, adenosine-5'-monophosphate; ADP, adenosine-5'-diphosphate; ATP, adenosine-5'-triphosphate; IMP, inosine-5'-monophosphate; cyclic AMP, cyclic adenosine-3',5'-phosphate.

phorylase kinase, which phosphorylates the phosphorylase-*b* by means of ATP. The conversion of phosphorylase-*a* into phosphorylase-*b* is catalyzed by the enzyme phosphorylase phosphatase (phosphatase) which effects the splitting of phosphate from phosphorylase-*a*. The phosphate is bound by a seryl residue of phosphorylase and enzymatic activity is confined to the phosphorylated state. According to our present knowledge the effect of phosphatase is not only characterized by the cleavage of phosphate but also the halving of molecular weight of phosphorylase-*a* (Keller, Cori, 1953; Graves et al., 1960) since tetrameric phosphorylase-*a* is transformed into dimeric phosphorylase-*b*. Consequently, the effect of phosphatase can be represented by the following equation:

$$\underset{\text{(tetramer)}}{\text{phosphorylase-}a\text{-}} \xrightarrow{\text{phosphorylase phosphatase}} \underset{\text{(dimer)}}{2 \text{ phosphorylase-}b} + 4\, P_i$$

According to Hurd and his coworkers (Hurd et al., 1966) the liberation of P_i from the tetrameric phosphorylase-*a* does not take place in one step and thus a mixture of phosphorylase molecules is formed as a result of phosphatase action. The mixture is composed of molecules containing four, three, two and one phosphate groups, as well as molecules free of phosphate. It will be shown that the above mechanism of phosphatase action is not correct because, according to our experience, phosphatase is able to dephosphorylate the dimeric *a* form only.

The regulation of the inactivation of phosphorylase-*a* by phosphatase has so far remained an unsolved problem, as the amount of phosphatase found in the tissues is rather constant. However, the intermediates of glycogenolysis considerably influence the rate of phosphatase reaction; thus the inactivation of phosphorylase-*a* is inhibited by P_i, glucose-1-P, fructose-di-P, ribose-5-P and glycerolphosphate (Cori, Cori, 1945; Sutherland, 1951; Cowgill, 1959; Fischer et al., 1963; Hurd et al., 1966a; Bot, Dósa, 1967; Holmes, Mansour, 1968), whereas it is enhanced by glucose-6-P, glucose and glycogen (Hurd et al., 1966a; Bot, Dósa, 1967; Holmes, Mansour, 1968). Beside the intermediates some nucleotides as AMP, ADP, ATP and IMP can also inhibit the phosphatase reaction (Sutherland, 1951; Fischer et al., 1963; Krebs et al., 1964; Nolan et al., 1964; Bot, Dósa, 1967), while cyclic AMP is ineffective (Krebs et al., 1964). NaF is also a potent inhibitor of the phosphatase reaction (Sutherland, 1951; Keller, Cori, 1955; Cowgill, 1959).

In a short communication (Bot, Dósa, 1967) we have shown that the inactivation of phosphorylase-*a* by phosphatase is hindered by AMP even at a concentration as low as the physiological one. However, phosphorylase-*a* is inactivated in vivo in the muscle though AMP is present. According to Krebs and his coworkers (Krebs et al., 1964) this phenomenon can be attributed to the action of adenylate deaminase, which decomposes AMP and thus renders the action of phosphatase possible. The complete decomposition of AMP is, however, not probable. We observed that at low concentration glucose-6-P can moderate the inhibition caused by AMP while at higher concentration the inhibition is complete-

ly eliminated. In view of this fact, one may assume the existence of a regulatory mechanism according to which the inactivation of phosphorylase-*a* is controlled by the concentration ratio of AMP and glucose-6-P or, simply, by the concentration of the latter.

In our present communication it will be shown that AMP and glucose-6-P do not affect phosphatase but rather phosphorylase-*a*, which acts as substrate, and a conformational change of the latter is responsible for the inhibition stimulation of phosphatase reaction.

This fact can explain the action of some effectors which cause conformational changes in the phosphorylase-*a*. Namely glucose-6-P, glucose and glycogen stimulate the reaction, while AMP, glucose-1-P and P_i inhibit it.

We have already suggested (Bot et al., 1970) that a similar mechanism is encountered in the transformation of phosphorylase-*b* to phosphorylase-*a*, i.e. in the kinase reaction. According to Holzer (1969) the kinase and phosphatase reactions are enzymatically catalyzed chemical modifications of enzymes. Our results indicate that this chemical modification of phosphorylase in both directions is considerably affected by the conformation of the protein acting as substrate.

Materials and methods

Preparation of phosphorylase-a

Phosphorylase-*a* was prepared from crystalline phosphorylase-*b* with the enzyme kinase, according to the method of Krebs and Fischer (1962). Crystalline phosphorylase-*b* was prepared from rabbit muscle (Krebs, Fischer, 1962). Kinase was prepared as described by Krebs et al. (1964). The phosphorylase-*a* obtained by kinase reaction was recrystallized three times in 0.05 M Tris, 0.05 M mercaptoethanol buffer, pH 6.8, then it was treated with Norit to remove traces of AMP. The specific activity of the enzyme obtained by this method was 40 to 50 U/mg protein.

Preparation of phosphorylase phosphatase

Phosphatase was prepared by a slightly modified method of Keller and Cori (1955). The main steps of the preparation were as follows: rabbit muscle homogenate prepared with two volumes of water was centrifuged at 8000 *g*. The supernatant was filtered and the pH was adjusted to 5.9 with acetate buffer. A precipitate consisting mainly of kinase was formed during this treatment. The precipitate was removed by centrifugation and the pH of the supernatant was adjusted to pH 5.1 with acetate buffer. The precipitate containing the bulk of phosphatase was separated by centrifugation and dissolved in 0.08 M Tris buffer, pH 6.8. Then the pH of the solution was adjusted to 9.4 with 1 M Na_2CO_3. Some contaminating protein precipitated on heat treatment (1 hour at 37 °C) and was removed by

centrifugation. The supernatant was neutralized to pH 7.0 with acetate buffer. If any precipitate was formed it was removed by centrifugation. The supernatant adjusted to pH 8.0 with 1 M Na_2CO_3, served as phosphatase preparation.

Assay of phosphatase activity

The activity of phosphatase was determined by measuring the decrease in the activity of phosphorylase-*a* employed as substrate (Keller, Cori, 1955). A solution containing 20 to 80 units/ml of crystalline phosphorylase-*a* was preincubated at 30 °C for 20 minutes. The activity of phosphatase was measured in 0.8 ml total volume containing 8 to 32 units of preincubated phosphorylase-*a*, phosphatase in a suitable dilution and the effector under investigation. 0.02 M Tris – 0.02 M cysteine, pH 6.8 was employed as buffer solution. Phosphatase was diluted to effect not more than 50 per cent decomposition of phosphorylase-*a* during incubation, at 30 °C for 10 minutes. The reaction was stopped by the addition of 0.06 M EDTA – 0.08 M glycerophosphate – 0.1 M NaF buffer, pH 6.8, to reach a dilution in which the activity of residual phosphorylase-*a* could still be reliably measured. The dilution also decreased the concentration of the effector so that it did not influence the activity of phosphorylase-*a*.

The unit of phosphatase was taken as the thousandfold amount of the first order reaction rate constant (Keller, Cori, 1955).

Assay of phosphorylase-a activity

The activity of phosphorylase-*a* was determined according to the method of Cori et al. (1955). The incubation medium contained 16 mM glucose-1-P, 1 per cent glycogen, 40 mM Tris – acetate buffer, pH 6.8 and an amount of phosphorylase-*a* consuming not more than 25 per cent of the glucose-1-P present in the mixture.

The amount of P_i liberated from glucose-1-P was determined according to Taussky and Shorr (1953).

The unit of phosphorylase-*a* is defined as the amount of enzyme which liberates 1 μmole P_i from glucose-1-P in one minute.

Results

The mechanism of inhibition caused by AMP

The mechanism of the inhibition caused by AMP was determined by measuring the rate of phosphatase reaction in the presence of various amounts of AMP.

First the amount of phosphorylase-*a* as substrate was varied while the concentration of phosphatase was kept constant (Fig. 1A). Then the effect of AMP was investigated by varying the concentration of phosphatase in the presence of a constant amount of phosphorylase-*a* (Fig. 1B).

It is apparent from Fig. 1A that the inactivation of a small amount of phosphorylase-*a* was inhibited by even low concentrations of AMP, whereas higher concentrations were required to prevent the inactivation of a larger amount of phosphorylase-*a*.

The change in the concentration of phosphatase, however, did not affect the amount of AMP required for the inhibition of the reaction (Fig. 1B). It can be

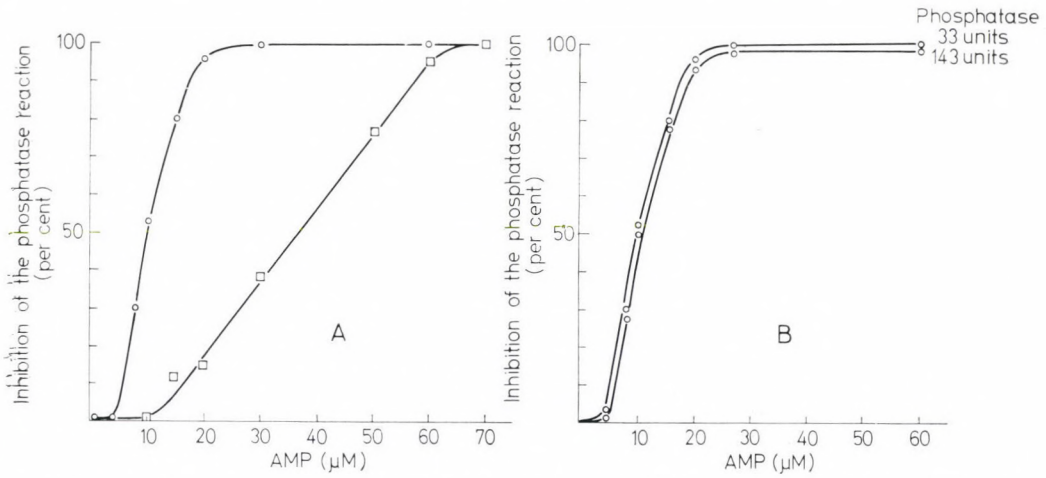

Fig. 1. Inhibition of the phosphatase reaction by AMP. 8 units (○——○) and 32 units (□——□) of phosphorylase-*a*, respectively, were incubated with phosphorylase phosphatase as described in Methods, in the presence of various amounts of AMP. The amount of phosphatase in 0.8 ml of incubation medium was 33 units (A) and 143 units (B) respectively. The dilution and activity assay of the unreacted phosphorylase-*a* were performed as described in Methods. The inhibition is related to the rate of the phosphatase reaction in the absence of AMP

seen that an increase in the concentration of phosphatase from 33 to 143 units did not diminish the inhibitory effect of AMP.

To further investigate the mechanism of inhibition caused by AMP we prepared the AMP complex of phosphorylase-*a* by compensation dialysis. It is well known that phosphorylase-*a* can bind 1 mole of AMP per subunit (DeVincenzi, Hedrick, 1967). The dialysis was performed against a solution containing AMP at a concentration which still did not inhibit the phosphatase reaction. The phosphorylase-*a*–AMP complex obtained by compensation dialysis was employed as a substrate in the phosphatase reaction (Fig. 2).

As shown in Fig. 2, the phosphorylase-*a*–AMP complex was either very slowly transformed into phosphorylase-*b* by phosphatase or no reaction took place at all. That the modification of phosphorylase-*a* by AMP was the real cause of the inhibition of phosphatase reaction, was proved by the subsequent addition of native phosphorylase-*a* to the reaction mixture. Phosphatase rapidly decom-

posed the native enzyme but it left unchanged phosphorylase-*a* bound in the complex. This experiment gave substantial support to the assumption that AMP changed the conformation of phosphorylase-*a* which was thus rendered inaccessible for phosphatase. The above experiment also proved that AMP bound to phosphorylase-*a* did not inhibit the action of phosphatase since the latter was able to transform phosphorylase-*a* subsequently added to the mixture.

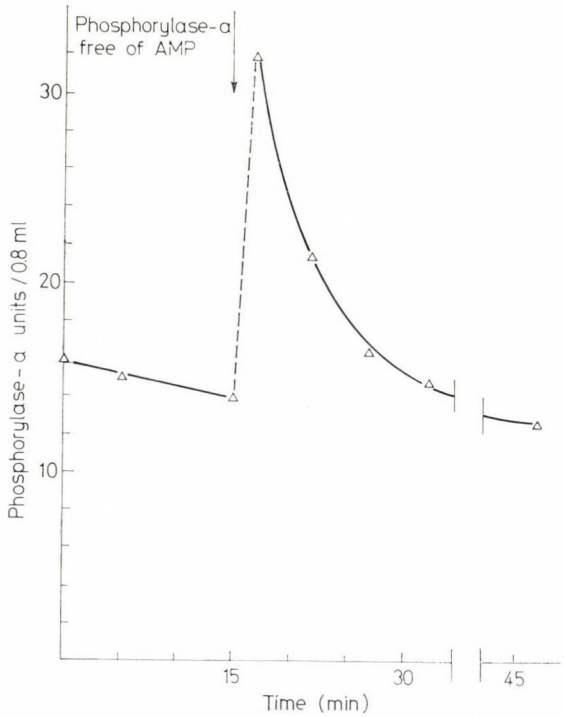

Fig. 2. The effect of phosphatase on phosphorylase-*a* in the presence and absence of AMP. 16 units of phosphorylase-*a* containing AMP was incubated with 45 units of phosphatase. 16 units of phosphorylase-*a*, not containing AMP, were added to the mixture after 15 min. Samples were taken at the indicated times and the activity of the unreacted phosphorylase-*a* was measured after dilution as described in Methods. Phosphorylase-*a* containing AMP was prepared by dialysis of a phosphorylase-*a* solution in 0.03 M Tris—0.01 M cysteine buffer pH 6.8, containing 4×10^{-6} M AMP against the same buffer for 24 hours. The dialysing solution was changed every 6 hours. During dialysis the E_{260}/E_{280} ratio of the phosphorylase-*a* solution increased from 0.60 to 1.53. This showed the accumulation of AMP in the phosphorylase-*a* solution

The mechanism of inhibition caused by glucose-1-P

It is well known that glucose-1-P also inhibits the inactivation of phosphorylase-*a* by phosphatase (Cori, Cori, 1945). Cori and Cori attributed this phenomenon to a "salt effect" as marked inhibition was only observed with at least 30

mM glucose-l-P. However, they also found that glucose-1-P inhibited the tryptic digestion of phosphorylase-*a* (Cori, Cori, 1945). This observation indicated that glucose-1-P altered the conformation of phosphorylase-*a*. According to more recent data (Fischer et al., 1963; Hurd et al., 1966a; Bot, Dósa, 1967) glucose-1-P present in a small concentration (1 mM) could also hinder the phosphatase reaction. Thus we studied the mechanism of the inhibition caused by glucose-1-P. The effect-increasing concentrations of glucose-1-P on the phosphatase reaction

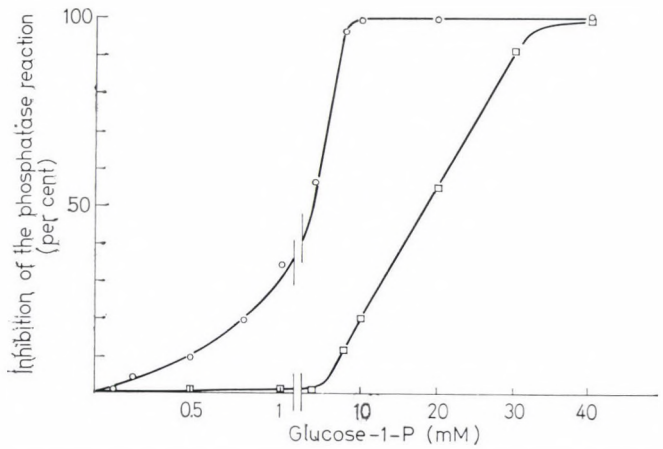

Fig. 3. Inhibition of the phosphatase reaction by glucose-1-P. 8 units (○——○) and 32 units (□——□) of phosphorylase-*a*, respectively, were incubated with phosphorylase phosphatase as described in Methods. The incubation medium contained 31 units of phosphatase in 0.8 ml. The dilution and the measurement of the activity of the unreacted phosphorylase-*a* was performed as described in Methods. The inhibition of the phosphatase reaction is related to the rate of the reaction in the absence of glucose-1-P

is shown in Fig. 3. Two different amounts of phosphorylase-*a* were employed in the experiments. It is seen in Fig. 3 that in the presence of a small amount of phosphorylase-*a* (8 units) the phosphatase reaction was inhibited to a considerable extent by 4 mM glucose-1-P, while 8 mM glucose-1-P completely abolished the reaction. However, 4 mM glucose-1-P was insufficient when a greater amount (32 units) of phosphorylase-*a* was employed and complete inhibition could be achieved only with at least 40 mM glucose-1-P.

The extent of inhibition caused by glucose-1-P was found to depend only on the amount of phosphorylase-*a*, while the quantity of phosphatase had no influence on the reaction. The hindrance caused by AMP showed similar characteristics. These facts support our assumption that the inhibition of the phosphatase reaction by glucose-1-P could also be attributed to a change in the conformation of phosphorylase-*a*.

The mechanism of stimulation and of the suppression of inhibition by glucose-6-P

As already mentioned in the introduction some observations indicate that the inactivation of phosphorylase-*a* by phosphatase is enhanced by glucose-6-P (Hurd et al., 1966a; Bot, Dósa, 1967; Holmes, Mansour, 1968). However, the mechanism of action of this metabolite is still unknown. We studied the effect of glucose-6-P on the phosphatase reaction. First the influence of the amount of phosphorylase-*a* on the effect caused by glucose-6-P was studied (Fig. 4).

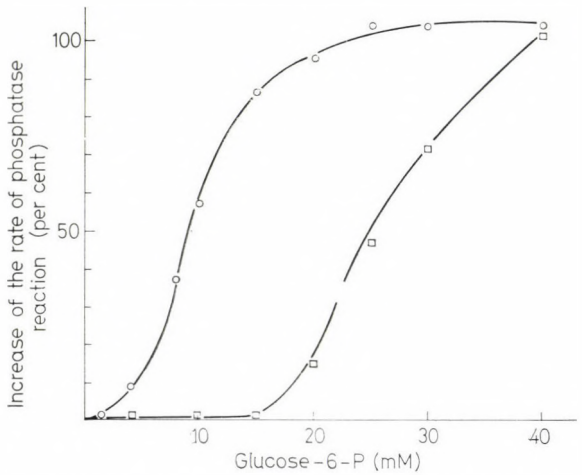

Fig. 4. Effect of glucose-6-P on the phosphatase reaction. 8 units (○——○) and 32 unit (□——□) of phosphorylase-*a*, respectively, were incubated with phosphorylase phosphatase as described in Methods. The incubation medium contained in 0.8 ml 21 units of phosphatase. The increase in the rate of reaction is related to the rate of reaction in the absence of glucose-6-P

Fig. 4 shows the activating effect of glucose-6-P in increasing concentrations in the presence of 8 and 32 units of phosphorylase-*a*. The rate of the reaction was considerably increased by even 10 mM glucose-6-P when 8 units of phosphorylase-*a* were employed as substrate, however, this concentration of glucose-6-P was found to be still ineffective in the presence of 32 units of phosphorylase-*a*. In the latter case a considerably higher concentration (30 mM) of glucose-6-P was required to produce the same effect.

The variation of phosphatase concentration had no influence on the stimulating effect of glucose-6-P just as it did not modify the effects of AMP and glucose-1-P. These results also lead to the conclusion that the variation of the rate of phosphatase reaction is caused by a change in the conformation of phosphorylase-*a*, in the present case on the effect of glucose-6-P.

The mechanism of action of glucose-6-P and its possible physiological role are being approached in our experiments which show that glucose-6-P wards off the inhibition by AMP of the phosphatase reaction. This phenomenon is shown in

Fig. 5. Glucose-6-P was employed in increasing concentrations in the presence of AMP.

It can be seen in Fig. 5 that the inhibitory effect of AMP gradually decreased by increasing the concentration of glucose-6-P. At a definite concentration ratio of glucose-6-P and AMP neither stimulation nor inhibition were found. If the concentration of glucose-6-P was higher than prescribed by this ratio the reaction rate increased even in the presence of AMP. This regulation may also have physiological significance since AMP and glucose-6-P can influence the phosphatase reaction even at the low concentrations encountered in the tissue.

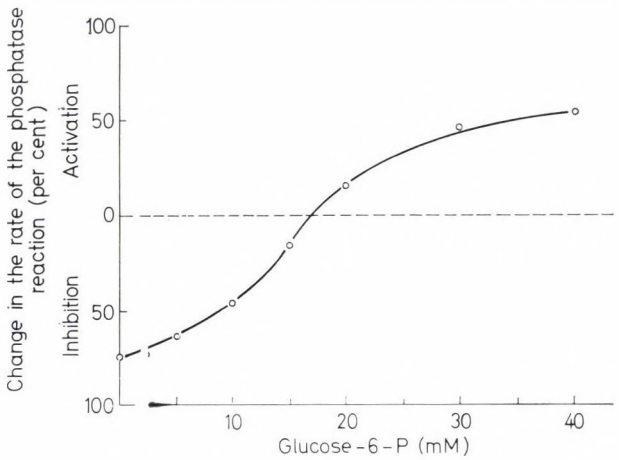

Fig. 5. Effect of glucose-6-P on the phosphatase reaction inhibited by AMP. 8 units of phosphorylase-*a* were incubated with 17 units of phosphorylase phosphatase in the presence of 15 μM AMP and various amounts of glucose-6-P. The incubation and the measurement of the activity of the unreacted phosphorylase-*a* were performed as described in Methods. The change in the rate of phosphatase reaction (inhibition or activation) is related to the rate of the reaction measured in the absence of effectors

The effect on the phosphatase reaction of some intermediates of glycogenolysis

Since the two consecutively formed intermediates of glycogenolysis, glucose-1-P and glucose-6-P, have opposite effects on the phosphatase reaction, it seemed to be of interest to examine the effect of some other important intermediates too. Among fructose phosphates Holmes and Mansour (1967) showed that fructose-di-phosphate inhibited the reaction. However, the effect of the other fructose phosphates and other intermediates has not yet been systematically investigated. The inhibitory effect of P_i, mannose-1-P and glycerophosphate has already been observed (Cori, Cori, 1945; Cowill, 1959; Holmes, Mansour, 1967). We were primarily interested in disclosing whether any other intermediate had a stimulating effect, similar to that of glucose-6-P. The results are shown in Table 1.

It is apparent from the data of Table 1 that the phosphatase reaction was more or less inhibited by the intermediates examined, except in the case of 3-P-

Table 1

Effect of some intermediates of glycogenolysis on the phosphorylase phosphatase reaction

8 units of phosphorylase-*a* were incubated with 18 units of phosphorylase phosphatase at the effector concentrations indicated in the table. The incubation, dilution, and the measurement of the activity of the unreacted phosphorylase were performed as described in Methods. The inhibition of the phosphatase reaction is related to the rate of the reaction measured in the absence of the effectors. Fructose-6-P, fructose-1-P, 2-P-glyceric acid, 3-P-glyceric acid were produced by REANAL (Budapest), Mannose-6-P was a product of Sigma (USA), UDPG was a product of Calbiochem (USA). All preparations were of reagent grade.

Effector	mM	Inhibition, per cent
fructose-6-P	2	28.6
fructose-6-P +glucose-6-P	2 10	0
fructose-1-P	2	25.8
fructose-1-P +glucose-6-P	2 10	23.0
fructose-di-P	2	48.6
fructose-di-P +glucose-6-P	2 10	15.8
mannose-6-P	2	65.6
mannose-6-P +glucose-6-P	2 10	14.6
UDPG	1	31.5
UDPG +glucose-6-P	1 10	31.5
UDPG +glucose-6-P	1 20	31.5
UDPG	2	76.4
2-P-glyc. acid	2	34.3
2-P-glyc. acid +glucose-6-P	2 10	2.9
3-P-glyc. acid	2	0

glyceric acid. Fructose-6-P and mannose-6-P also inhibited in contrast to glucose-6-P. Glucose-6-P decreased or suppressed the inhibition caused by fructose-6-P, fructose-di-P, mannose-6-P or 2-P-glyceric acid. However, it could not ward off the inhibition caused by fructose-6-P or UDPG. It is interesting to note that fructose-6-P inhibited the phosphatase reaction while glucose-6-P had an opposite effect. It is probable that most intermediates containing phosphate have no specific

effect but inhibit by their phosphate groups. However, the effect of glucose-6-P (stimulation and suppression of inhibiton) is highly specific.

Inhibition of the phosphatase reaction by NaF

It has been known for long that NaF is a potent inhibitor of the phosphatase reaction (Sutherland, 1951; Keller, Cori, 1955). However, in contrast to the other effectors investigated so far the amount of phosphorylase-*a* did not influence the inhibition by NaF. This phenomenon is shown in Fig. 6.

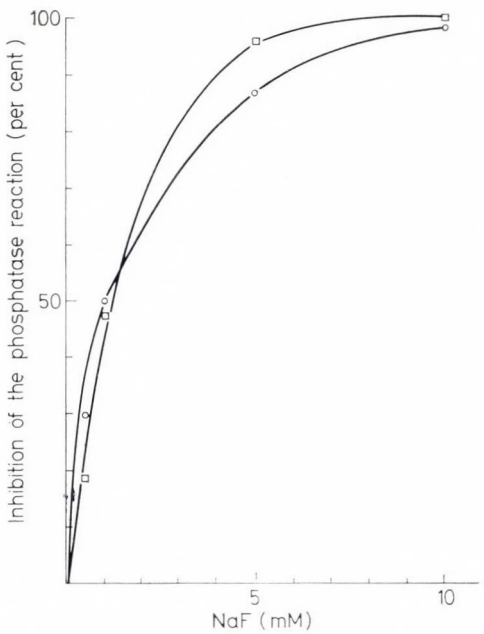

Fig. 6. Effect of NaF on the phosphatase reaction. 8 units (○——○) and 32 units (□——□) of phosphorylase-*a*, respectively, were incubated with 29 units of phosphorylase phosphatase. The incubation and the measurement of the activity of the unreacted phosphorylase-*a* were performed as described in Methods. The inhibition of the phosphatase reaction is related to the rate of the reaction measured in the absence of NaF

It is apparent in Fig. 6 that the same extent of inhibition was observed in the whole investigated range of NaF concentration with 8 and 32 units of phosphorylase-*a*. Thus the inhibition caused by NaF considerably differs from that of the other effectors as it presumably affects phosphatase directly and does not exert its effect by changing the conformation of phosphorylase-*a*. At the same time the mechanism of inhibition caused by NaF provides an indirect evidence for the fact that the other effectors do not directly influence phosphatase but rather phosphorylase-*a*.

The effect of tetramer formation by phosphorylase-a on the phosphatase reaction

The above experiments indicated that the conformational change of phosphorylase-*a* induced by the effectors did not only modify the activity of phosphorylase-*a* but also the rate of phosphatase reaction. However, the conformational change of phosphorylase-*a* can also take place without the influence of the effectors, when it is transformed from the dimeric into the tetrameric form at low

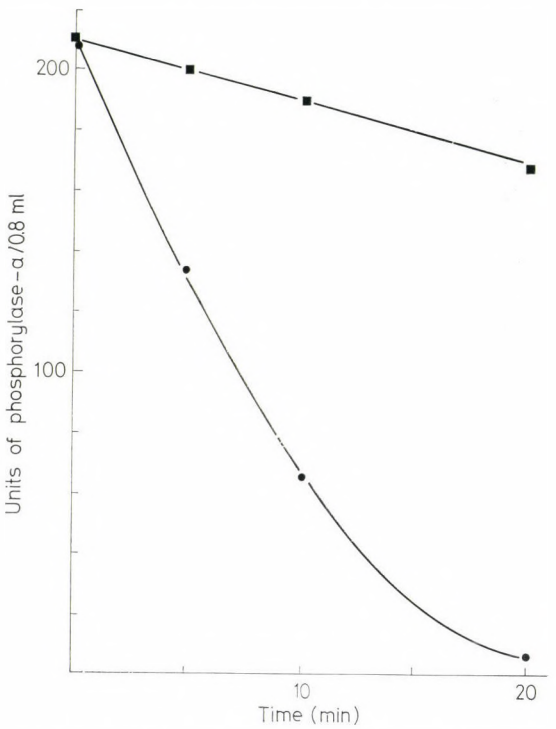

Fig. 7. Effect of the dimeric and tetrameric forms of phosphorylase on the phosphatase reaction. 210 units of dimeric phosphorylase-*a* (○——○) and a mixture containing 80% of the tetrameric form of phosphorylase-*a* (□——□), respectively, were incubated with 42 units of phosphorylase phosphatase in the presence of 0.5% glycogen. The incubation was performed at 18 °C. The dilution and the measurement of the activity of the unreacted phosphorylase-*a* were performed as described in Methods. The stabilization of the dimeric and tetrameric forms of phosphorylase-*a* was performed as described in the text

temperature and at appropriate protein concentration. About 80% of phosphorylase-*a* is in tetrameric form in a 2.2 mg/ml solution of phosphorylase-*a* at 18 °C. The formation of the tetramer can be prevented if glycogen is added to the solution before cooling, since the dimeric enzyme forms a complex with glycogen (Wang et al., 1965; Metzger et al., 1967). If glycogen is added to the solution after cooling down, the equilibrium between the dimeric and tetrameric phosphorylase

does not change in a short time. Thus the effect of phosphatase can be studied in the presence of two conformations of phosphorylase-*a* at 18 °C: with dimeric phosphorylase-*a*, if glycogen was added to the solution before cooling or with phosphorylase mainly in the tetrameric form if glycogen was added to the cold solution. The results of these experiments are shown in Fig. 7.

It can be seen in Fig. 7 that dimeric phosphorylase-*a* was much more rapidly transformed by phosphatase than the mixture containing mostly the tetrameric form.

This result suggests that dimeric phosphorylase-*a* is the substrate of phosphatase while tetrameric phosphorylase-*a* cannot be attacked by the phosphatase. Thus the conformational change of phosphorylase-*a* effected by cooling could also influence to a great extent the rate of the phosphatase reaction too. As it has been shown the specific activity of phosphorylase-*a* is greatly decreased by tetramer formation (Wang, Graves, 1964). Thus the various changes in the conformation of phosphorylase-*a*, whether caused by effectors or not, can be considered as modifications of the protein acting as substrate. This phenomenon affects not only the activity of the enzyme itself but also its enzymic transformation, i.e. its chemical modification.

Discussion

The dephosphorylation of phosphorylase-*a* by phosphatase and the simultaneous loss of enzymic activity of the former takes place in such an enzymic reaction where the substrate is also a high molecular weight enzyme protein.

Phosphorylase-*a*, which acts as substrate, is known to be an allosteric enzyme and many ligands change its conformation. These changes are reflected in the increase or decrease of the activity of phosphorylase-*a*. Obviously, the conformation of phosphorylase-*a* changes on the effect of these ligands even if phosphorylase-*a* is serving as substrate for phosphatase. However, it remains a question, whether such a change in the conformation also influences the rate of dephosphorylation by phosphatase. i.e. whether the conformational change of phosphorylase-*a* affects the inactivation reaction caused by phosphatase. Although several authors have assumed this to be the case (Nolan et al., 1964; Helmreich et al., 1965; Bot, Dósa, 1967) no direct evidence of it has so far been provided.

Our present experiments following several lines of approach seem to prove that the changes in the conformation of phosphorylase-*a* also affect the rate of the phosphatase reaction. This was shown by the fact that the effect of both AMP and glucose-1-P depended to a great extent on the amount of phosphorylase-*a* present in the solution while it was independent of the amount of phosphatase. On the other hand the inhibition by NaF was found to be independent of the amount of phosphorylase-*a*. All these phenomena can be explained by assuming that NaF directly affects phosphatase, whereas AMP and glucose-1-P influence phosphorylase-*a* by modifiying its conformation to an extent depending on the concentra-

tion of phosphorylase-*a*. This assumption is also supported by the fact that both AMP and glucose-1-P affects the enzymic activity of phosphorylase-*a* through a conformational change whereas NaF has no such effect. The importance of the conformation of phosphorylase-*a* in the phosphatase reaction is amply proved by the fact that phosphorylase-*a* obtained by compensation dialysis and containing AMP could not be transformed, while the native enzyme could be transformed in the same medium.

Beside this obvious and attractive explanation one may also suppose that the effectors bound to phosphorylase-*a* promote or inhibit the binding of the enzyme to the active center of phosphatase, without any changes in the conformation. However, the changes induced by the effectors in other properties of phosphorylase-*a* (enzymic activity, dimer–tetramer equilibrium, digestibility by trypsin) weigh in favour of conformational changes occurring parallel with the binding of effectors.

Our results are in agreement with the observations of Nolan et al. (1964), who claim that AMP inhibits the dephosphorylation of phosphorylase-*a*, but not that of a phosphodecapeptide obtained by tryptic digestion, as this peptide has lost the ability to bind AMP.

Among the intermediates tested glucose-6-P showed unique features as it was the only one which stimulated the phosphatase reaction and decreased or completely suppressed the effect of certain inhibitors.

The opposing effects of glucose-6-P and AMP may also have a regulatory role. AMP enhances the activity of phosphorylase-*a* in the glycogenolysis and, at the same time, it protects the enzyme from inactivation by phosphatase. If glucose-6-P is accumulated during the decomposition of glycogen, this not only moderates the ability of phosphorylase-*a* to decompose glycogen but also renders the inactivation of phosphorylase-*a* by phosphatase possible. Thus the conformational changes of phosphorylase-*a* induced by AMP and glucose-6-P affect both the enzymic activity of phosphorylase-*a* and its decomposition by phosphatase.

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The Effect of Substrate Analogues on the Iodine Sensitivity of Penicillinase from B. cereus

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The iodine-induced inactivation process of penicillinase was studied in the presence of methicillin. This substance was found to have a favourable effect on one of the two iodine-induced inactivation reactions described earlier of penicillinase, but has no effect on the other. In appropriate concentrations cephalothin is capable of warding off the effect of methicillin. With the help of methicillin it was possible to demonstrate that the two iodine-induced inactivation reactions may proceed independently of each other.

Introduction

It is known from the work of Citri et al. (Citri et al., 1961, 1962; Garber et al., 1962) that penicillin and its derivatives have a certain influence on the conformation of penicillinase. The majority of penicillins (e.g. methicillin, oxacillin) enhance the iodine sensitivity of penicillinase, while various cephalosporins and benzylpenicillin reduce the iodine sensitivity of the enzyme being in the iodine sensitive state. We have demonstrated in our earlier work (Csányi et al., 1970a) that the iodination reaction, by means of which the changes in penicillinase conformation can be detected, takes place in two steps. The following experimental conditions favour first reaction: 0 °C, pH 9, 10^{-3} N iodine when 30 to 35% of the enzyme activity will disappear within a few tenth of a minute. For the second step pH ranges above 9 are more favourable.

The aim of our present work has been to investigate which of the two reactions is influenced by the various substrate analogues which either reduce or enhance the iodine sensitivity of penicillinase.

Materials and methods

In the experiments the exopenicillinase produced by strain 569/H of B. cereus was used. The purification of the enzyme has been described in an earlier communication (Csányi et al., 1970).

The sodium salt of 6(2,6-dimethoxybenzamido)-penicillanic acid (Methicillin) is a product of Beecham Res. Lab. Ltd., the sodium salt of 7(thiophene-2-

acetamido)cephalosporic acid (Cefalotin, Keflin) is produced by Eli Lilly and Co. and the potassium salt of penicillin G by Biogal Works.

The experimental conditions for the iodine treatment of penicillinase have been analyzed in our earlier paper (Csányi et al., 1970a). We only wish to recall that iodine treatment has always been started by the addition of the enzyme and the treatment was performed at 0 °C in 0.05 M phosphate buffer. The activity assay of penicillinase has also been described in an earlier paper (Csányi, 1961).

The symbols introduced by us before will be used here, too: the residual enzyme activity after iodine treatment at pH 6 is expressed in percentage of the activity of the untreated enzyme and is denoted by A_{R6}, the activity measured after iodine treatment in a pH 9 medium is also expressed in the percentage of the activity of the untreated enzyme and is denoted by A_{R9}. Activities A_{R6} and A_{R9}, as described earlier, indicate the two steps in the inactivation of penicillinase (Mile et al., 1970a).

Results

Inactivation of penicillinase by iodine in the presence of methicillin

We have described in our earlier communication that in the presence of some 10^{-3} N iodine at 0 °C 30–35% of penicillinase is inactivated at pH 9 and not more than 5–10% at pH 6. At both pH values inactivation proceeds very rapidly and the reaction is practically complete in 1 minute (Csányi et al., 1970a). The kinetics of the inactivation process was studied in the presence of 100 μg/ml of methicillin with 7×10^{-3} N iodine at both pH 6 and 9. The results are shown in Fig. 1. It appears that at pH 6 about 70% of the enzyme is inactivated in less than

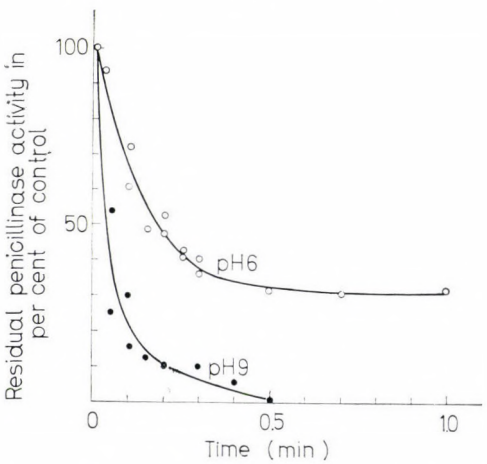

Fig. 1. Inactivation of penicillinase by iodine in the presence of methicillin. 300 U of peni cillinase dissolved in 5 ml of 0.05 M phosphate buffer was treated with 7×10^{-3} N iodine at 0 °C in the presence of 100 μg/ml of methicillin at pH 6 and 9. The residual activity is expressed in percentage of the activity of the untreated control.

30 seconds, while at pH 9 the enzyme is completely inactivated within the same period of time. In the subsequent iodine treatments in the presence of methicillin incubation time was always 30 seconds.

Fig. 2 shows the effect of methicillin concentration of the inactivation of penicillinase by 7×10^{-3} N iodine at pH 6. It appears that in the presence of methicillin the rate of iodination reaction increases up to a certain limit value. Figs 3 and

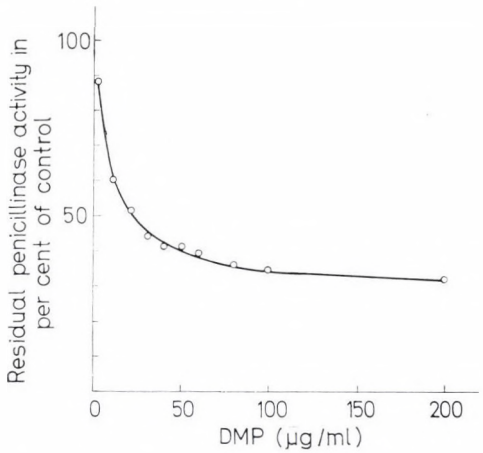

Fig. 2. Effect of methicillin concentration on the inactivation of penicillinase by iodine. 300 U of penicillinase was treated at pH 6 with 7×10^{-3} N iodine for 30 seconds at different methicillin (DNP) concentrations. The residual activity is given in percentage of the activity of the untreated samples

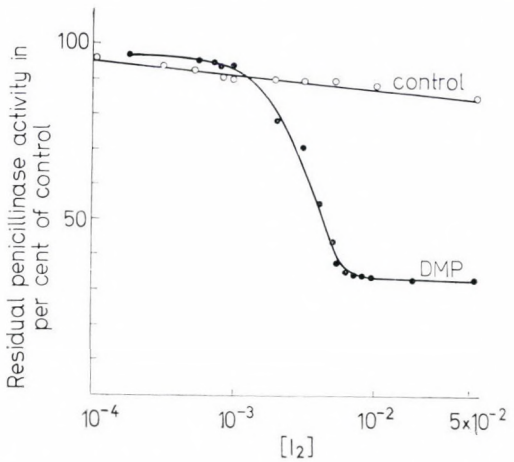

Fig. 3. Effect of iodine concentration on the inactivation of penicillinase in the presence of methicillin at pH 6. 300 U of penicillinase was treated with various concentrations of iodine at pH 6 in the presence of 100 μ/ml of methicillin at 0°C for 30 seconds. The residual activity is expressed in percentage of the activity of the untreated samples

4 demonstrate the effect of iodine concentration on the inactivation process of the enzyme in the presence of 100 μg/ml of methicillin at pH 6 and 9, respectively.

Analysis of the experimental results reveals that the iodine-inactivation reaction of penicillinase in the presence of methicillin follows a course different from that without methicillin. At pH 6 the native enzyme is resistant even to very high iodine concentrations (Csányi et al., 1970a), while in the presence of methicillin marked inactivation (amounting to 70%) occurs at iodine concentrations above 10^{-3} N. It is interesting to note that the remaining 30% of enzyme activity

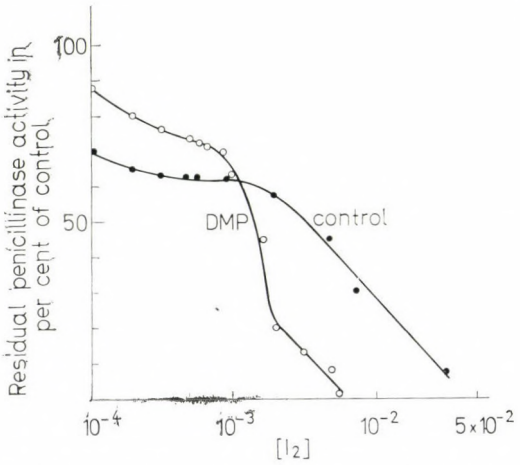

Fig. 4. Effect of iodine concentration on the iodine-induced inactivation of penicillinase at pH 9 in the presence of methicillin. 300 U of penicillinase was treated with various concentrations of iodine at pH 9 in the presence of 100 μg/ml of methicillin (DMP) at 0°C for 30 seconds. The residual activity is expressed in percentage of the activity of the untreated sample

is not lost even at high iodine concentrations (Fig. 3). When inactivation is induced at pH 9 two phenomena can be observed. Below 10^{-3} N iodine concentration methicillin appears to reduce the inactivating effect of iodine, while at iodine concentration higher than 10^{-3} N inactivation proceeds rapidly even at pH 9 and reaches 100% already at relatively low iodine concentration values (Fig. 4).

These results have led us to the working hypothesis according to which methicillin has practically no effect on the "first" reaction of the inactivation of penicillinase by iodine. By the "first" reaction we mean the 30–35% inactivation of the native enzyme at pH 9 in the presence of 10^{-3} N iodine. Moreover, it appears from the experiment illustrated in Fig. 4 that in the presence of methicillin this reaction is less pronounced. On the other hand methicillin has a marked effect on the "second" reaction of inactivation which does not take place at pH 9 in the presence of 10^{-3} N iodine, but only at higher pH values or with higher iodine concentrations. Should this hypothesis be true this would mean at the same time that the two reactions of iodine inactivation proceed independently of each other and it depends on the experimental conditions which of them will dominate.

It appears from Fig. 2, which shows the effect of methicillin concentration, that only a very slight inactivation takes place at pH 6 in the presence of 7×10^{-3} N iodine in a medium free of methicillin, but as methicillin concentration is raised the degree of inactivation also increases until it reaches a value of about 70%. If our suggested working hypothesis is correct then it would follow from the experiment illustrated in Fig. 2 that the residual penicillinase activity after iodine treatment in the presence of increasing methicillin concentrations will have to be increasingly more "sensitive" to iodine in the absence of methicillin, but at pH 9. This should be so, because according to our hypothesis the residual activity after iodine treatment in the presence of methicillin is due to the lack of the "first" reaction at pH 6 even in the presence of methicillin. On the other hand this "first" reaction takes easily place when iodine treatment is performed at pH 9 without methicillin.

This expected consequence of our hypothesis could be checked experimentally. The experiment was carried out as follows: a given quantity of penicillinase

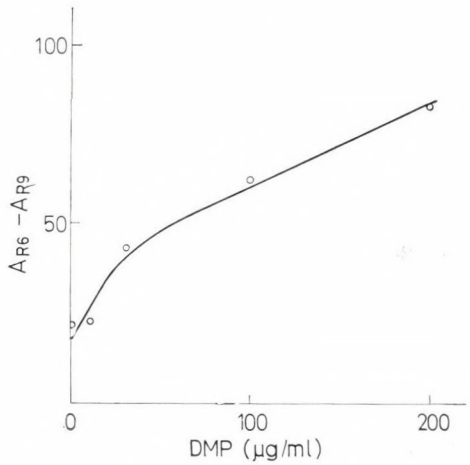

Fig. 5. The effect of iodine treatment at pH 6 in the presence of methicillin on the A_{R6} and A_{R9} activities of the enzyme. Penicillinase was treated with 7×10^{-3} N iodine at 0°C for 30 seconds in the presence of various methicillin concentrations. After iodine treatment the solution was diluted 100-fold and iodine treatment repeated with 10^{-3} N iodine at pH 6 and pH 9 at 0°C for 60 seconds. The values of A_{R6} and A_{R9} activities were calculated from the residual activities and their differences are plotted vs. methicillin concentration during the first iodine treatment

was treated at pH 6 in the presence of increasing methicillin concentrations with 7×10^{-3} N iodine, the iodine was then removed and the mixture was diluted 100-fold its initial volume (to reduce methicillin concentration). Iodine treatment was repeated at pH 6 and pH 9 with 10^{-3} N iodine. That is the A_{R6} and A_{R9} activities of the methicillin-treated enzyme were determined in accordance with the definition given in Materials and Methods. The differences between A_{R6} and A_{R9} activities (this difference equals the enzyme activity which has been destroyed

in the "first" reaction) measured after iodine treatment in the presence of a certain given methicillin concentration are plotted in Fig. 5 as a function of methicillin concentration. It is quite clear that the difference between A_{R6} and A_{R9} increases as the methicillin concentration is raised, that is the proportion of enzyme activity which is rapidly inactivated at pH 9 in methicillin-free medium increases. Hence this experiment strongly supports our hypothesis.

The effect of cephalothin on the iodine-induced inactivation reactions of penicillinase

In addition to methicillin, experiments were carried out with other analogues too, such as oxacillin and pyrazocillin, and similar results were obtained. These analogues belong to the group classified by Citri et al. as iodine sensitivity-enhancing compounds. The other group of compounds contain the readily hydrolys-

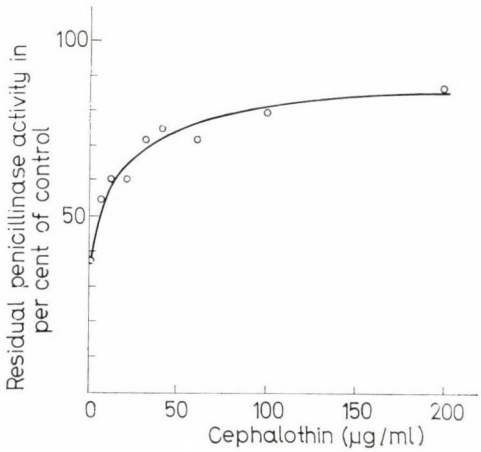

Fig. 6. Effect of cephalothin on iodine sensitivity produced by methicillin. 300 U of penicillinase was treated with 7×10^{-3} N iodine in the presence of 100 μg/ml of methicillin and various concentrations of cephalothin at pH 6 and 0°C for 30 sec. The residual activity is expressed in percentage of the activity of the untreated samples

able penicillins which tend to reduce the iodine sensitivity of penicillinase (Garber et al., 1962). From many aspects it is cumbersome to work with hydrolysable analogues (among others their hydrolysis produces compounds which react with iodine). Cephalosporines, which hydrolyze much more slowly, belong to this group and they also reduce the iodine sensitivity of penicillinase. We have chosen cephalothin from this group and examined whether this compound was capable of warding off the effect of methicillin. The result of the experiments is shown in Fig. 6. It appears that in adequate concentrations cephalothin is capable of warding off the effect of methicillin at pH 6, i.e. it abolishes the enhancement of iodine-induced inactivation.

Discussion

We have reported in our earlier communications that the inactivation of penicillinase by iodine takes place in two steps: the "first" reaction proceeds even at low (10^{-4} N) iodine concentrations at pH 9, while the "second" reaction takes place at pH 9 or at higher pH values, but only at several orders of magnitude higher iodine concentrations (Csányi et al., 1970a). By treating penicillinase in strong alkali we have confirmed that the two reactions are not the consequences of the different iodine sensitivities of two interconvertible enzyme conformations (Mile et al., 1970a).

Iodination experiments carried out with isotopic iodine have proved that all penicillinase molecules are iodinated in the course of the "first" inactivation reaction and one di-iodotyrosine per molecule is formed. The formation of di-iodotyrosine leads to the 30–35% reduction in enzyme activity (Mile et al., 1970b).

The experiments described in this paper seem to support the assumption that the two iodine-induced inactivation reactions of penicillinase may proceed quite independently of each other. The presence of methicillin favours the "second" inactivation reaction, which takes place even at pH 6 in the presence of methicillin, whereas without methicillin this reaction proceeds only at pH values higher than 9. Since methicillin is capable of forming a specific bond with penicillinase, we have assumed that this bonding brings about a conformational change which enables the "second" reaction to occur also at lower pH values. The effect of cephalothin, a representative of the other group of substrate analogues, is the opposite of that of methicillin, as demonstrated also by Citri et al. (1962).

The relative independence of the two iodine-induced inactivation reactions may be explained by several alternative hypotheses. The iodinated groups modified in course of the two reactions may influence the active centre of the enzyme independently of each other. Iodination of one group results in a 30–35% decrease in activity, while iodination of the other group is responsible for the 65–70% decrease in activity. However, it is quite conceivable that the penicillinase molecule possesses two independent active centres. This has already been suggested earlier in the study of the specific effect of pyrasocillin on the activity of penicillinase (Csányi et al., 1970b). Further experiments are needed to solve the problem which of these two, or other, possible explanations are valid.

We are indebted to Professor Bruno F. Straub for his continued, most helpful interest and criticism; thanks are also due to Mr I. Balázs, for his devoted technical assistance.

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Effect of Electrolytes on the Activity and Iodine Sensitivity of Penicillinase from B. cereus

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The effect of various electrolytes on the activity and iodine sensitivity of penicillinase was studied. Higher concentrations of potassium hydrogen phosphate and potassium iodide activate the enzyme, while KI in concentrations higher than 0.4 M reversibly inhibits enzyme activity. Enzyme activity is also inhibited by borate ions. Inhibition by borate is reversible, but higher dilutions are needed to reverse this inhibition than would follow from the concentration dependence of inhibition. In higher concentrations the electrolytes affect the progress of the pH resistant iodine-induced inactivation reaction of penicillinase. At iodine concentrations higher than 10^{-3} M inactivation is enhanced. In the presence of borate the iodine-induced inactivation of the enzyme at pH 9 starts only at extremely high iodine concentrations.

Introduction

The penicillinase of B. cereus is known to be a protein of extremely flexible structure. Under different experimental conditions significant conformational changes can be observed in the penicillinase; these conformational changes can be followed by measuring the iodine sensitivity of the enzyme (Citri, 1958; Citri et al., 1960). Our earlier investigations have shown that this iodination reaction, which is suitable to the measuring of the conformational changes of penicillinase, takes place in two independent steps (Csányi et al., 1970; Mile et al., 1970; Ferencz et al., 1970). Under experimental conditions which favour the first reaction (0 °C, pH 9, 10^{-3} M of iodine) 30–40% of the enzyme activity disappears within a minute. The second step is favoured above pH 9. Competitive inhibitors have no influence on the first reaction, but accelerate the second (Ferencz et al., 1970). We have already indicated in our first paper about iodine-induced inactivation reactions that the iodine sensitivity of penicillinase is greatly affected by the presence of various electrolytes (Csányi et al., 1970). In this paper we describe our pertaining experiments.

Materials and methods

In the experiments the exopenicillinase produced by strain 569/H of B. cereus was used. The enzyme was purified as described earlier (Csányi et al., 1970). The conditions of incubation with iodine are given in the description of experiments. Enzyme activity after iodine treatment was measured, if not indicated otherwise, by diluting 1 ml of the incubation mixture to 50 ml with a 1 : 1 mixture of 0.5% gelatin and 0.25 M phosphate buffer, pH 6.5, and incubating the mixture with 0.06 g of the sodium salt of penicillin G at 30 °C for 5 minutes. The reaction was stopped by the addition of 20 ml of inhibitor solution (0.5 M sodium tungstate and 1.0 M sodium acetate). This was followed by the usual iodometric measurement (Csányi, 1961).

Results

Effect of electrolytes on the activity of penicillinase

When studying the effect of various buffers on the iodine sensitivity of the enzyme we have observed that certain electrolytes have a marked influence on the catalytic activity of penicillinase.

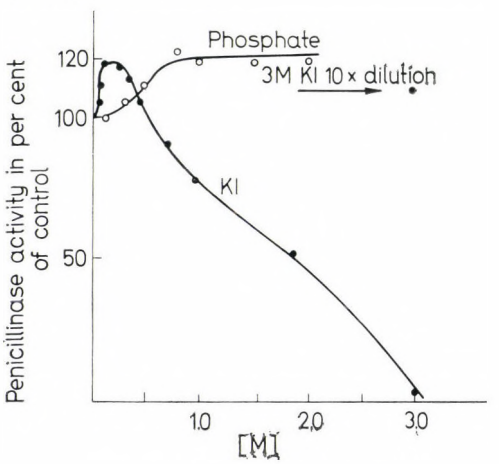

Fig. 1. Effect of potassium hydrogen phosphate and potassium iodide on the activity of penicillinase. 800 U of penicillinase was incubated in 20 ml of a mixture containing 0.06 g of the sodium salt of penicillin G dissolved in 2 ml of 0.25 M phosphate buffer and phosphate or iodide in concentrations as indicated. The phosphate was a solution of KH_2PO_4 whose pH value had been adjusted to 6.5 with KOH. In the case of samples containing KI the solution contained an additional 0.0005 N thiosulfate to remove free iodine (thiosulfate had no effect on enzyme activity even in several orders higher concentrations). The enzyme was incubated at 30 °C for 5 minutes and the reaction was stopped by the addition of 20 ml of the inhibitor. In case of higher phosphate concentrations the inhibitor was made up with hydrochloric acid to have a pH of 4. Enzyme activity values measured after the 10-fold dilution of 3 M KI are also plotted. The controls contained beside penicillin G dissolved in 2 ml of 0.25 M phosphate buffer no other additives. The values of enzyme activity are expressed in percentage of the controls

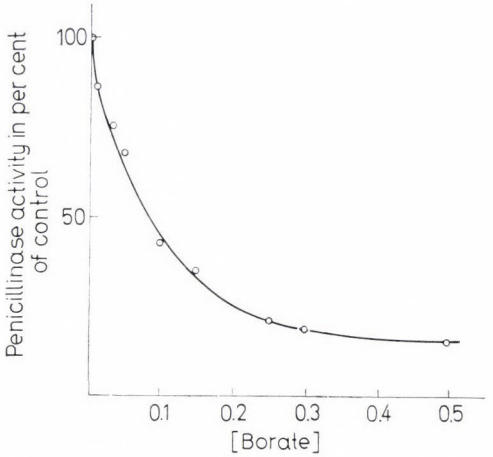

Fig. 2. Effect of borate on the activity of penicillinase. 500 U of penicillinase was incubated in 20 ml of a solution which contained 0.06 g of the sodium salt of penicillin G dissolved in 2 ml of 0.25 M phosphate buffer and various concentrations of borate as shown in the Figure. The pH of the mixture was 6.5. Incubation was performed at 30 °C for 5 minutes. The activity is expressed in percentage of the control sample, which contained no borate.

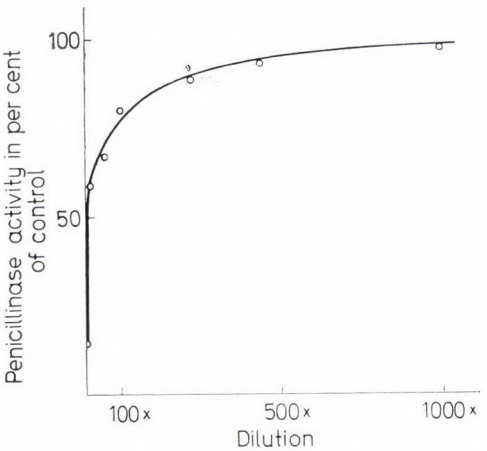

Fig. 3. Effect of dilution on the activity of penicillinase incubated in borate pH 6. 500 U of penicillinase was incubated in various volumes of 0.25 M borate buffer, pH 6, at 30 °C for 10 minutes. The samples were then diluted in proportions shown in the Figure to obtain 20 ml final volume. The diluting solution contained enough phosphate to ensure a final phosphate concentration of 0.1 M and pH 6.5. The samples were then incubated with 0.06 g of the sodium salt of penicillin G at 30 °C for 5 minutes, after which the reaction was stopped by the addition of 20 ml of the inhibitor solution and iodine consumption was measured. The activities were expressed in percentage of the samples which had not been treated with borate

Fig. 1 shows the effect of potassium iodide and of "potassium dihydrogen phosphate" (prepared from KH_2PO_4 by adjusting the pH of the solution to 6.5 with KOH). If the incubation mixture used for measuring the activity of penicillinase is made up with various concentrations of these electrolytes, then, depending on the concentration and type of the electrolyte, an increase or inhibition of enzyme activity can be observed as compared to the activity measured in the usual incubation mixture. With 1 M or more concentrated phosphate solutions a 20% activity increase was measured. A similar increase in activity was also found with 0.4 M KI, while in KI solutions of higher concentration enzyme activity gradually

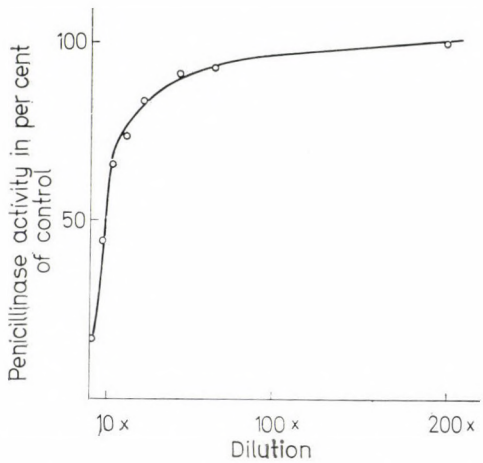

Fig. 4. Effect of dilution on the enzyme activity of penicillinase incubated in borate pH 9. Experimental conditions were the same as in Fig. 3, but pre-incubation in borate was performed at pH 9

decreased and reached practically 0 in 3.0 M solution. The inhibitory effect of KI on activity is fully reversible, on appropriate dilution the initial activity is recovered.

A very marked inhibition of enzyme activity was found in the presence of borate ions (Fig. 2). This inhibition by borates is also reversible, but the dilution necessary to regain the initial activity is far greater than that which follows from the borate concentration dependence of the inhibition; after dilution the conformation changes instantaneously. The inhibitory effect of borate is also concentration dependent. In more alkaline media an even higher dilution is required to regain the initial activity than in acidic media (Figs 3 and 4). Preliminary experiments have shown that part of the free tyrosines of the enzyme when dissolved in borate becomes "buried". Probably the profound conformational change caused by borate ions is responsible for the observed drop in activity just as for the anomalous behaviour towards dilution.

The effect of electrolytes on the iodine sensitivity of penicillinase

The two reactions of the iodine-induced inactivation of penicillinase were studied generally in phosphate buffer. It appeared necessary to examine the effect of the concentration of the phosphate buffer on iodine-induced inactivation at pH 6 and 9. We have found that when 10^{-3} N iodine is used phosphate concentrations between 0.05 and 0.8 M seem to have no particular effect on inactivation: at pH 6 the degree of inactivation is 5 – 10%, at pH 9 the usual 30 – 40%. We have demonstrated earlier that in the presence of methicillin, which has a specific

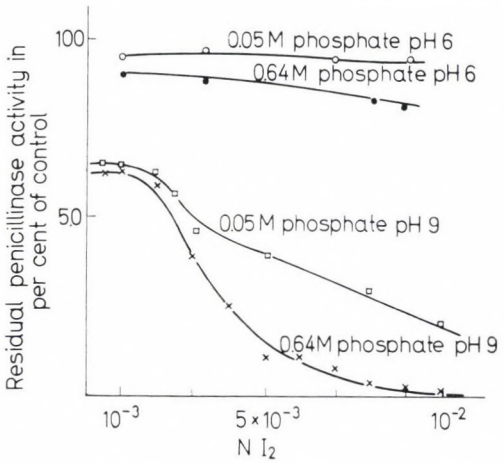

Fig. 5. Effect of iodine concentration on the iodine-induced inactivation of penicillinase in diluted and concentrated phosphate solutions. 400 U of penicillinase was treated in 1 ml of the incubation mixture with iodine at concentrations shown in the Figure, in 4 parallel series at pH 6 and 9 in 0.05 and 0.64 M phosphate buffer at 0 °C for 1 minute. After incubation in the presence of iodine the reagent was removed by an equivalent amount of thiosulfate dissolved in 0.1 ml and the residual activity was measured after 50-fold dilution of the mixture

effect on penicillinase, the inactivation by iodine is strongly affected by iodine concentration (Ferencz et al., 1970). The same phenomenon was observed when polyethyleneglycol, which raises the iodine sensitivity of penicillinase, was used (Csányi et al., in preparation).

We have therefore studied the effect of iodine concentration on inactivation taking place within one minute at two different phosphate concentrations (0.05 and 0.64 M), at pH 6 and 9 (Fig. 5). It appears from the figure that at pH 6 iodine concentration has no particular effect on the inactivation of the enzyme. However, when iodination is carried out at pH 9, in concentrated phosphate solutions and above 10^{-3} N, iodine concentration, the enzyme will be inactivated to a far greater extent than at lower (0.05 M) phosphate concentrations.

The specificity of this effect with respect to the applied ions was also investigated. Penicillinase was treated with 7×10^{-3} N iodine in solutions containing various concentrations of the different ions (Fig. 6). It is clear that the electrolytes

have different effects on the iodine-induced inactivation of the enzyme. The effect of sodium chloride appears to be particularly anomalous. We are probably dealing here with such fine effects affecting enzyme conformation which, with our present knowledge, can hardly be interpreted.

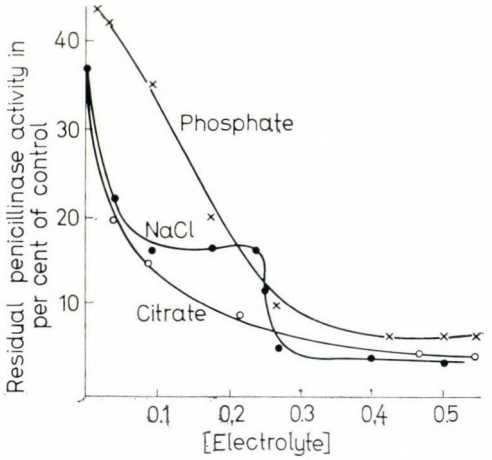

Fig. 6. Effect of electrolyte concentration on the iodine-induced inactivation of penicillinase at pH 9 in the presence of 7×10^{-3} N iodine. 400 U of penicillinase was treated with 7×10^{-3} N iodine at pH 9 at electrolyte concentrations shown in the Figure. Sodium citrate and sodium chloride were dissolved in the phosphate buffer. After the removal of iodine the residual activity was measured after a 50-fold dilution of the mixture and expressed in percentage of the activity of the untreated samples

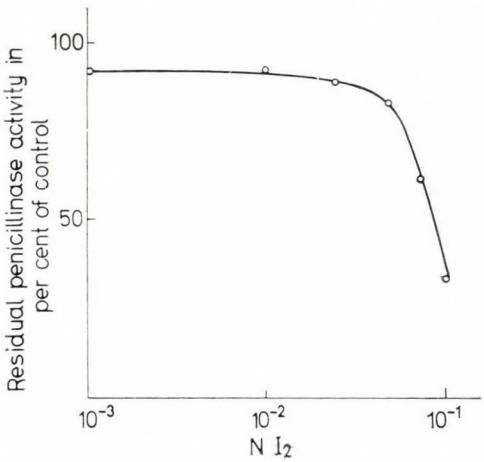

Fig. 7. Effect of borate on the iodine-sensitivity of penicillinase. 10 000 U of penicillinase was incubated in 0.042 M borate buffer, pH 9, with various concentrations of iodine at 0 °C for 1 minute. After the removal of iodine, part of the mixture was diluted 1000-fold and the enzyme activity was measured. The control sample was treated in the same way but contained no iodine

Since it is highly probable that the various electrolytes, which also influence the activity of the enzyme, do this by changing the enzyme conformation, it appeared worth-while to investigate the effect of borate ions, which influence the most strongly though in a reversible manner the inactivation of penicillinase, on the iodine-induced inactivation of the enzyme. The enzyme dissolved in 0.042 M borate buffer was treated with various concentrations of iodine at pH 9, the incuba-

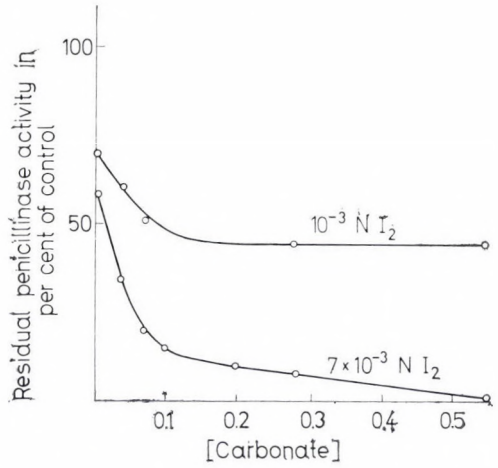

Fig. 8. Effect of carbonate concentration on the iodine-induced inactivation of penicillinase at pH 9 by 10^{-3} and 7×10^{-3} N iodine. 500 U of penicillinase was treated in 2 ml of Na_2CO_3—$NaHCO_3$ buffer of concentrations shown in the Figure at 0 °C with 10^{-3} and 7×10^{-3} N iodine, respectively, for 1 minute

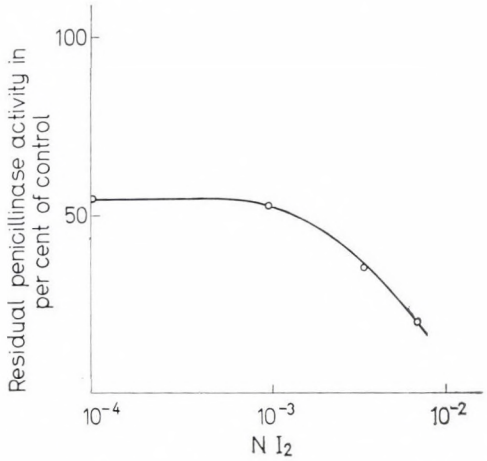

Fig. 9. Effect of iodine concentration on the iodine-induced inactivation of penicillinase in a 0.055 M carbonate buffer pH 9. 500 U of penicillinase was treated for 1 minute in 2.0 ml of a 0.055 M Na_2CO_3—$NaHCO_3$ buffer at 0 °C with iodine at concentrations shown in the Figure

tion mixture was diluted 1000-fold and the residual activity measured. The result of the experiment is shown in Fig. 7. It appears from the figure that in relatively dilute borate solutions, where enzyme activity drops merely a few per cent, the pH-sensitive iodine-induced inactivation of penicillinase will no longer take place even at iodine concentrations as high as 10^{-2} N. At this high pH value, too, the inactivation of the enzyme starts only at an iodine concentration of about 10^{-1} N. Hence borate, which inactivates penicillinase, brings about conformational change that greatly protects the enzyme against the inactivating effect of iodine.

The phosphate buffer applied so far in our experiments enabled the use of mixtures with identical electrolyte composition even under different experimental conditions (Csányi et al., 1970). However, for experiments at pH 9 this buffer is not quite satisfactory, since in this pH range it has a low buffer-capacity. Studying the effect of various electrolytes we have found that around pH 9 phosphate may be replaced by carbonate, since this ion has approximately the same effect on the iodination reaction as phosphate ion; the anomalies observed with borate, chloride and iodide do not occur with carbonate and the buffer-capacity is also satisfactory. Figures 8 and 9 show the effect of various carbonate and iodide concentrations on the iodine-induced inactivation at pH 9.

Discussion

It is well known that electrolytes influence protein conformation. With certain enzymes a specific ligand bond can be demonstrated. In these cases certain ions alter the steric structure of the protein molecule by being bound at definite points (Friedberg et al., 1969; di Prisco et al., 1969; Sameyima et al., 1969). In the case of other enzymes a non-specific, general ion effect exists, that is the enzyme will assume its active conformation only at a definite ionic strength (di Prisco et al., 1966; Arima et al., 1968).

In the case of penicillinase the various ions have a marked effect on enzyme conformation, as shown by the above experiments, in which enzyme activity is enhanced or inhibited by electrolytes. The data collected so far are not sufficient to decide whether we are dealing here with an entirely non-specific ion effect or in certain cases a specific ligand effect is also involved. It seems probable that when the pH-resistant reaction is enhanced (Figs 5 and 6) the ion effect is non-specific. High electrolyte concentrations will enhance inactivation at pH 9 only above a definite iodine concentration, in full agreement with the data obtained in the presence of methicillin (Ferencz et al., 1970) and of polyethyleneglycol (Csányi et al., in preparation). The effect of the last two substances is specific beyond doubt: methicillin – the competitive inhibitor of penicillinase – is bound to the enzyme and in the case of polyethyleneglycol, too, it was possible to demonstrate a bond between the latter and the enzyme. It seems therefore probable that in all three cases (concentrated electrolyte, methicillin, polyethlyleneglycol) a specific conformation develops which in itself is not iodine-sensitive, but is converted into an

iodine-sensitive state at high iodine concentrations. High iodine concentration alone is not sufficient to form the iodine-sensitive conformation.

The inhibition observed in the presence of borate seems to be associated with some specific ligand effect, since activity inhibition occurs at considerably lower concentrations than in the presence of other salts and even at very low concentrations profound conformational changes can be detected by the measurement of iodine sensitivity. The anomalous behaviour of borate inhibition towards dilution also seems to indicate a more specific effect. Obviously, more experiments are needed to clarify this problem.

The authors wish to thank Prof. Bruno F. Straub who followed this work with great interest, and Mr I. Balázs for his valuable technical assistance.

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The Effect of a Paramagnetic Center of 2.03 Average *g*-Value on d-Glyceraldehyde-3-Phosphate Dehydrogenase

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(Received September 22, 1970)

On the effect of nitrogenoxide and ferrous ion a paramagnetic center of 2.03 average *g*-value was formed in d-glyceraldehyde-3-phosphate dehydrogenase.

The SH- and histidyl groups of the enzyme took part in the formation of the paramagnetic center as coordination ligands.

The number of paramagnetic centers of the enzyme increased in time because first the most reactive and most readily accessible ligands reacted only. After the modification of the steric structure of the protein paramagnetic complexes were formed also on the sterically hindered ligands.

The formation of paramagnetic center inhibited the activity of the enzyme and this inhibition was competitive with the substrate whereas it was of a mixed type with the coenzyme and phosphate ion.

Introduction

A paramagnetic center of 2.03 average *g*-value has been observed in the ESR spectrum of yeast cells (Vanin, Nalbandyan, 1965; 1966) and localized in the mitochondrium (Lisovskaya et al., 1970a). This center could be also detected in transplantable tumor cells (Saprin et al., 1968), in the tissue of rodents in the early stage of carcinogenesis (Vithayathil et al., 1965), moreover in normal rabbit liver tissue. The study of the ESR spectra of cell components revealed that under certain conditions a part of the iron and sulphur contained in the so-called "non-heme iron" proteins could be detected in the form of this center (Vanin et al., 1967). The complex formed during carcinogenesis was shown to be bound to extramitochondrial protein fractions containing SH groups (Woolum, Commoner, 1970).

New paramagnetic centers, among others the one of 2.03 average *g*-value, appeared in various tissues and cells after incubation in nitrogen oxide (Azhipa et al., 1966; Vanin, Chetverikov, 1968; Lisovskaya, Vanin, 1969). This phenomenon could be explained by the fact that NO formed a paramagnetic complex with ferrous ion in the presence of coordination ligands (McDonald et al., 1965). In samples of biological origin the iron bound in non-heme structures (non-heme iron) can only act as ferrous ion in the complex while the sulphur contained in SH-groups and the nitrogen bound in imidazol groups or in free amino-groups can

serve as coordination ligands. Thus the complex is called non-heme iron nitrosyl or iron-nitric oxide or g 2.03 paramagnetic center. As the ESR signal of this center was easily detectable the incubation in nitrogen monoxide was suggested for the detection of non-heme iron proteins (Vanin, 1967). Indeed, this method has been successfully employed to reveal functional differences in the nitrogen fixation of microorganisms and the respiration of yeast cells (Ivleva et al., 1969; Liskovskaya et al., 1970).

Thus the above paramagnetic center is not only of biological interest but it has a methodical significance as well. The study of this paramagnetic center is rather difficult since the biological objects investigated so far (cell suspension, tissue homogenate) were too complex, whereas the chemical models (cysteine-Fe-NO complex) could hardly disclose its biological role. The paramagnetic center was also formed on peptides and isolated proteins on the effect of Fe^{++} and NO (Vanin, 1967). The SH-groups were found to be involved in the center and their signal in the ESR spectrum could clearly be identified. Indirect evidence led to the assumption that another part of the spectrum was the signal of the complex formed on the imidazol groups (Woolum et al., 1968). However, the number and type of SH- or imidazol groups taking part in the formation of the complex are unknown and it is not clarified either how this influences the function of the molecule. Since the knowledge of these facts would not only indicate the limits of applicability of the method in the detection of non-heme iron, but would also answer the question whether this method could be used for the investigation of structure and function, we endeavoured to study the paramagnetic center on a simple biological model, a crystalline enzyme. Glyceradehyde-3-phosphate dehydrogenase was adopted, as this enzyme had been thoroughly investigated, its amino acid sequence, mechanism of action and its probable active center were fairly well known, even the changes in its steric structure on the effect of various blocking agents has been characterized (cf. relevant chapters in the textbooks and reviews: Dévényi et al., 1969; Sund, 1970).

Materials and methods

D-glyceraldehyde-3-phosphate dehydrogenase (GAPD) was prepared from pig muscle and recrystallized four times (Elődi, Szörényi, 1956). Glyceraldehyde-3-phosphate (GAP) was prepared from fructose-1,6-diphosphate (Reanal) as described by Szewczuk et al. (1961). NAD was a product of Reanal, diethylpirocarbonate (DEP) was produced by Schuchardt, monoiodoacetic acid by Light, p-chloro-mercuri-benzoate (p-MB) was a product of British Drug Houses, Ltd. All other chemicals were reagent grade commercial preparations.

The paramagnetic complex was prepared according to Woolum et al. (1968). The pH of the ascorbic acid solution needed for the generation of NO was adjusted to 6.0–6.8 with 5 N NaOH. In ESR experiments the solutions of the enzyme, ascorbic acid, ferrous sulfate and sodium nitrite were mixed and the mixture was

immediately transferred into capillary tubes and then incubated. The reaction was stopped by dipping the capillaries into liquid nitrogen.

The formation of paramagnetic center could not be executed with enzyme dissolved in a buffer solution as the buffers usually employed also formed paramagnetic centers with nitrogen monoxide and ferrous ion. $(NH_4)_2SO_4$ behaved in a similar manner, therefore the experiments were performed with enzyme dissolved in distilled water and gel-filtered on a Sephadex G-50 column equilibrated with distilled water. Since several of the reagents used could also form paramagnetic centers the mixture was also dialyzed or gel-filtered after treatment with iodoacetic acid, p-MB or DEP, and the complex was prepared thereafter.

Above 0 °C the spectra were registered by means of a JES-ME-3X ESR Spectrometer, while the refrigerated samples were analyzed in a JES-P-10 ESR Spectrometer. The ESR signal was registered simultaneously with a Mn^{++}—MgO control, the third line of which is at $g = 2.03$. The amplitudes of the signals of the different samples were normalized to the Mn^{++} line. Thus the measurements could be compared quantitatively. The relative height or amplitude of the signal is the distance between the maximum and minimum of the spectrum as projected to the ordinate. The amplitude of the signal is, strictly speaking, proportional to the concentration of the paramagnetic center only after the double integration of the spectrum. However, as a first approximation, we calculated the relative height of the signal as a quantitative datum and plotted in the figures, since the line-width did not change with pH, temperature or enzyme concentration.

Enzymic activity was measured as described previously (Keleti, Batke, 1965). The SH-groups were determined by titration with p-MB (Boyer, 1954), whereas DEP was employed for the determination of histidines (Ovádi et al., 1967).

For the inhibition experiments GAPD was dissolved in distilled water at pH 6.0. The paramagnetic center was prepared by incubation of 3.75 ml of a 12.0 mg/ml GAPD solution with a mixture of 0.25 ml of a 180 mg/ml ascorbic acid solution pH 6.0, 0.25 ml of a 3.2 mg/ml $FeSO_4$ solution and 0.25 ml of a 180 mg/ml $NaNO_2$ solution at 0 °C (Woolum et al., 1968). After incubating for 10 minutes the mixture was diluted 300 to 400 fold with glycine buffer, pH 8.5, or tris buffer. The activity was assayed with 0.1 ml of diluted mixture in 3 ml final volume in silica cells of 10 mm light path in a Hilger UVISPEK or Opton PMQ II spectrophotometer.

Results

The signals of paramagnetic centers obtained with 10 mg/ml cysteine, 30 mg/ml histidine and 30 mg/ml enzyme, respectively, are shown in Fig. 1. The spectra were registered on refrigerated samples. The half-width of the histidine spectrum (the distance between the two most distant extreme values on the first derivative spectrum, cf. Hedvig, Zentai, 1969 p. 51) is about 57 Gauss indicating a not

completely resolved triplet. The signal of cysteine has a half-width of 40 Gauss only, an unresolved doublet. The spectrum of the enzyme is composed of these two signals, its half-width is somewhat wider, 60 to 65 Gauss, and the signal of cysteine predominates in it. In liquid enzyme samples the width of the spectrum is the same.

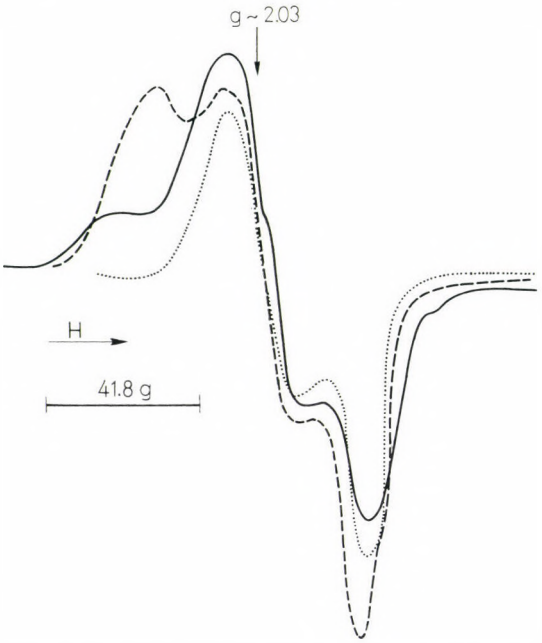

Fig. 1. ESR signal of the $g \approx 2.03$ center obtained with cysteine, histidine and GAPD in refrigerated samples. The paramagnetic complex was prepared as described in Materials and Methods. ——— the signal of 30 mg/ml GAPD in the 2nd hour; - - - the signal of 25 mg/ml histidine; . . . . . the signal of 10 mg/ml cysteine both in the 5th minute. The arrow under H shows the direction of the increasing magnetic field. The *g*-values changed in the opposite direction. It is apparent that the signal due to histidine has also a peak at higher *g*-values, by which it can be distinguished from that of cysteine

Fig. 2 shows the time course of the change in the amplitude of the signal obtained with the amino acids and the enzyme, as well as the decrease of enzyme activity with time. The signal of cysteine decreased slowly whereas that of histidine very rapidly. The signal of the enzyme rapidly increased for thirty minutes, then the increase slowed down and the signal reached a plateau value after two hours. The amplitude of the signal after two hours was about tenfold the value measured after five minutes. The center could be maintained for a prolonged time only in a closed capillary tube because of its sensitivity to oxygen. The complete signal-form of the enzyme samples developed in 5 to 10 minutes; the peak characteristic of histidine and observable at lower field strength ($g \approx 2.04$) appeared beside the signal

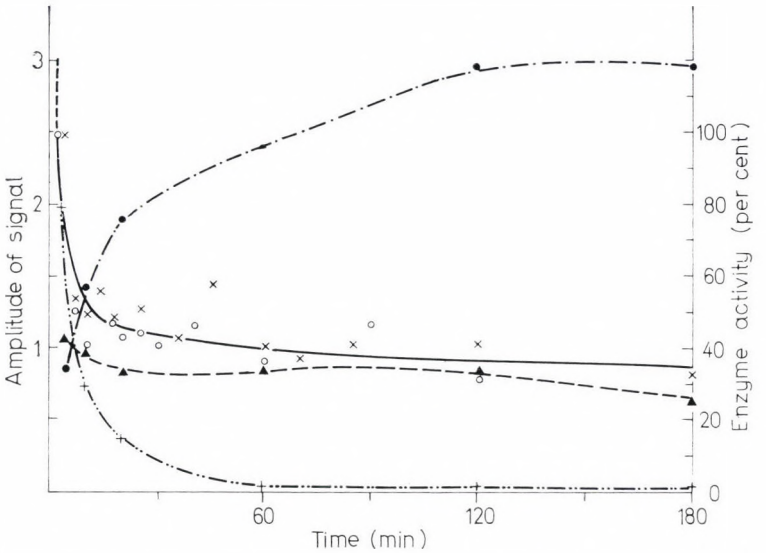

Fig. 2. Time course of the change in the amplitude of the signal of the $g \approx 2.03$ paramagnetic complex. The amplitude of the signal of 30 mg/ml GAPD, ●·—·●; 25 mg/ml histidine, +··—··+; and 10 mg/ml cysteine ▲—▲. The enzyme activity in per cent is shown on the ordinate on the right side. After mixing the components as described in Materials and Methods samples were taken from the mixture at various times and the activity of the samples was measured after a 300-fold dilution in glycine buffer. The first point was measured 2 to 3 minutes after the preparation of the mixture. The activity of this sample was taken as 100%. The two different symbols (×, o) refer to enzyme activities measured in two separate experiments

of cysteine after 5 to 10 minutes only. On further incubation the form of the signal did not change, which pointed to the fact that only the number of paramagnetic complexes, not their character, had changed.

More than 70 per cent of the enzymic activity is lost on the effect of the paramagnetic center and this inhibition developed in about 10 minutes. The inhibition attained its maximum between 7 and 10 minutes. The activity only changed to a slight extent during the following one to three hours.

These data led us to the conclusion that first the SH-groups of the enzyme participated in the formation of the paramagnetic radical followed by the reaction of the histidyl groups. These reactions were responsible for the inhibition of enzymic activity. Further on the steric structure of the enzyme changed as a secondary process, as a result of which, paramagnetic centers were formed on more SH- and histidyl groups, but the enzyme activity did not further decrease.

The development of the signal of the enzyme at various pH values is shown in Fig. 3. In alkaline medium the ESR signal was smaller and it was characteristic of cysteine. The complex was less paramagnetic than in the previous cases. The ESR spectra remained in the protein fraction after gel-filtration of the sam-

ples on a Sephadex-column, which proved that the center was formed on the enzyme.

Fig. 4 shows the signal height of the complex formed on the enzyme at various temperatures. The amplitude of the signal of the enzyme denatured by heat at 60 °C did not vary in time, whether the enzyme was incubated at 60 °C or it was put into a water bath at 60 °C for 3 minutes after incubation at 0 °C. The amplitude of the signal was greater at the 5th minute in the heat-denatured sam-

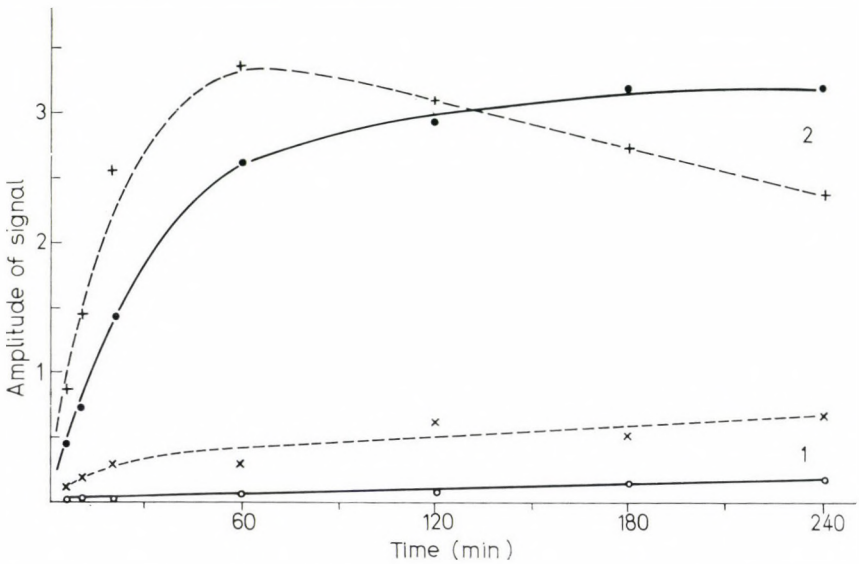

Fig. 3. Time course of the change in the amplitude of the signal of the complex of $g \approx 2.03$ at various pH values. The pH of the mixture was adjusted with 5 N NaOH or 0.1 N TRIS or 0.1 N HCl solutions

1) Alkaline range × — — × pH 8; o ——— o pH 9
2) Acide range + — — + pH 6; ● ——— ● pH 6.8

ples than in the native enzyme, but later on it did not increase and remained characteristic of cysteine as in the first minutes. The amplitude of the signal of samples incubated at 0 °C and then denatured by heat decreased as compared to that of the native and the peak characteristic of histidine disappeared.

Since denaturation was carried out at a temperature different from that at which the native enzyme was studied the possibility had to be excluded that the decrease in the amplitude of the spectrum could be due to the difference in temperature. The temperature dependence of the signal of the heat-denatured enzyme is shown in Fig. 5 in the temperature range −140° to + 98 °C. The variation in the amplitude of the signal of the complex followed Curie's law, but the temperature dependence was negligible between 0 ° and 60 °C.

Fig. 6 shows the time dependence of the signal of the enzyme treated with 10 mole equivalents of monoiodoacetic acid, 4 mole equivalents of $AgNO_3$

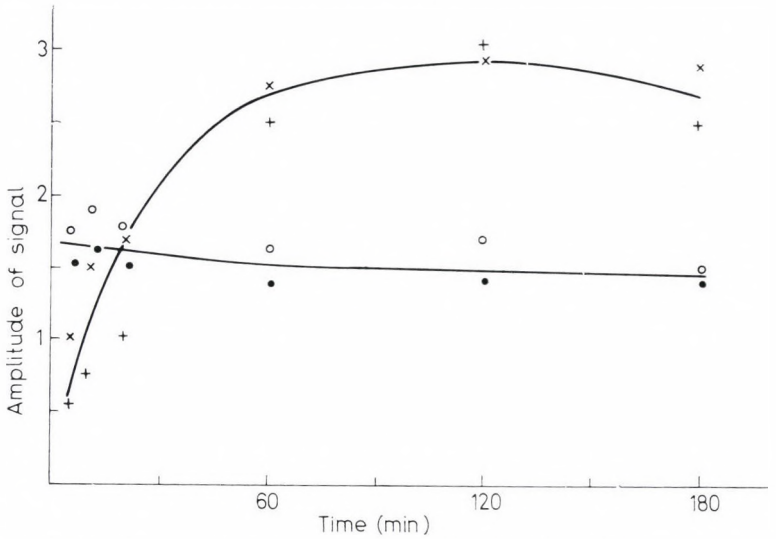

Fig. 4. The effect of heat denaturation on the amplitude of the $g \approx 2.03$ signal of GAPD. +, × samples incubated at 0 °C (control); ● — ● samples incubated at 60 °C; o — o enzyme incubated at 0 °C. The latter preparation was immersed in a water bath at 60 °C for 3 minutes before refrigeration. The duration of incubation is indicated on the abscissa. The preparation and measurement of the complex were performed as described in Materials and Methods, i.e. the spectra were registered on refrigerated samples independently of the temperature of incubation

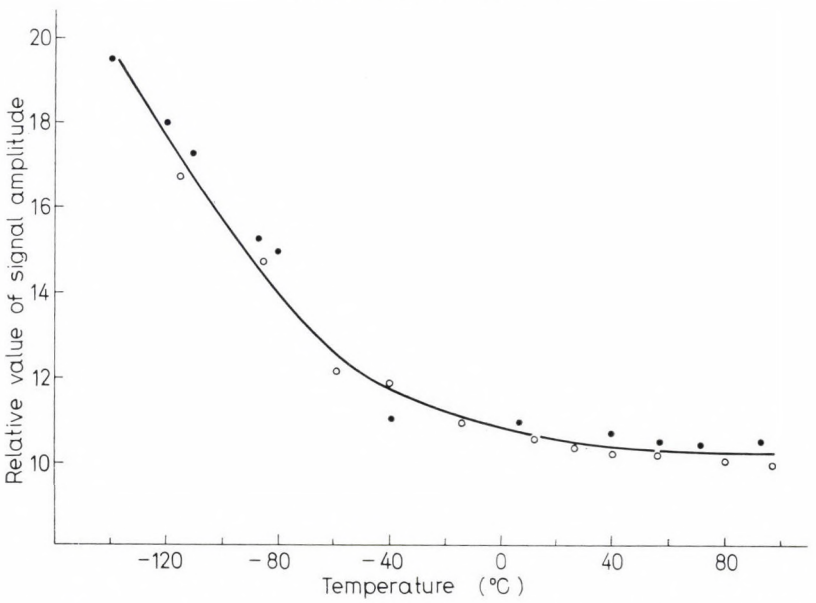

Fig. 5. Relative values of the amplitude of the signal obtained with denatured enzyme as a function of temperature. The complex was denatured in a water bath at 60 °C. The spectrum of the complex was measured at the temperatures indicated on the abscissa

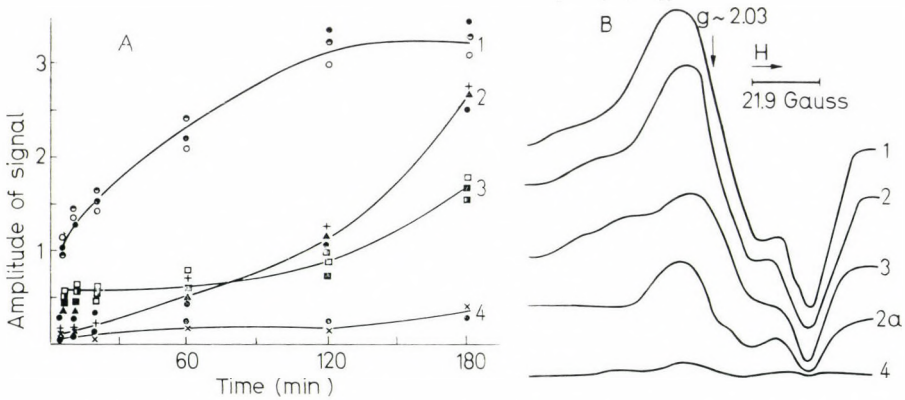

Fig. 6. Time course of the amplitude of the signal of the enzyme complex incubated with various blocking agents and without treatment, respectively. After the blocking carried out as described in Methods the pH of the enzyme solution was adjusted to 6, and then the complex was prepared. A) The various symbols represent independent experiments. 1. Native enzyme; 2. Carbethoxylated enzyme, 3. Enzyme treated with monoiodoacetic acid or silver ions; 4. Mercaptidated enzyme. B) ESR spectra measured in refrigerated state. 1. Native enzyme after incubation for 180 min; 2. Carbethoxylated enzyme after 180 min of incubation and 2 a refrigerated at the 120th minute of the incubation; 3. Carboxymethylated enzyme refrigerated at the 180th minute; 4. Mercaptidated enzyme after 180 min of incubation

100 mole equivalents of DEP and 20 mole equivalents of p-MB, respectively. All these blocking agents hindered the development of the signal of the complex as compared to the native enzyme. The mercaptidation (p-MB-treatment) caused a permanent inhibition of the development of the ESR signal. If the development of the complex was started when the mercaptidated enzyme had already become opalescent or it had precipitated, the signal characteristic of histidine was observed in the spectrum. The carbethoxylation (DEP-treatment) of the enzyme inhibited the appearance of spectra characteristic of histidine and cysteine only temporarily. The enzyme treated with DEP exhibited a small cysteine-type signal which gradually increased, and then around the 180th minute the histidine-type signal suddenly appeared. Carboxymethylation (monoiodoacetic acid-treatment) and Ag ion decreased only the signal characteristic of cysteine. In this instance the amplitude of the histidine signal was equal to that obtained with the native enzyme, while the amplitude of the signal characteristic of cysteine was only one half of that. On further incubation the intensity of the spectra decreased (not shown in the figure). Some characteristic spectra are shown in the insert of the figure.

Among the reagents required for the formation of the paramagnetic center, $FeSO_4$ (in tris buffer in the presence of arsenate) strongly innhibited the activity of the enzyme. Under similar circumstances, or in the presence of phosphate ion, ascorbic acid had the same effect. $NaNO_2$ alone did not inhibit the activity of the

enzyme in any of the cases studied. Likewise, inhibition could not be detected either with $FeSO_4$ or with ascorbic acid when these reagents were applied separately and the enzyme activity was measured in glycine buffer in the presence of

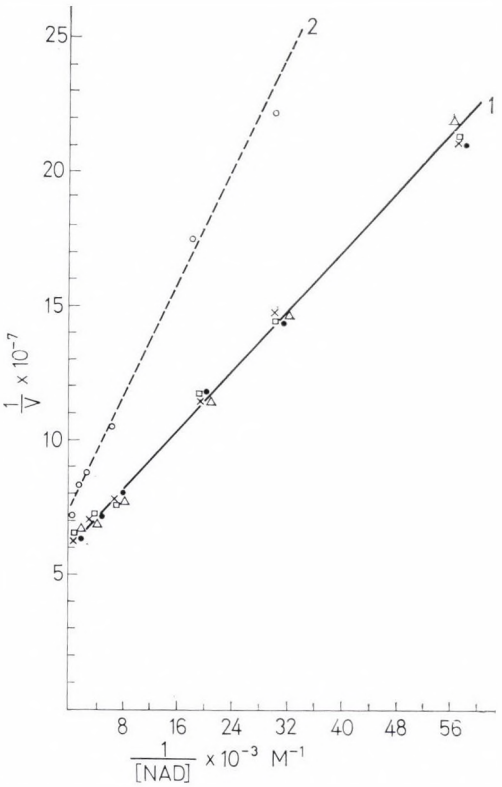

Fig. 7. Inhibitory effect of the paramagnetic center with respect to NAD as plotted according to Lineweaver and Burk. The paramagnetic center was prepared as described in Methods. The mixture was diluted with 0.1 M glycine buffer, pH 8.5, after incubation for 10 minutes. The measurement of activity was performed at a final GAPD concentration of 1 to 3 μg/ml. The results are normalized to the same protein concentration. GAP: 1.38×10^{-3} M; phosphate ion: 1×10^{-2} M. v = moles of NADH formed per ml in 15 sec. 1. ×: native GAPD; □: GAPD + $FeSO_4$; ●: GAPD + ascorbic acid; △: GAPD + $NaNO_2$. 2. o GAPD containing paramagnetic center

phosphate ion (Fig. 7). Therefore we investigated the effect of the paramagnetic center in glycine buffer and in the presence of phosphate.

The inhibition of enzyme activity caused by the paramagnetic center was of a mixed type with both NAD (Fig. 7) and phosphate ion (Fig. 8), whereas the inhibition was competitive with the substrate (Fig. 9).

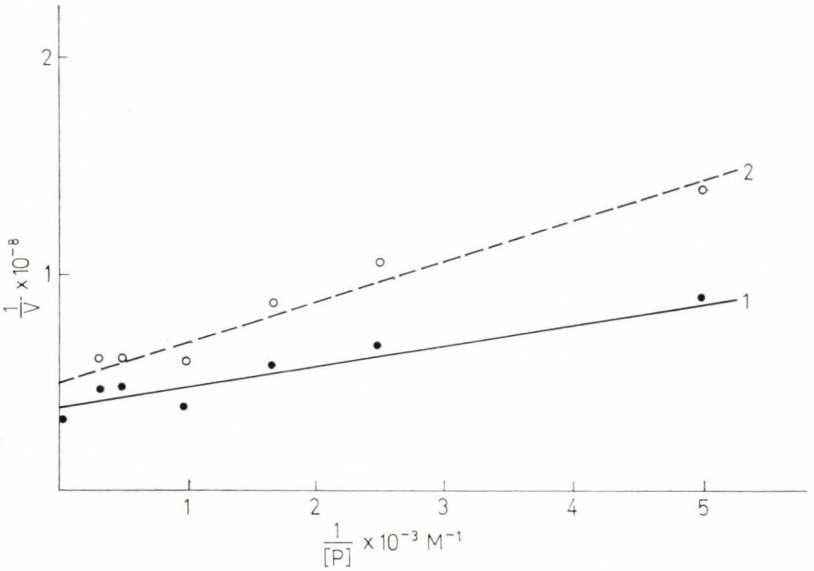

Fig. 8. Inhibitory effect of the paramagnetic center with respect to phosphate ion as plotted according to Lineweaver and Burk. The experimental conditions and the symbols are the same as in Fig. 7. NAD: 1.48×10^{-3} M; GAP: 1.38×10^{-3} M

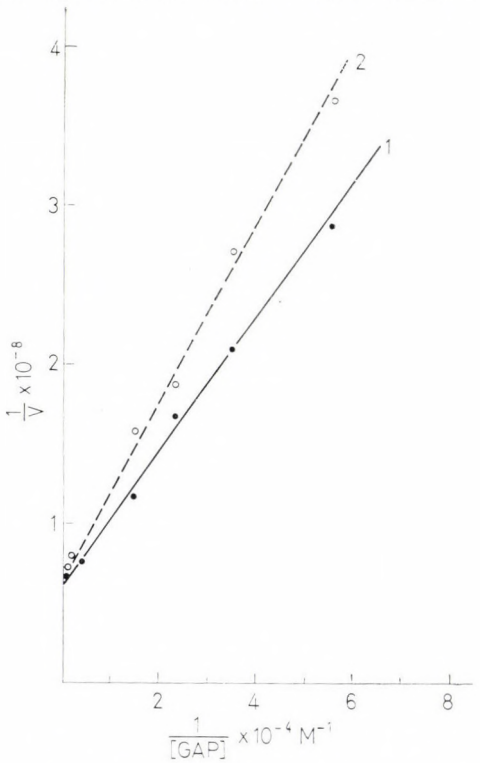

Fig. 9. Inhibitory effect of the paramagnetic center with respect to GAP as plotted according to Lineweaver and Burk. The experimental conditions and the symbols are the same as in Fig. 7. NAD: 1.48×10^{-3} M; phosphate ion: 1×10^{-2} M

Since the oxidized substrate forms an acylenzyme with the SH-group of GAPD and the paramagnetic center exhibits competitive inhibition with the substrate, we examined whether the number of SH-groups titratable with p-MB changed on the effect of the paramagnetic center. Native GAPD contains 16 SH-groups per molecule. Ten minutes after the formation of the paramagnetic center approximately half of the SH-groups disappeared (Fig. 10). The titration performed in the second hour also showed that 8 out of the 16 SH-groups were blocked.

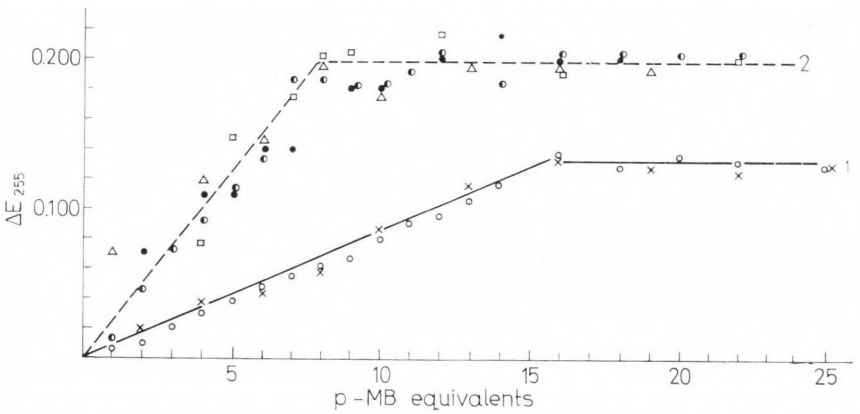

Fig. 10. Determination of the free SH-groups of GAPD. A solution containing about 300 μg/ml protein was incubated with various amounts of p-MB as indicated in the Figure, in 0.1 M glycine buffer, pH 8.5, at 0 °C for 1 hour. 1: native GAPD; 2: GAPD containing paramagnetic center. Since the absorbance of the paramagnetic center was great at 255 nm the differences in absorption were read partly after dilution with buffer and partly after gel-filtration on a 15×1.5 cm Sephadex G-50 column. The different symbols indicate independent experiments normalized to the same protein concentration

Since histidine is carbethoxylated in the reaction with DEP, i.e. a covalent bond is formed, it was probable that DEP abolished the paramagnetic center even if it was bound to histidine. Indeed, as a maximum 23 to 24 histidine residues could be detected with DEP on the surface of both the native enzyme and that containing the paramagnetic center.

Discussion

The study of the spectra of the complexes formed with the enzyme and with the two amino acids, respectively, confirmed the results of Vanin (1967) and Woolum et al. (1968) obtained with peptides. The enzyme GAPD studied by us is composed of four subunits; each of them contains 4 SH and 11 imidazol groups (Harris, Perham, 1968). Thus both ligands have to be considered as participants in the formation of the paramagnetic center. If both SH and imidazol groups are present, the signal of the former is predominant in the ESR spectrum.

The ESR signal of the amino acid complex was narrower in liquid medium than in the refrigerated state and showed a superfine structure. The enzyme complex did not show a superfine structure either in the liquid or in the frozen state and the width of the signal was the same in the two cases. The phenomenon indicates that the complex was formed on the protein molecule since the motion of the centers bound to the enzyme got "frozen". Therefore the signal was also broadened in liquid medium and the anisotropy of the g-tensor could be seen from the spectra (cf. Hedvig, Zentai, 1969). The appearance of an unresolved doublet in the signal of the cysteine complex led to the conclusion that the center was axially symmetrical, whereas the triplet signal of the histidine comples pointed to the existence of different g-values in all the three main directions (Chetverikov et al., 1969; Kneubühl, 1960; Woolum et al., 1968).

The signal obtained with compounds of small molecular weight (urea, guanidine-HCl, etc.) and with amino acids decreased in time, while the signal of the protein complex was more stable. The disappearance of the paramagnetic centers is assumed to be caused by recombination as a diamagnetic center is formed from two paramagnetic ones (McDonald et al., 1965). This process is inhibited in the case of centers bound to macromolecules, since the collision of such centers is rare. This phenomenon accounts for the fact that the histidine complex formed on the protein molecule is also stable. However, the gradual increase of the signal cannot be explained by the stability of the centers. According to our knowledge, there are no data in the literature about such an increase of the signal. One of the four SH-groups of a subunit of GAPD is especially reactive and two of them are located on the surface of the molecule, whereas the other two SH-groups are located inside the molecule (Racker, 1954; Friedrich, Szabolcsi, 1967; Boross et al., 1969). In the case of native GAPD only 6 out of the 11 histidyl groups of a subunit are found to be reactive (Ovádi et al., 1967; Ovádi, Keleti, 1969). Thus the groups which may participate in the formation of the paramagnetic center are partly buried inside the molecule. We assume that the increase of signals is brought about by the unmasking of buried groups due to the gradual unfolding of the enzyme molecule during the complex formation.

These were two ways to prove this hypothesis. The first possibility was to destroy the structure of the protein by denaturation and then to observe the formation of the complex by means of the ESR signal. We tried a number of denaturing agents but they could not be used if the complex was prepared according to Woolum et al. (1968), since paramagnetic complexes were also formed on the denaturing agents (guanidine hydrochloride, urea, dimethylformamide, dioxane) and thus the evaluation of the spectra was rendered impossible. In these solutions an intensive signal could be detected even in the absence of the proteins, rapidly decayed in time. In the presence of protein the ESR signal also disappeared rapidly and the complex was transformed into a diamagnetic form. Other denaturing agents (sodium dodecyl sulfate) could prevent the formation of the paramagnetic complex. Denaturation by heat was the only method that could be applied. In the first minutes the signal of the GAPD samples denatured by heat was indeed

greater than that of native GAPD, however, it did not change in time. On the other hand, the signal of the complex formed on the native protein decreased after heat treatment. This phenomenon was also observed by Vanin (1967) on lyophilized samples. Since the amplitude of the signal changes only slightly from 0 to 60 °C according to Curie's law, the decrease in the number of paramagnetic complexes was caused by a sudden conformational change of the protein. The complete disappearance of the histidine peak showed that this paramagnetic center was also labile on the protein as it completely disappeared on denaturation or lost its paramagnetic character.

The second possibility for the investigation of the role of the masked groups in the formation of the complex was to block the SH-groups with monoiodoacetic acid or silver ions or p-MB, while the imidazol groups with diethyl pyrocarbonate (DEP). With monoiodoacetic acid or 4 equivalents of Ag^+ only the four reactive SH-groups of the native GAPD molecule could be blocked (Racker, 1954; Boross, 1965), whereas p-MB could block all the SH-groups (Boyer, Segal, 1954). In fact, the treatment of the enzyme with monoiodoacetic acid or silver ion did not prevent the appearance of the cysteine signal, only decreased it by fifty per cent. The treatment with monoiodoacetic acid or with silver ions did not change the steric structure of the enzyme. In these cases four free, non-reactive SH-groups are on the surface of the enzyme beside the four blocked reactive ones.

Hence we interpret the fact that the cysteine-type signal slowly developed after the above treatment and attained only one half of the value obtained in the case of the native enzyme by assuming that the paramagnetic centers were formed only on the four accessible but non-reactive SH-groups. Consequently, the four reactive SH-groups of the native enzyme form complexes first, whereas the reaction of the other four free SH-groups is slow. The change in time of the amplitude of the signal is thus caused by the fact that the formation of the complex is much more rapid on the reactive SH-groups than on the non-reactive ones.

After treatment with 20 mole equivalents of p-MB no signal, or only that of the histidine complex could be detected. The mercaptidation of all SH-groups of the enzyme causes profound changes in the steric structure. In this case no more SH-groups can be unmasked. It seems probable that p-MB sterically hinders the histidine groups in the non-precipitated enzyme and they become accessible only after precipitation.

The blocking with DEP mainly inhibits the development of the signal in the beginning, but later the whole spectrum characteristic of the enzyme can be detected, though the amplitude of the signals is smaller. DEP can only block the imidazol groups located on the surface of the enzyme, but some of these are near the reactive SH-group hindering its accessibility (Ovádi, Keleti, 1969).

However, carbethoxylation changes the steric structure of the enzyme which is proved by the fact that both the SH-groups and some of the previously hindered histidine groups could react during incubation and formed paramagnetic centers.

The number of the SH-groups decreases during the formation of the com-

plex as detected by titration with p-MB. However, we failed to demonstrate the same for histidyl groups during this process by means of titration with DEP. DEP forms a covalent bond with histidine and thus the complexes are probably destroyed by the reagent. The fact that 8 out of the 16 SH-groups disappeared during the formation of the complex is in agreement with the results of carboxymethylation and of the treatment with silver ions. In both modification reactions only the free SH-groups of the enzyme seem to react. It should be noted that the number of SH-groups titrated after two hours does not provide full evidence. After such prolonged incubation the protein was precipitated during gel-filtration and the titration with p-MB could be performed in 8 M urea only. We could not decide whether more SH-groups did not react during the prolonged incubation or the paramagnetic center was removed from the SH-groups reacting later on the effect of denaturation, or on the effect of urea.

The mere finding that only the free SH-groups reacted does not exclude that the formation of the paramagnetic center causes a change in the steric or quaternary structure of the enzyme. The complete extinction of the excimer fluorescence of the enzyme on the effect of the paramagnetic center (Keleti, 1970) points to this phenomenon.

The results of the enzymological investigations are in agreement with the above statements. The formation of the paramagnetic center causes a competitive inhibition with the substrate only, as the substrate reacts during its oxidation, with the reactive SH-group. A mixed type inhibition was found with the coenzyme and with the phosphate ion, i.e. a competitive component was also involved. This phenomenon can be explained on the one hand by the fact that both substances are bound to the active center containing the reactive SH-group, thus steric hindrance may also occur. On the other hand the paramagnetic center can also be formed on histidine which could participate in the binding of phosphate ion. The fact that total inhibition of the enzyme not reached even after a long incubation can be probably explained by assuming that the paramagnetic centers were partly removed from the enzyme by the competitive GAP and by NAD and phosphate exhibiting a competitive component in experiments made in the presence of optimal substrate concentrations. However, it is also possible that the glycine buffer employed in the measurement of enzyme activity removed paramagnetic centers from the enzyme.

At any rate, these observations do not exclude the hypothesis of Woolum and Commoner (1970) according to which the center may have a regulative role in the activity of certain enzymes even under physiological conditions.

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Thin Layer Ion Exchange Chromatography on Resin-Coated Chromatoplates

III. Testing of Methionine and Lysine in Plant Seeds

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(Received January 15, 1971)

A simple screening method is described for testing methionine and lysine in plant seeds on resin-coated chromatoplates. The method is suitable for the examination of a great number of samples within a short time.

In plant selection – especially in the preliminary phase – the determination of lysine and methionine content of seed samples is a difficult task. The analysis of a great number of samples requires sophisticated equipment and considerable time. An analysis with the standard single-column analyser technique with three buffers lasts three and a half hours (Dévényi, 1969), while it can be performed in 75 minutes by means of rapid programming (Dévényi, 1971). However, even the latter method is very time consuming if the analysis of several thousand samples is required.

The thin layer chromatographic technique on resin-coated chromatoplates (Dévényi, Zoltán, 1970; Dévényi, 1970) offers a means for comparative investigations. By this method the lysine and methionine content of a great number of samples can be estimated under relatively simple experimental conditions and in a short time.

Materials and methods

Dowex 50×8 *type chromatoplates*

Chromatoplates of 20×20 cm in size were used in the Na^+ cycle (Dévényi, Zoltán, 1970). The plates were equilibrated with sodium citrate (pH 3.28, 0.02 N Na^+) according to the method previously published (Dévényi, 1970). In some of our experiments chromatoplates "IONEX-25" (Macherey, Nagel & Co., Düren, W. Germany) were used.

Composition of the eluting buffers is shown in Table 1.

Table 1

Eluting buffers

pH	Na^+ N	Citric acid monohydrate g	NaOH g	HCl 37% sp. gr. 1.19 ml	glycerol ml
3.28	0.2	14.1	8.0	12.3	100
5.28	0.35	24.6	14.0	6.5	100

Final volume is 1000 ml in deionized water

Fodder samples

The fodder samples tested were kindly supplied by Professor E. Kurnik (Research Institute of Forage Crops, Iregszemcse). Samples 28, 29, 30 and 32 were enriched fodders, samples 18, 21 and 25 were plant seed homogenates.

Hydrolysis

Hydrolysis of the samples was performed with 6 N hydrochloric acid in nitrogen atmosphere at 105 °C for 48 hours. The samples to be hydrolysed in 2 ml 6 N hydrochloric acid contained 8 mg protein. The hydrolysates were directly applied to the chromatoplates and thus decomposition during evaporation was excluded. For methionine 20 microliters of hydrolysate per 1 cm produced bands of suitable intensity whereas 10 microliters were sufficient for the test of lysine.

Development of the chromatoplates

The development of chromatoplates was performed as described earlier (Dévényi, 1970).

Quantitative determination of methionine and lysine

The rapid single-column procedure was employed (Dévényi, 1971).

Results and discussion

The optimal experimental conditions for the separation of methionine from other amino acids on chromatoplates are very similar to those applied in the amino acid analyser technique, since the chromatoplates are coated with Dowex 50×8 type resin. Optimal separation was obtained in sodium citrate buffer, pH 3.28, 0.2 N Na^+ at 50 °C. The separation was found to be incomplete at room temperature, similarly to column chromatography, whereas the evaporation and condensation of the conventional buffers prevented the formation of sharp bands at higher temperature. However, excellent separation and good reproducibility were achieved at higher temperature (50 °C) in the presence of additives, e.g. 10 to 20% glycerol, which lowers the tension of the buffer solution. The time required

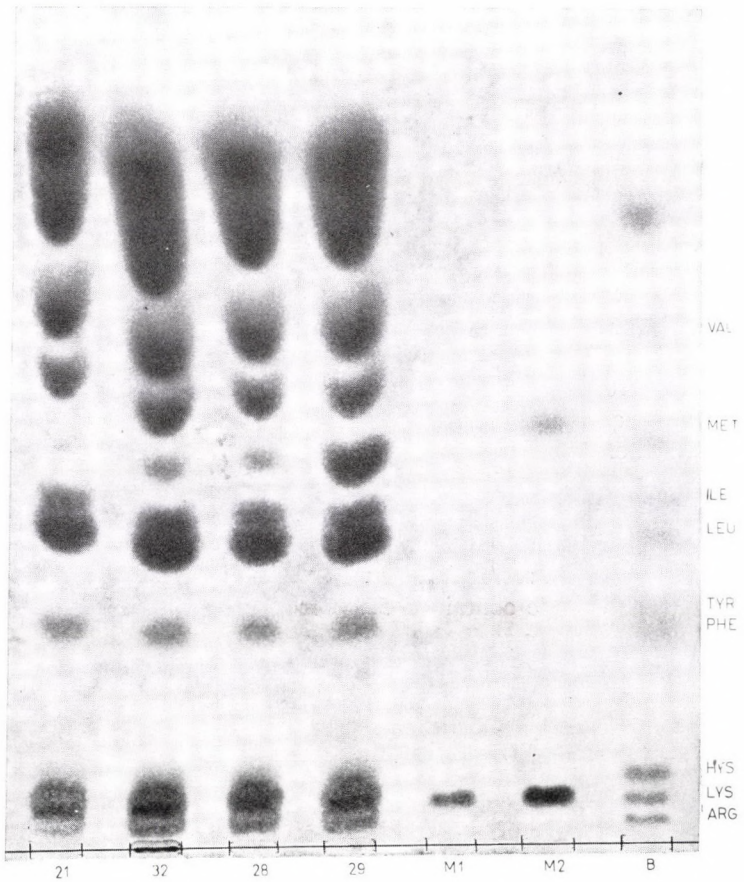

Fig. 1. Thin layer ion exchange chromatogram of acid hydrolysates of plant seeds and fodders at pH 3.28 (methionine-test). The experimental conditions are described in the text. M = LYS + MET, B = Amino acid calibration mixture. Sample numbers as referred to in Table 2

Table 2

Methionine and lysine content(+) of different seeds and fodders

Number	Source of protein in the fodder	Per cent amino acid in the protein	
		methionine	lysine
18	Vigna sinensis L.	0.5	6.7
21	Cicer ariethinum G—172	0.8	8.2
25	Helianthus annus L. VNIIMK 6540	0.5	3.8
28	Pisum sativum L.	2.6	7.4
29	Pisum sativum L.	5.5	7.6
30	Pisum sativum L.	0.5	9.1
32	Glycerine soja L. ISz7	2.6	5.7

(+) The quantitative analyses were carried out by the rapid single column procedure (Dévényi, 1971).

for satisfactory separation was about 5 hours. To ensure an adequate flow of the buffer, a filter paper strip was fixed to the upper edge of the layer by means of a rubber band, similarly to the equilibration of the chromatoplates.

Fig. 1 shows a typical chromatogram. It is seen that methionine is very sharply separated from both isoleucine and valine. The various seed sample hydrolysates contained equal amounts of protein (samples containing 0.08 mg of protein in 20 microliter solution were employed). This is apparent from the nearly identical intensity of the leucine and tyrosine + phenylalanine bands. However, the intensities of the bands corresponding to methionine are markedly different. Sample 21 contains a very small amount of methionine (corresponding to 0.8% methionine in the protein, see Table 2), while in sample 29 the methionine band is very intensive (this sample contained 5.5% methionine). Thus, the intensity of the methionine bands offers preliminary information of the amount of this amino acid in the sample. Samples of equal protein content can be classified as "low", "medium" or "high" in respect of their methionine content. This technique could be of use in the preliminary selection of a great number of seed samples. After this selection, the samples of special importance could be quantitatively analysed.

A similar selection can be made on the basis of lysine content of the samples. Fig 2 shows a chromatogram representing various plant seeds differing in their lysine content. The protein content of samples was the same (samples containing 0.04 mg protein in 10 microliter solution were employed). The buffer used in this case was sodium citrate, pH 5.28, containing 10% glycerol. The temperature was 50 °C. It can be seen that there is a marked difference in the intensities of the lysine bands corresponding to 3.8, 6.7 and 9.1% lysine content, respectively (see Table

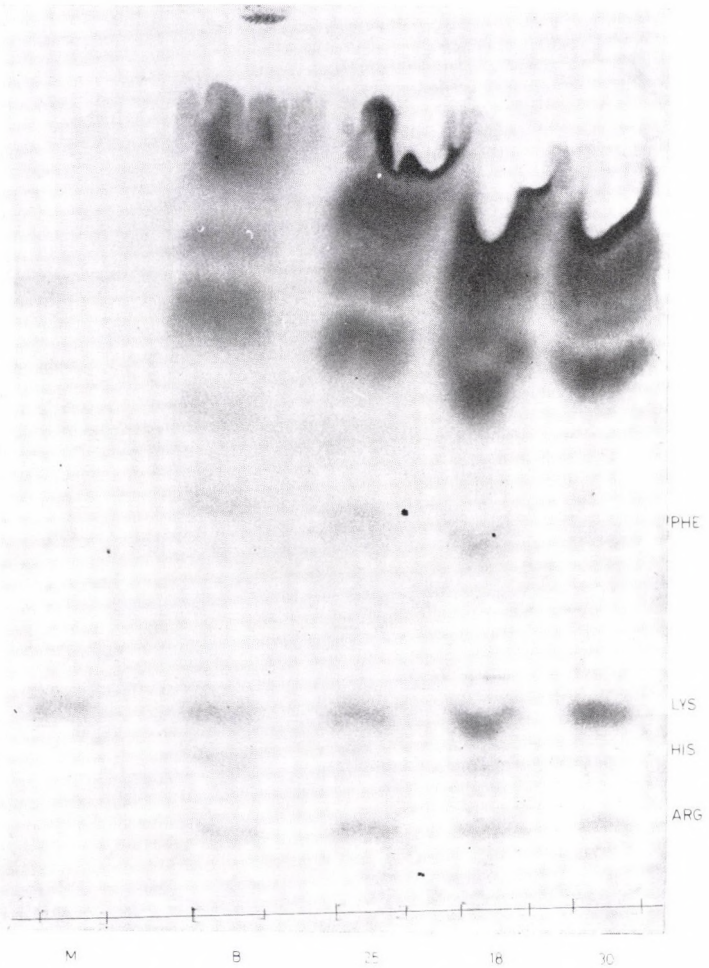

Fig. 2. Thin layer ion exchange chromatogram of acid hydrolysates of seeds and fodders at pH 5.23 (lysine-test). The experimental conditions are described in the text. M = LYS + MET, B = Amino acid calibration mixture. Sample numbers as referred to in Table 2

2). The time required for the separation was about 3 hours. The method used was the same as for methionine. Since the separation of basic amino acids is very sharp, the chromatoplates can be overloaded in the case of very low lysine content (e.g. corn, maize, etc.).

Because of its simplicity the above method appears to be suitable and useful in the selection of plant seeds in the routine characterization of feedstuffs etc., followed by quantitative determination if necessary.

The authors wish to thank Professor Bruno F. Straub for his advice, help and interest. We are further indebted to Professor E. Kurnik and Mrs Szánthó (Research Institute of Forage Crops) for valuable discussions.

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Amino Acid Analyzer Programming for the Rapid Determination of Methionine and Lysine

(Short Communication)

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(Received January 15, 1971)

The time required for a complete analysis and regeneration is three and a half hours on a standard, automatic amino acid analyzer by a single-column procedure with three buffers (Dévényi, 1969). The analysis can be performed in less time by employing a special equipment (Ertinghausen et al., 1969). However, a complete amino acid analysis is not required in many instances. In several cases it is sufficient to determine some specific components only. This is the case in the field of plant selection, in food and fodder chemistry, etc. In the last cases the determination of methionine and lysine is of utmost importance. The time required for these analyses can be considerably shortened by using shorter columns and an adequate programming.

In our experiments a BioCal Type BC 200 amino acid analyzer was employed. The filling was Chromex UA8 spherical resin (Reanal, Budapest). The reagents were produced by Reanal, except ninhydrine and methyl cellosolve, which were both Merck products. The experimental conditions and the programming of the analyzer are summarized in Table 1.

Table 1

Experimental conditions and programming for the determination of methionine and lysine

Column height	22 cm
Filling height	14 cm
Flow rate of buffer	100 ml/hour
Flow rate of ninhydrine	50 ml/hour
Temperature	50 °C
Buffer A	sodium citrate, 0.2 N Na^+, pH 3.28
Buffer B	sodium citrate, 0.8 N Na^+, pH 4.25
Buffer back pressure	10 to 12 atm
Programming	0 to 25 min. Buffer A
	25 to 62 min. Buffer B
	62 to 65 min. 0.2 N NaOH
	65 to 75 min. Buffer A

A
B
MET
LYS
VAL
ILE
NH3
HIS
0.5
0.4
0.3
0.2
0.1
0

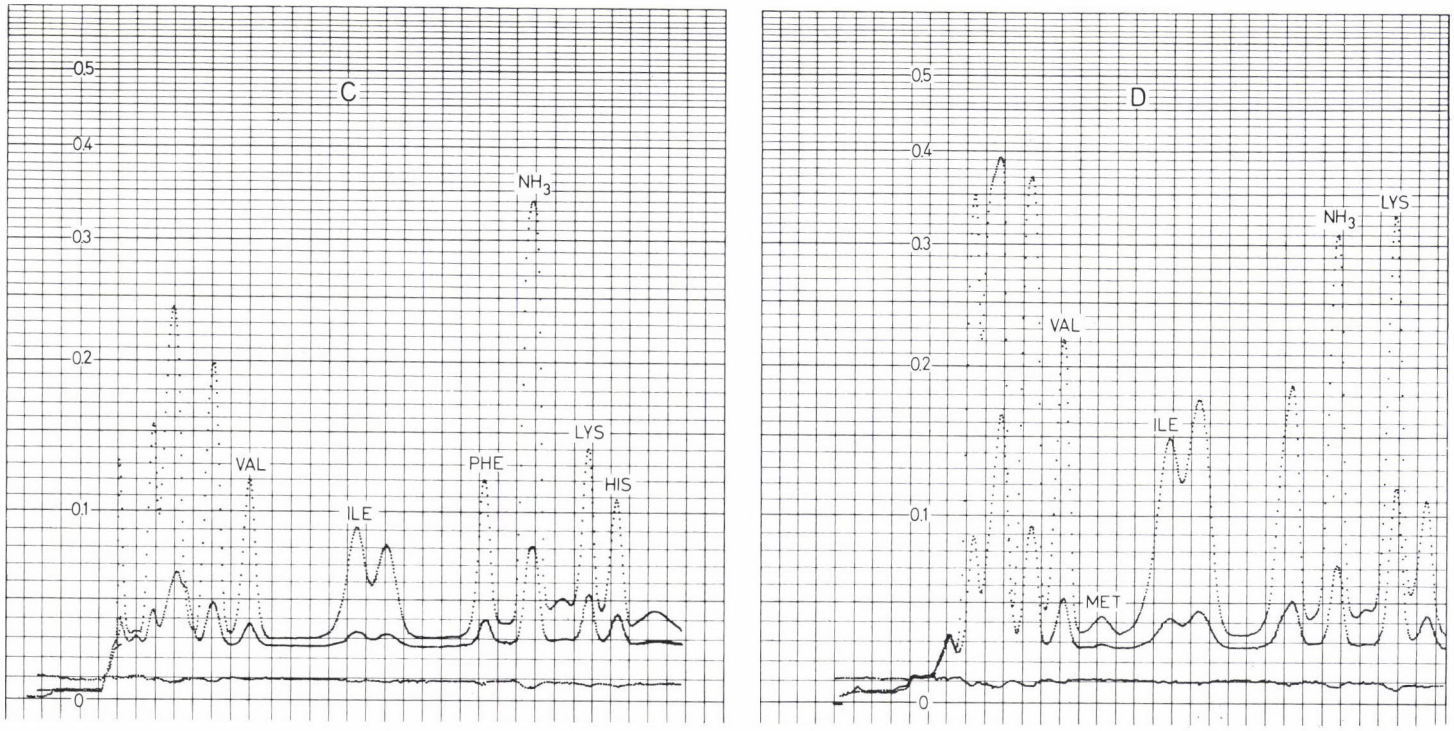

Fig. 1. Rapid determination of methionine and lysine with automatic amino acid analyzer. *A:* calibration mixture (0.125 μmole of all amino acids but lysine and methionine, 0.5 μmole of lysine and methionine), *B:* hydrolysate of peas enriched in methionine, *C:* calibration mixture after performic acid oxidation (it is apparent that tyrosine was also decomposed during the oxidation of methionine), *D:* hydrolysate of peas of low methionine content

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The chromatogram represented in Fig. 1A shows a calibration mixture analyzed by the aid of the new programming.

The amount of each amino acid was 0.125 μmole except that of methionine and lysine. These two were used in a fourfold amount (0.5 μmole) to secure a better localization of them. It is apparent on the chromatogram that methionine can be sharply separated from valine and isoleucine, while lysine is well separated from histidine and ammonia. The chromatogram represented in Fig. 1C was obtained with a calibration mixture oxidized with performic acid and thus freed from methionine. Tyrosine was also decomposed during oxidation. The fact that this change is also detectable on the chromatogram is characteristic of the excellent resolution of the method. The shortening of the column leaves the first section of the acidic and neutral amino acids (Asp, Thr, Ser, Glu, Pro, Gly, Ala and Cys) unresolved. This phenomenon is, however, indifferent as far as the determination of methionine and lysine is concerned, moreover it is advantageous since at this expense the time required for the analysis can be shortened.

The chromatograms in Fig. 1B and Fig. 1D represent the acidic hydrolysates of peas enriched in methionine and of peas of low methionine content, respectively.

The above results prove that the determination of methionine and lysine content of a given sample can be performed in 75 minutes on a standard analyzer, with a reproducibility and accuracy characteristic of the instrument, by shortening the resin filling to 14 cm, employing an adequate programming with two buffers and a regeneration with sodium hydroxide after the experiment.

The author is indebted to Mrs J. Báti for the invaluable assistance in performing the experiments.

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Detection and Determination of Tryptophan by Ion Exchange Chromatography

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A thin layer ion exchange chromatographic method has been developed for the rapid determination of tryptophan content in alkaline hydrolysates of peptides and proteins. For the quantitative analysis of hydrolysates a rapid programming of standard amino acid analyzers is presented.

For the determination of tryptophan content of peptides and proteins almost exclusively spectrophotometric methods are available (Holiday, 1936; Goodwin, Morton, 1946; Bencze, Schmidt, 1957; Edelhoch, 1967). However, these methods have several drawbacks. First, these determinations are influenced by all substances that absorb in the wavelength region in question, and such substances (e.g. nucleotides, a coenzyme) may accompany proteins. Secondly, the spectrophotometric methods are unsuitable to the study of substances of plant origin (seeds, leaves, seedlings, etc.) Thirdly, in amino acid sequence analysis the amount of available material is mostly not enough for the spectrophotometric assay. Apart from all these, there is no such method which would ensure, in a uniform manner with all proteins, the unfolding, which is indispensable for the spectrophotometric analysis.

As it is known, tryptophan is destroyed during acid hydrolysis. Under appropriate conditions it is stable on alkaline hydrolysis. However, the analysis of alkaline hydrolysates by the traditional chromatographic procedures (paper and thin-layer chromato graphy, classical analyzer technique) can be performed only after desalting, wh ich means time-consuming preparational work.

This obstacle can be circumvented in qualitative and semi-quantitative analyses, with thin-layer ion exchange chromatography (Dévényi, Zoltán, 1970; Dévényi, 1970; Ferenczi et al., 1971) or in case of quantitative analyses by an appropriately adopted amino acid analyzer technique by the use of buffers of high molarity.

Materials and methods

Dowex 50×8 *type chromatoplates*

Chromatoplates, 10×10 cm in size, were used (Dévényi, Zoltán, 1970) in Na^+-cycle, without previous equilibration.

Amino acid analyzer

BioCal BC 200 type analyzer was used with Chromex UA8 spheric resin (Reanal, Budapest).

Chemicals

Ninhydrin and methyl cellosolve were Merck preparations, all other chemicals were produced by Reanal (Budapest). The authors are indebted to Dr. P. Előd for the kind supply of N-acetyl tryptophan and glycyl-tryptophan.

Amylase

Hog pancreas amylase was prepared according to the method of Hatfaludi et al. (1966). The preparations were recrystallized three times.

Alkaline hydrolysis

This was performed mainly as described by Kuiken et al. (1947), in the following way: 5 mg of peptide or protein were dissolved in 2 ml of 4 N NaOH, flushed with nitrogen, then after sealing the tubes were kept at 105 °C for 5 hours. After cooling, 3 ml of 0.2 N sodium citrate buffer, pH 3.28, and 1 ml of 37% HCl were added to the hydrolysate. An aliquot of the hydrolysates acidified in this way was applied *directly* to the chromatoplates or the analyzer.

In quantitative determinations the analyzer was calibrated with tryptophan treated in the above manner (cf. Table 1).

Buffer

Both to thin layer ion exchange chromatography and analyzer technique 0.1 N sodium citrate, pH 6, buffer containing 1.4 M NaCl and 10% methyl cellosolve was used.

Results and discussion

On Dowex 50 × 8 type chromatoplates, in Na^+-cycle, developed with 0.1 N sodium citrate buffer, pH 6, containing 1.4 M NaCl and 10% methyl cellosolve tryptophan is the amino acid that shows the least mobility. As it is clearly seen on the chromatogram in Fig. 1A, already on a 10 × 10 cm chromatoplate, with about 8 cm running distance, it can be readily separated from the basic amino acids, which lie nearest to tryptophan on the basis of their relative Rf-values. The acidified alkaline hydrolysate (cf. Methods) can be directly applied to the chromatoplates; the salt introduced this way does not interfere with the anal-

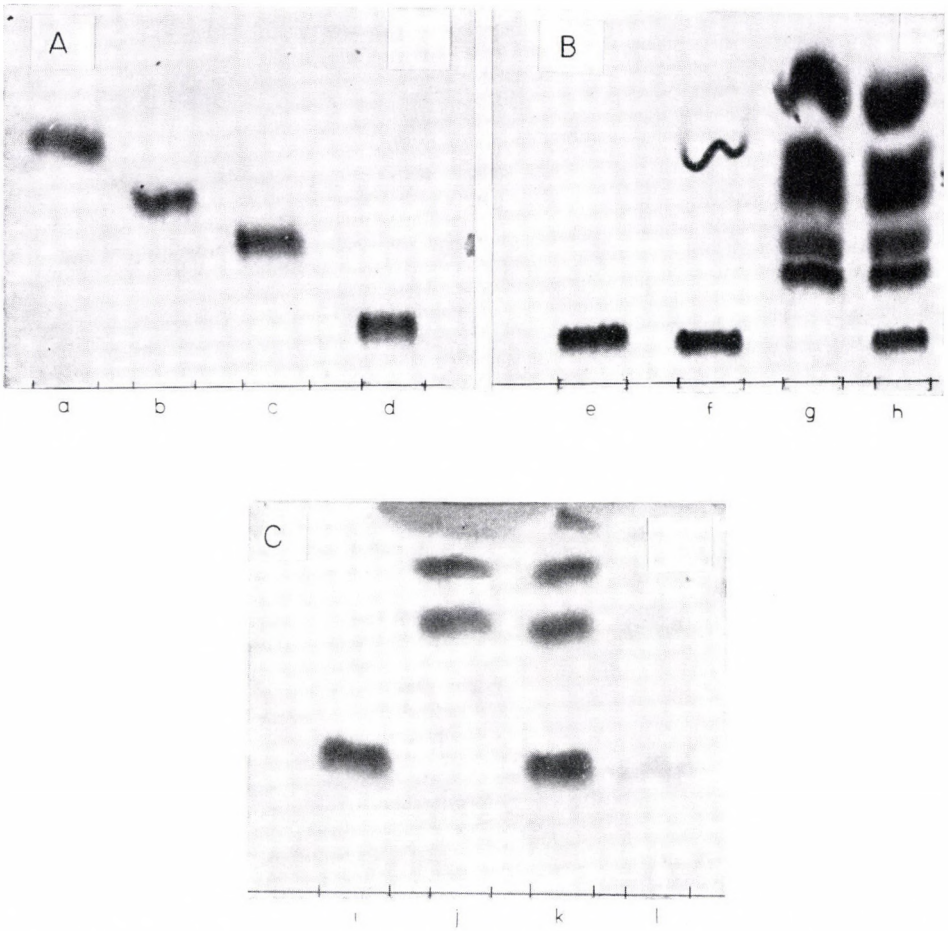

Fig. 1. Detection of tryptophan on Dowex 50×8 type resin-coated chromatoplate. 1A: a = LYS b = HIS, c = ARG, d = TRP; 1B: e = hydrolysate of N-acetyl tryptophan, f = hydrolysate of GLY—TRP, g = TRP-free calibration mixture, h = calibration mixture containing TRP; 1C: i = alkali-treated TRP, j = TRP-free calibration mixture, k = calibration mixture containing TRP, l = alkaline hydrolysate of 30 μg of amylase. For experimental conditions see text

ysis from the point of view of tryptophan. Chromatogram 1B shows the alkaline hydrolysates of N-acetyl tryptophan and glycyl tryptophan. In the latter case it is conspicuous that the salt front migrates with amino acids of high R_f-value, thus it does not disturb the detection of tryptophan, which exhibits low R_f-value. The duration of run was 20 to 25 min.

If the material to be analyzed contains only a small amount of tryptophan and therefore the chromatoplate has to be heavily loaded with the alkaline hydrolysate, it is advisable to fix a filter paper wick to the upper edge of the chromato-

Table 1

Programming of analyzer for the determination of tryptophan

Column height	22 cm
Filling height	14 cm
Flow-rate of buffer	100 ml per hour
Flow-rate of ninhydrin	50 ml per hour
Temperature	50 °C
Buffer back-pressure	8—12 atm
Buffer	0.1 N sodium citrate pH 6, containing 1.4 M sodium chloride and 10% methyl cellosolve
Integration constant of tryptophan	10.25
Integration constant of NaOH-treated tryptophan	9.34

plate by means of a rubber band and to turn the major part of the wick to the other side of the chromatoplate. Chromatography is then continued until the front migrated 3—4 cm into the wick (at room temperature about 90 min). In this way the interfering effect of the large amount of salt and other amino acids can be excluded (cf. Fig. 1C).

It follows from the nature of the method that it can be readily applied in sequence analysis, in the rapid testing of plant materials, and in several other similar fields. In contrast to the traditional possibilities of chromatography it renders possible the direct analysis of acidified alkaline hydrolysates containing much salt.

We elaborated a rapid analyzer programme for quantitative determinations. The experimental conditions are summarized in Table 1.

Under the adopted circumstances tryptophan is eluted after the elution of all amino acids, as a homogeneous peak (Fig. 2). On the 14 cm column the amino acids of the standard calibration mixture are eluted in 16 min by the pH 6 buffer of high molarity (Fig. 2A). The tryptophan (1 μmole) treated as in alkaline hydrolysis and then added to the calibration mixture is sharply separated from the other amino acids (Fig. 2B). The overall time requirement of the analysis is only 25 min.

The applicability of the method was tested by the analysis of hog pancreas amylase. Table 2 summarizes the data of four independent measurements.

Since less than 10% of tryptophan is degraded under the condition of hydrolysis (cf. Table 1), calculations were based on the integration constant of alkali-treated tryptophan. As it can be seen in Table 2, as calculated for a molecular weight of 53 000, 17.1 moles of tryptophan were found per mole of hog pancreas amylase. This value is in excellent agreement with that of Elődi and Krysteva (1970), obtained by spectrophotometric measurement.

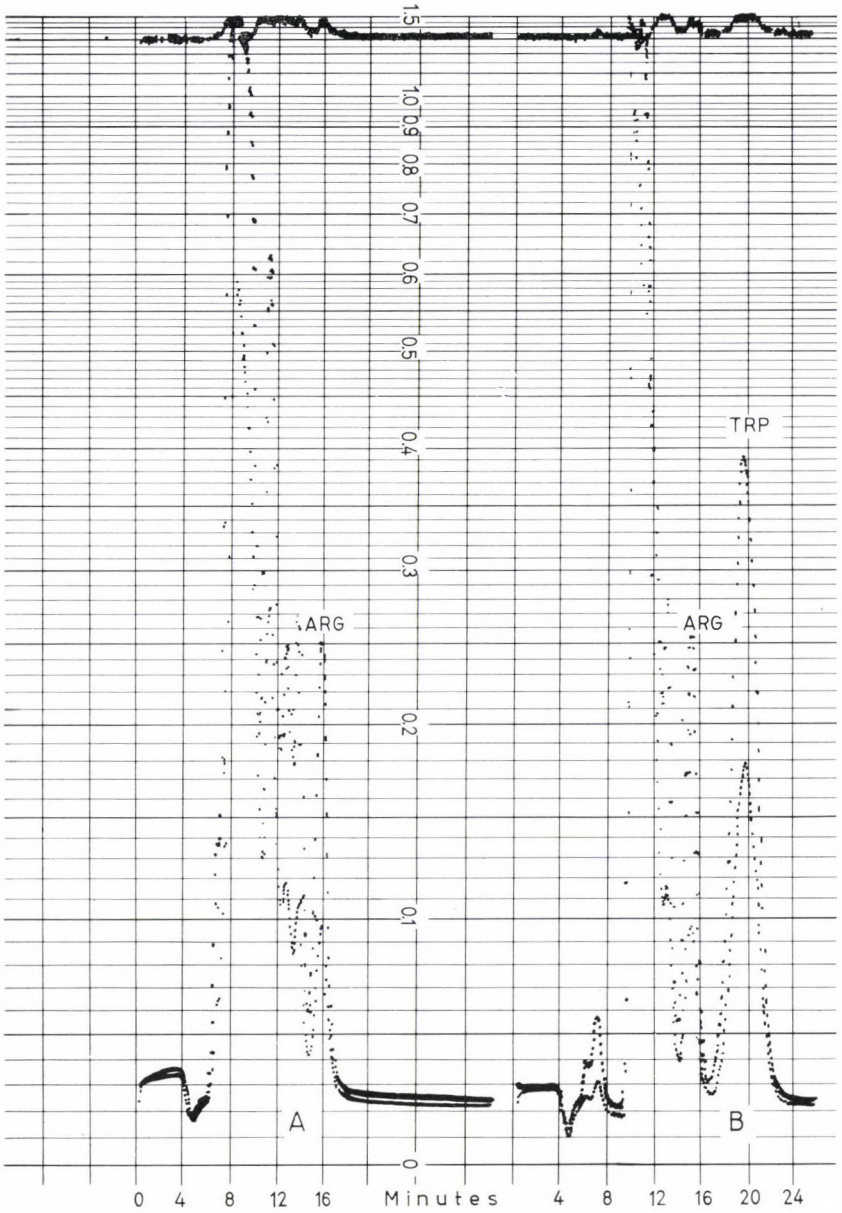

Fig. 2. Quantitative determination of tryptophan with automatic amino acid analyzer. 2A = TRP-free calibration mixture; 2B = calibration mixture containing 1 μmole of TRP. For experimental conditions see Table 1

The data in Table 2 suggest that in case of a purified protein the simple, alkaline hydrolysis used by us can be successfully applied with the aid of an alkali-treated calibration mixture.

Table 2

Determination of tryptophan content of hog pancreas amylase from an alkaline hydrolysate with amino acid analyzer

Four different samples of amylase were hydrolyzed and analyzed.

Amount of amylase analyzed, mg	Measured tryptophan, μmoles	Residue/mole (M. W. 53 000)
0.80	0.26	17.2
0.64	0.21	17.4
0.72	0.23	16.9
0.72	0.23	16.9
	Average:	17.1

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A Reliable Index of Tissue Metabolic Activity during the Initial Phase of Rat Liver Regeneration

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The chemical composition, oxygen consumption and anaerobic glycolytic potential of regenerating liver tissues were investigated following partial hepatectomy.

It was shown that liver regeneration was accompanied by profound changes in the chemical composition of the tissue, characterized by higher total lipids and lower protein and glycogen content. The net effect of these alterations was a significant increase in dry matter content of the regenerating liver tissue. The changes were most pronounced during the initial phase of regeneration.

Since there is a change in dry matter composition the conventional Q-value (metabolic activity/mg dry matter/hour) is unsuited to serve as an index of tissue metabolic activity during liver regeneration. It is proposed that specific metabolic activity be used as an index of tissue metabolic performance during regeneration.

The significant deviations in the numerical values of tissue metabolic activity, depending on whether calculated as Q-value or as specific metabolic activity, are presented.

Since Warburg introduced the Q-value into biochemical terminology – defined as metabolic activity /mg dry weight/ hour (Warburg, 1924) – the use of this term has been generally accepted. Accordingly, Q-values served most frequently as an index of metabolic performance also in studies on the metabolic characteristics of regenerating liver; e.g. both oxygen consumption and glycolytic potential of the regenerating liver tissue have been repeteadly expressed in terms of Q-values (Perkinson, Irving, 1956; Kidd et al., 1944; Noll, 1963).

However, the chemical composition of liver and so the composition of its dry material are also markedly changed during regeneration (Brues et al., 1936; Harkness, 1952). This change is especially pronounced during the initial phase of the process. Under such circumstances the reliability of Q-values as an index of metabolic activity could be seriously questioned.

This paper is to show that "specific metabolic activity" is a more relevant index of tissue metabolism during regeneration than the conventional Q-values.

Methods

Rats were used throughout the experiments. Two-third partial hepatectomy was performed according to Higgins and Anderson (1931). Groups of 10–12 animals were killed by decapitation and the livers analysed at 6-hour intervals following the partial surgical removal of the organ.

Dry weight was determined by drying a small piece of the liver – usually about 150 mg – at 90 °C overnight.

Protein content was determined by the micro-Kjeldahl method, by assuming 15.1 per cent nitrogen (Smith, Stockell, 1954).

Total lipid was estimated by weighing the residue left after the evaporation of a repeated chloroform: ether extract according to the method of Sperry (1954).

Glycogen was determined with anthrone reagent according to Bloom et al. (1951).

Oxygen consumption of liver slices was measured manometrically. Slices were prepared free hand, blotted to remove excess moisture, and weighed. The slices were transferred into the main compartment of a Warburg vessel that contained 3 ml of oxygenated Krebs – Ringer-phosphate buffer, pH 7.4 (Umbreit et al., 1959). The buffer contained 0.01 M glucose. The central well contained 0.2 ml of 30% KOH and a roll of filter paper. The gas phase was replaced with O_2 and the flasks were equilibrated in a water bath (37.5 °C) for 10 minutes. Readings on the manometers were taken at 10 minute intervals for 1 hour.

Anaerobic glycolytic potential of liver slices was also determined manometrically. Slices of 150 – 200 mg fresh weight were transferred into manometric vessels immersed into cracked ice and containing 3 ml of a Krebs – Ringer-0.25% $NaHCO_3$ medium with 0.01 M glucose and 0.02% of sodium pyruvate. Pyruvate was added to the medium to prevent the inhibition of glycolysis that would have inevitably occurred due to the accumulation of $NADH_2$ (Woods et al., 1963). Both the medium and the manometer vessels were flushed with a mixture of 95% nitrogen and 5% CO_2 before starting incubation. The temperature of the bath was kept constant at 37.5 °C throughout the experiments.

The results were analysed statistically by the Student "t"-test.

Results

The results showed that the chemical composition of liver changed substantially during the initial phase of regeneration induced by partial ablation of the liver (Fig. 1).

The dry matter content of the tissue considerably increased in the 12 hours following surgery, and remained high during the subsequent 12 hours. Normalization occurred after 40 – 42 hours only.

The change in protein content followed a different course. The first 24-hour period of regeneration was characterized by lowered protein concentration. Then normalization commenced and became complete after about 30 hours postoperatively.

The most pronounced change occurred in the lipid content of the tissue; approximately a 4-fold increase could be demonstrated by the 12th postoperative hour. This elevated level persisted during the subsequent 12-hour period. This was then followed by a slow diminution, bringing the values down to the normal range around the 48th hour.

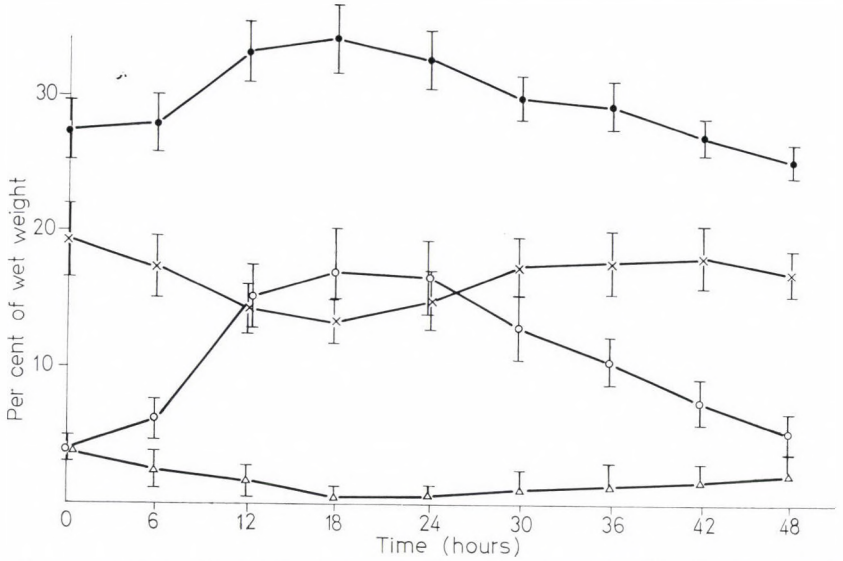

Fig. 1. Percentual change in the chemical composition of regenerating liver tissue as calculated on a wet weight basis. The points represent the average of 10—12 measurements. S. D. values are represented by vertical lines. ● —— ●, dry matter; × —— ×, protein; ○ —— ○, total lipid; △ —— △, glycogen

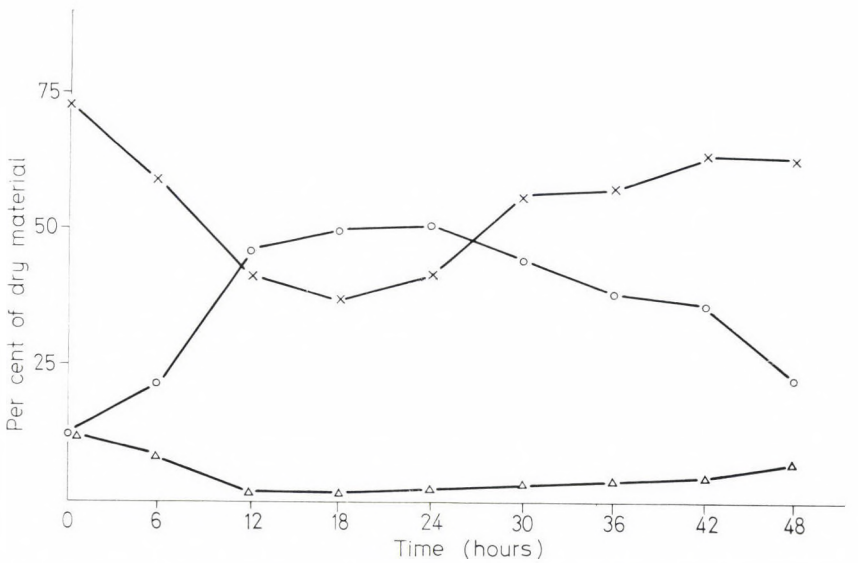

Fig. 2. Change in the composition of dry material of regenerating liver tissue. × —— ×, protein; ○ —— ○, total lipid; △ —— △, glycogen

Glycogen was diminished almost to zero after 18 hours. Although a moderate rise was characteristic of the subsequent period, the values remained below normal throughout the whole period of observation.

The relative changes in the composition of dry material are indicated in Fig. 2.

Wheras protein constituted approximately 70% of the dry material under normal circumstances, its proportion diminished to about 40% by the 18th hour following partial hepatectomy.

Lipids accounted for about 15% of the dry material in the normal liver but became the main constituent in the regenerating liver. As early as 18 hours following surgery, lipids amounted to about 50% of the dry matter. The quick elevation of this constituent was followed by a similarly quick period of diminution.

Glycogen represented about 15% of the dry material in the intact liver. This constituent disappeared almost entirely from the liver by the 18th hour and remained low throughout the entire period of the examination.

Owing to the drastic changes occurring in the composition of dry matter indicated above, dry weight appeared unsuited to be used as a basis for the calculation of metabolic performance in regenerating liver tissue.

Both oxygen consumption and glycolysis are processes maintained by serial enzymic reactions. Thus, as an analogy to "specific enzymic activity", an expression commonly used in the biochemical literature, the introduction of the terms "specific oxygen consumption" and "specific glycolytic activity" might be useful. We propose these terms instead of the conventional Q-values to express the metabolic activity of regenerating liver. What is new in these terms is that the metabolic performance of regenerating liver is related to a protein basis instead of the dry weight.

Comparative data obtained by applying both calculations are indicated in Table 1.

As it is seen, not only the absolute values but also the extent of changes differed markedly depending on the method of calculation. For example, conventional Q-values indicated 61 and 53% decrease in oxygen consumption at the 12th and 36th hour, respectively. The corresponding values, calculated on a protein basis were 36 and 44% only. Practically no change could be detected in the oxygen consumption in terms of Q-values – either at the 24th or 48th hour. On the contrary, 80 and 20% increase was revealed, respectively, by the use of protein reference.

The values of glycolytic activity differed even more profoundly. While Q-values suggested a moderate (4 and 14%) decrease in the glycolytic potential during the first two periods of observation, a substantial (61 and 38%) increase was detected on the protein basis. Similarly, the 112 and 20% increases indicated by the Q-values for the last two periods of the observation, respectively, corresponded to 160 and 30% increase when calculated on a protein basis.

Table 1

O_2-consumption and glycolytic potential of regenerating liver tissue expressed in terms of Q-value (QO_2 and $Q^{N}_{C_2}O_2$) or specific metabolic activity ($QO_2/p.$ and $Q^{N}_{C_2}O_2/p.$)

Time after partial hepatectomy, hours	Q_{O_2} ± S. D.	% change compared to the normal	$Q_{O_2}(p)$* ± S. D.	% change compared to the normal	N_2 Q_{CO_2} ± S. D.	% change compared to the normal	N_2 $Q_{CO_2}(p)$* ± S. D.	% change compared to the normal
0 (normal)	10.9 ±1.7	—	15.2 ±2.2	—	26.0 ±2.9	—	36.2 ±4.1	—
12	4.2 ±0.06	−41	9.8 ±1.4	−36	24.7 ±3.0	−5	58.0 ±7.3	+60
24	12.8 ±1.6	+17	27.7 ±3.4	+82	22.5 ±2.8	−13	50.2 ±6.1	+38
36	5.1 ±0.07	−53	8.6 ±1.1	−44	55.5 ±6.7	+112	94.2 ±10.1	+160
48	11.9 ±1.6	+9	18.2 ±2.7	+19	30.8 ±3.9	+18	46.7 ±5.4	+29

* (p) = values calculated on a protein basis

Discussion

Metabolic activities of tissues of similar chemical composition can be reliably compared in terms of conventional Q-values. This condition holds in every case where the functional capacity of a tissue is changed without alteration in the chemical composition. However, such comparisons may be misleading if the chemical composition of the tissues also changes to a considerable extent. It is obvious that numerically identical metabolic activities measured in two separate tissues may represent markedly different metabolic activities, if the concentrations of the cellular constituents responsible for the metabolic process in question differ in the two tissues. This is exactly the case when the metabolic performances of intact and regenerating liver tissues are to be compared in the early phase of regeneration. It is well known that the chemical composition of liver changes during regeneration following a partial two-third hepatectomy (Brues et al., 1936; Gurd et al., 1948; Harkness, 1952).

Thus there are two alternative routes that can be followed, theoretically at least, when the metabolic performances of intact and regenerating livers are to be reliably compared. One possibility is to find a chemical constituent the concentration of which is kept constant throughout the regeneration process. This, however, may hardly be successful since almost all tissue components are mobilized to sat-

isfy the requirements of the extraordinary active proliferation during the initial phase of regeneration in this tissue.

Consequently, we propose the other route to follow: to introduce the term "specific metabolic activity". This term is essentially identical with the conventional Q-value with the exception that protein content instead of dry weight, is used as a reference in the calculations.

It is true, of course, that protein concentration also changes during regeneration. However, it appears reasonable to assume that metabolic activity follows more closely the alterations of protein concentration than that of dry material. As it has been shown the composition of dry matter undergoes pronounced alterations during the initial phase of regeneration; it mainly consists of enzymatically inert substances such as neutral lipid, while there is a simultaneous decrease in the enzymatically active proteins.

Beside technical errors committed in the majority of measurements of glycolytic potential of the regenerating liver (cf. Rosenthal, 1932; Noll, 1963; Burk et al., 1967) the disregard of changes occurring in the chemical composition of the regenerating liver and, in turn, the uncritical use of Q-values as an index of the metabolic activity, may have provided the basis for claims according to which the regeneration of liver is a process of proliferation with unaltered glycolytic potential (Norris et al., 1942; Burk, 1942).

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Urea Production by Rat Liver Slices at Various Urea Concentrations in the Medium

(Short Communication)

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Clinical observations indicated that the blood urea level followed a saturation-type curve in time in uraemic patients (Menyhárt, unpublished data). It has also been shown that B_6-vitamin deficiency provoked experimentally in rats was regularly accompanied by a substantial rise in blood urea level and the urea production of liver slices taken from such animals was depressed to a significant extent (Caldwell, McHenry, 1954).

In the light of these data it appeared to us as if urea concentration in the medium that surrounds liver cells would influence the urea production capacity of the liver tissue. The experiments presented in this paper were aimed at testing this hypothesis.

Male and female albino rats, 180 to 230 g in weight were used.

Urea production was measured in liver slices incubated in a medium completed with various concentrations of urea. The preparation, pre-treatment and incubation of slices were performed according to Krebs and Henseleit (1932). The pre-treatment of slices included the washing out of preformed urea.

Urea was determined manometrically by measuring CO_2 liberated by urease treatment. Urease (Fluka, Switzerland) was allowed to act under acidic conditions (in acetate buffer, pH 5.0). Due to the relatively large amount of urea added to the medium, the mixture had to be diluted tenfold prior to manometric measurements. Double blanks were run simultaneously, with a solution containing all of the constituents except the slices. Values of blank readings were subtracted from those obtained with the medium containing the slices. This was then expressed as Q_{urea} (μl urea-CO_2 liberated/mg dry weight/hour).

The dry weight of the specimens was determined by keeping a small piece of liver, usually around 150 mg, at 105 °C overnight.

Data, if required, were statistically analyzed by Student's "t"-test.

Table 1 demonstrates the results of a typical experiment. These data were collected under conditions when slices were incubated without urea (column 1), with 3.3 mM urea (column 2) and with 33.0 mM urea (column 5). Manometer readings were made on a tenfold diluted medium. As the wet weight of slices was nearly identical, the urea production of the slices (columns 1, 4 and 7) can be di-

Table 1

Liberation of urea-CO_2 from different media

Urea—CO_2 liberated from the medium containing									
1		2	3		4	5	6		7
slices only		3.3 mM urea, no slices CO_2 µl	slices + 3.3 mM urea		difference between 3 and 2 µl (3-2)	33.0 mM urea, no slices CO_2 µl	slices + 33.0 mM urea		difference between 6 and 5 µl (6-5)
wet wt mg	CO_2 µl		wet wt. mg	CO_2 µl			wet wt. mg	CO_2 µl	
201	24.3	17.1	198	26.5	9.4	173.7	199	174.8	1.1

Table 2

Q_{urea}-values as a function of urea concentration in the medium

mM urea in the medium	0	3.3	6.6	13.2	19.8	26.4	33.0
Number of columns	1.	2.	3.	4.	5.	6.	7.
	1.90	1.20	1.03	0.36	—	—	—
	1.56	0.98	0.35	0.28	—	—	—
	1.70	0.92	0.42	0.52	—	—	—
	1.90	1.20	0.50	0.28	—	—	—
	1.66	1.18	0.33	0.32	—	—	—
	1.90	1.24	0.82	0.50	—	—	—
	1.98	—	0.88	0.52	—	—	—
	2.00	—	—	—	0.30	0.20	—
	2.10	—	—	—	0.16	0.16	—
	2.02	—	—	—	0.32	0.18	—
	2.80	—	—	—	0.12	0.16	—
	2.04	—	—	—	0.16	0.12	—
	2.10	—	—	—	0.30	0.12	—
	2.04	—	—	—	0.18	0.02	—
	1.86	—	—	—	0.28	—	—
	2.00	—	—	—	0.34	—	0.07
	2.04	—	—	—	—	—	0.04
	2.40	—	—	—	—	—	0.00
	1.80	—	—	—	—	—	0.06
	1.30	—	—	—	—	—	0.00
	1.80	—	—	—	—	—	0.03
	1.50	—	—	—	—	—	0.00
$n =$	22	6	7	7	9	8	7
$\lambda =$	1.927	1.20	0.618	0.397	0.240	0.120	0.028
p_{1-4}	0.005						
p_{4-7}	0.005						

rectly compared. As it is seen, urea production of slices incubated without urea amounted to 24.3 μl of urea-CO_2, whereas 9.1 and 1.1 μl were produced by slices incubated in the presence of 3.3 and 33.0 mM urea, respectively.

The Q_{urea} values as a function of urea concentration of the medium are indicated in Table 2. By increasing the urea concentration of the incubation medium the urea production of slices decreased. At high urea concentrations urea production was greatly inhibited or even entirely abolished. This is indicated by the extremely low Q_{urea} values and by a rising frequency of zero urea production, as seen in the last two columns of Table 2.

These data indicate that the urea concentration prevailing in the medium exerts a regulatory effect on the rate of urea production by liver slices. In vivo experiments also support the inhibitory effect of urea on the urea cycle (Menyhárt, unpublished data).

The physiological significance, and exact nature of the observed effect of urea are under investigation in our laboratory.

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Informofer-like Protein in Polyribosomes

(Preliminary Communication)

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It has been shown that in eukaryotic cells the mRNA of polyribosomes is in association with protein as ribonucleoprotein. EDTA treatment of polyribosomes results in the separation of this mRNP from the ribosomes (Perry, Kelly, 1968; Henshaw, 1968; Cartouzou et al., 1969; Burny et al., 1969). At a certain salt concentration the mRNP dissociates into RNA and protein, with the ribosomes remaining intact (Perry, Kelly, 1968). Starting from this observation we have treated rat liver polysomes with EDTA and 0.8 M KCl successively, and have separated the released proteins by gel-filtration. As a result we found that the first post-ribosomal peak contained a protein having the same electrophoretic mobility as component B of informofer proteins (Tomcsányi et al., 1970).

Fig. 1 presents the Sephadex G-200 elution profile of polysomes treated with EDTA and KCl. Three protein peaks corresponding to the protein fractions separated from the ribosomes can be discerned. The protein content of the fractions amounts to 6–8% of the total protein content of the polysomes. Polyacrylamide gel electrophoresis of the lyophilized proteins obtained from a fraction of the first peak yielded only one heavily stained band, three diffuse and one sharp, lightly stained bands (Fig. 2 B). The electrophoretic mobility of the main band is the same as that of component B of informofer proteins (Fig. 2 C). The second peak represents the main band and numerous fast components, most of which have the same mobility as ribosomal proteins. The third peak represents only ribosomal proteins. The last-mentioned two data are not shown in the figure.

There are some data in the literature which point to the similarity between mRNP separated from polysomes and mRNP (30 S particle) isolated from the nucleus. Their density, measured by CsCl density gradient centrifugation, is about 1.4 g/cm^3 (Perry, Kelly, 1968; Henshaw, 1968; Burny, 1969; Samarina et al., 1968). On treatment with 0.7 M KCl the 30 S particle completely separates into RNA and protein; similarly, treatment with 0.55 M LiCl results in the separation of the mRNP of polysomes (Perry, Kelly, 1968). According to our results polysomes contain an informofer-like protein. These data indicate that the informofer not only separates mRNA from the chromatin but also plays a role in the nucleo-cytoplasmic transport and attachment to ribosomes of mRNA. Our gel-filtration method not only ensures the separation of ribosomal and infor-

mofer-like proteins but also provides the proteins in larger amounts for preparative purposes. Studies on the further purification of informofer-like protein are in progress in our laboratory. After our results had been presented (Tomcsányi et al., 1970) and during the preparation of this manuscript, two papers appeared, by Schweiger and Hannig (1970) and by Olsnes (1970), who obtained similar results with different methods.

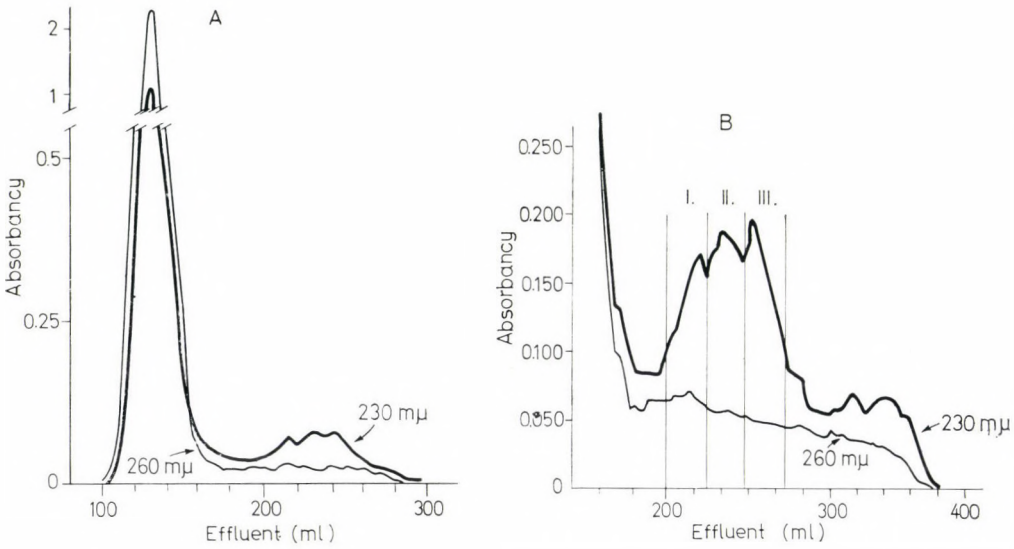

Fig. 1. Sephadex G—200 elution profile of polyribosomes separated with EDTA and KCl. Total polysomes were prepared according to Blobel and Potter's (1967) method with minor modifications. Rat livers were homogenized in sucrose-TKM buffer pH 7.2 (0.25 M sucrose, 0.05 M Tris, 0.025 M KCl, 0.005 M $MgCl_2$) to minimize the extraction of nuclear RNP. The post-mitochondrial supernatant was centrifuged at 150 000 *g* (average) for 2 hr (Janetzki VAC 60 z, 8×11 angle rotor). The pellet was suspended in TKM buffer, pH 7.5, to give a concentration of 10 mg/ml. 0.25 M K-EDTA, pH 7.5, was added to the suspension up to a final concentration of 0.05 M, and after 15 min 2.5 M KCl and 1 M $MgCl_2$ were added up to final concentrations of 0.8 M and 0.01 M, respectively. After a further 10-min standing (0 °C) the suspension was applied to a Sephadex G—200 column (50 cm × 3 cm) equilibrated with a solution containing 0.8 M KCl, 0.05 M Tris, 0.01 M $MgCl_2$, pH 7.5, and was then eluted with the same solution. The eluate was collected in 4-ml fractions and the absorbance was determined at 230 and 260 mμ. The protein fractions eluted after the ribosomes were pooled as indicated by the vertical lines, then dialyzed against a large volume of distilled water and finally lyophilized. A, Elution diagram of 6.5 mg polysomes; B, Same as A, but 25 mg polysomes. Only the proteins are shown. 260 mμ; 230 mμ

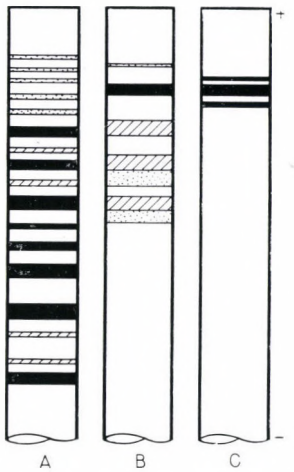

Fig. 2. Polyacrylamide gel electrophoresis of ribosomal proteins, of proteins separated from polysomes, and of informofer proteins. Preparation of the ribosomal and informofer proteins and subsequent electrophoresis were carried out according to Molnár (1969), except that 7.5% gels were used. The proteins released from the polysomes were dissolved in 10% sucrose containing 6 M urea and 0.05 M K-acetate, pH 6.8, and electrophoresed. A, Ribosomal proteins; B, Lyophilized protein after gel-filtration (peak I); C, Informofer proteins. A current of 5 mA per tube was applied for 4 hr

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Group Separation:
A New Method Facilitating Isolation of Peptides from Digests of High Molecular Weight Proteins

(Preliminary Communication)

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It is well known that the main difficulty in the sequence analysis of high molecular weight proteins is the separation and purification of their fragments because of the complexity of the digests. For this reason attemps have been made in our laboratory to develop a procedure by which the components of complex mixtures of peptides could systematically be separated into groups prior to the purification of the individual peptides. The promising results of the experiments obtained mainly with the enzymatic digests of ovalbumin showed that some of the new techniques could find immediate application particularly in the isolation of overlapping peptides or when the sequence around particular amino acid residues is studied.

Principle. A digest of a protein consisting merely of soluble fragments can easily be separated by paper electrophoresis or ion exchange chromatography into three – acidic, neutral and basic – fractions. The peptides which contain a particular amino acid residue, e.g. methionine, can generally be found in any of these groups. The distribution of the peptides containing methionine among the acidic, basic and neutral fractions is governed by the relative number of acidic and basic amino acid residues present in the individual fragments. By suitable modification of the charged groups or selectively converting neutral amino acid residues into acidic or basic ones, however, this distribution pattern can be altered, thus making possible for the desired peptides to form an easily separable group. Once the group of the particular peptides has been separated further purification becomes much simpler since the complexity of the mixture to be fractionated is greatly reduced.

Lysine and arginine peptides. If all side chain carboxyl groups in the fragments of a protein were blocked the basic fraction comprised all lysine and arginine peptides and the rest of the fragments accumulated in the neutral group. For blocking the carboxyl groups in proteins, the carbodiimide method of Hoare and Koshland (1967) was used. The (reduced and carboxymethylated) protein was coupled with methylamine as previously described (Furka, Sebestyén, 1969). Proteins containing cystine or cysteine were previously converted to S-carboxymethylated derivative. Separation of the basic fraction was carried out by passing the chymotryptic or subtilisin digest through a Dowex 50×2 column equilibrated

and eluted with 2 M pyridine. The lysine and arginine peptides were adsorbed, the neutral and histidine peptides passed through.

A different method has been developed to block the side chain carboxyl groups in peptides. The carboxymethylated protein was digested with chymotrypsin. The freeze-dried digest was stirred in dry methanol containing 0.1 N hydrogen chloride at 4°C for 48 hr. By this treatment all carboxyl groups – including the α-carboxyls – were esterified. After evaporation of the solvent in vacuo the mixture was digested with chymotrypsin in a pH-stat at 22°C for about 20 minutes. The pH of the mixture was kept at 6.0 by adding 0.1 N ammonium hydroxide. Redigestion resulted in the rapid removal of the blocking groups from the α-carboxyls leaving the side chain ester groups unaffected. The freeze-dried digest was dissolved in water and applied to a Dowex 1 column previously saturated with carbon dioxide. The lysine and arginine peptides passed through the column, the rest of the mixture absorbed on the resin.

The use of blocking of carboxyl groups together with maleylation offered a possibility even for the separation of the lysine peptides from the arginine-containing ones. The carboxymethylated protein was coupled with methylamine then treated with maleic anhydride as described by Butler et al. (1969). The chymotryptic digest was first separated into basic, neutral and acidic fractions. The basic fraction consisted of the arginine peptides. The neutral and acidic peptides were separately dissolved in pyridine – acetic acid buffer at pH 3.5; then heated for 6 hr at 60°C (Butler et al., 1969) to unblock the amino groups. The freeze-dried mixtures were dissolved in 2 M pyridine and applied to a Dowex 50 column. Thus, from the originally acidic fraction the lysine peptides could be recovered in the basic form, and adsorbed on the resin. The originally neutral mixture yielded fragments containing both lysine and arginine residues.

The lysine and arginine peptides could be divided into even more groups (five instead of three) in the following experiment. A sample of a carboxymethylated protein was maleylated then digested with chymotrypsin. The digest was first separated into acidic, neutral and basic fractions. The basic fraction consisted of arginine peptides. The acidic and neutral fractions were subjected separately to the unblocking procedure. From the mixtures a part of the lysine peptides and fragments containing both lysine and arginine could be separated. Then a second sample of carboxymethylated protein was directly digested with chymotrypsin. The digest was separated into basic, neutral and acidic fractions. The second and third fractions included those, so far missing, fragments which contained besides lysine or arginine one or more aspartic or glutamic acid residues. These two fractions were treated separately with methanolic-HCl then redigested with chymotrypsin. Thus two additional groups of lysine and arginine peptides could be separated.

Methionine and histidine peptides. In order to achieve further group separations either blocking of the basic groups in the protein or elimination of the lysine and arginine residues from the peptides was needed. The second possibility has been chosen. The carboxyl groups in a carboxymethylated protein were blocked by coupling with methylamine. The modified protein was digested with trypsin and

chymotrypsin. After additional digestion with carboxypeptidase B the mixture was freeze-dried, dissolved in 2 M pyridine, then applied to a Dowex 50 × 2 column. The lysine and arginine residues were released from the fragments by carboxypeptidase B and adsorbed on the resin. As a consequence, all peptides including the histidine-containing ones, passed the column. The freeze-dried mixture of the neutral peptides was divided into two parts in order to separate the histidine and methionine-containing fragments.

One part of the mixture was subjected to high voltage paper electrophoresis at pH 5.5 in pyridine acetate buffer. The histidine-containing fragments migrated toward the cathode and separated from the bulk of the neutral peptides.

The second sample was dissolved in 0.2 M pyridine acetate buffer, pH 3.5, iodoacetamide added in 10-fold molar excess then incubated at 37°C for 5 hr. The excess of the reagent was removed by extracting three times with peroxide-free ether. As a result of this treatment one positively charged group formed in each methionine residue (Lawson et al., 1962). Thus the basic methionine peptides could easily be separated from the unchanged neutral fragments.

Cysteic acid and serine phosphate peptides. When the carboxyl groups in phosphoproteins and oxidized proteins were blocked by coupling with methylamine, the serine phosphate and cysteic acid residues were unaffected. Thus, as a result of digesting these proteins with chymotrypsin, trypsin and carboxypeptidase B an additional separable acidic group was formed comprising the serine phosphate or cysteic acid peptides.

Notes. 1. It is not yet possible to locate the position of the asparagine and glutamine residues in the isolated peptides. 2. The blocked carboxymethyl cysteine is probably destroyed during acid hydrolysis and cannot be recovered. 3. Not all variants of the procedures are descibed in the paper. In special cases simplifications can be made. 4. Preliminary studies show that cysteine peptides can be separated in the aminoethylated form, too. The cystine peptides appear in a different group. 5. Aspartic acid, glutamic acid, tyrosine and tryptophan peptides are also suitable for group separation.

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Comparative Crystallographic Analysis of Different Bones with X-ray Diffractometry

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The authors have examined human and animal bones with comparative X-ray diffractometry. The diffractograms apparently very similar have sensitive index-numbers, characteristic of the given examined bone, as proved with the aid of simple measurement and calculation. On the basis of these index numbers it is possible to describe the crystallographic characteristics of the bones, their similarities and dissimilarities.

The examination method developed by the authors is suitable for determining the crystallographic compatibility of different kinds of bones used in bone transplantation, as well as for elucidating the connections between the mechanical and mineral characteristics of the bone. The method is useful in the crystallographic analysis of decomposition processes of fundamental importance in bone metabolism.

Introduction

The mineral substance of the bone was examined with the method of X-ray diffraction by several authors (Amprino, Engström 1952; Engström, Finean, 1953; Finean, Engström, 1953). In some cases also a comparative analysis of bone crystals with X-ray diffraction was performed though in a rather limited field only (Müller et al., 1966; Posner et al., 1965).

The biologically significant comparative crystallographic examination of different kinds of bones can only be performed if there does exist such a difference in X-ray diffraction patterns which is appropriate to reveal any possible crystallographic difference between different bones. In the literature at our disposal no unequivocal data could be found concerning this question. For this reason it was the aim of our work to find and demonstrate differences in the X-ray diffraction patterns of various bones in the hope that such differences may give a profound insight into the fundamental physiological and pathological processes of the bone on the one hand, and into the problems of bone transplantation on the other.

Material and method

Eleven kinds of bone were comparatively analyzed crystallographically (Table 1).

Table 1

Some important crystallographic characteristics of the examined bones

Bone specimens	Direction axis where the bone is more ordered	Degree of order	Shape of the crystal
A	*c*	high	stubby
B	*c*	strong	slightly elongated
C	weakly in both directions	weak	stubby
D	*c*	medium	slightly elongated
E	*c*	medium	strongly elongated
F	*c*	medium	strongly elongated
G	*c*	medium	slightly elongated
H	*c*	weak	somewhat elongated
I	*c*	weak	weakly elongated
J	*c*	weak	somewhat elongated
K	the same both in directions *a* and *c*	weakly medium	fairly elongated

A. Part of desantigenized corticalis from the anterior middle third of the tibia of a one year old calf. B. Part of desantigenized spongiosa from the proximal part of the tibia of a one year old calf. C. Desantigenized cortical bone of calf taken from the anterior middle third of the tibia. This bone was transplanted into man but the transplantation was not successful. A 0.5 mm thick peripheral part of the removed bone was examined. D. Par of human femoral spongiosa conserved in Merthiolate solution. E. Part of human tibial spongiosa conserved in Merthiolate solution. F. Part of native corticalis from the femur diaphysis of a two years old dog. The bone was transplanted into the quadriceps of a dog and removed two months later. A 0.5 mm thick peripheral part of the removed bone was examined. H. Cortical native bone from the femoral diaphysis of a three months old mouse. I. Cortical native bone from the femoral diaphysis of a three months old rat. J. Cortical native bone from the femoral diaphysis of a four months old guinea pig. K. Cortical native bone from the femoral diaphysis of a three months old rabbit.

The bone specimens were first cleaned from soft tissues by physical methods. The fat was extracted with petrol after grinding the bone to an average size of grains of 200 micra. The bone powder obtained in this way was pulverized forth until arriving at an average fineness of 20 micra and examined thereafter.

The examination was performed with the aid of a Philips Mueller-Mikro 111 diffractometer. Cu K_α radiation, monochromatized with a nickel filter, was used. The technique of recording agreed with the powder diffractometer technique generally used in the examination of rocks and minerals.

Results

The diffractograms of native bones contain generally relatively few, weakly resolved reflections. The shape of the reflection lines is broadened and blending into each other (Fig. 1).

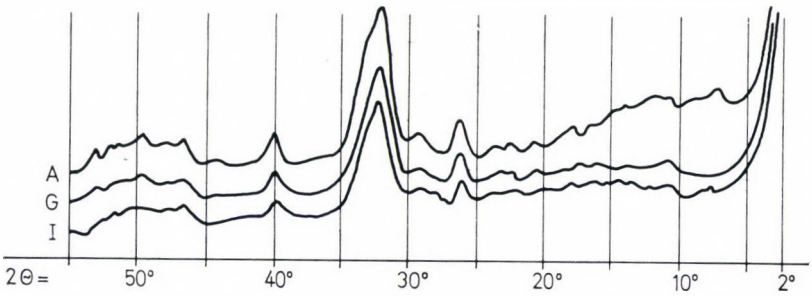

Fig. 1. Diffractograms of native bones from the corticalis of rat, dog and calf. The lines of diffraction are slightly resolved. The key-letters of the figures agree with those in Table 1

The apatites crystallize in the hexagonal crystal system, so they can take a needle or disc shape, depending on the formation temperature and pressure. The change in shape can be followed qualitatively by comparing the intensity of the reflections of a prism and a basal pinacoide plane:

$$\frac{I_{(hk0)}}{I_{(002)}} = R$$

R = ratio of sveltess.

In our examinations the $I_{(30\bar{3}0-21\bar{3}0-11\bar{2}2)}$ combined intensity of prism-bipyramid reflection was used instead of $I_{(hk0)}$, i.e. pure intensity of prism-reflection. This combined reflection has proper intensity and is, as a whole, undisturbed. According to the examinations of Mosebach (1965) the coincidence of bipyramid reflection with the prism reflections does not alter the sign of the change of the latter, only decreases its steepness. Thus it is right to use such a complex line.

A conclusion to the shape of apatite crystals of bone may be drawn from the above mentioned R sveltess ratio if it is plotted against the normalized intensity of the well-resolved basal pinacoide reflection.

$$\frac{I_{(hkl)}}{\Sigma I_{(hkl)}} = I^N{}_{(hk1)}$$

The relation is illustrated in Fig. 2. The hyperbolic connection expresses how the needle shaped crystals become stubby. This change in habit may develop in two ways:

a) the length does not change with the increase in thickness,
b) the length decreases with the increase in thickness.

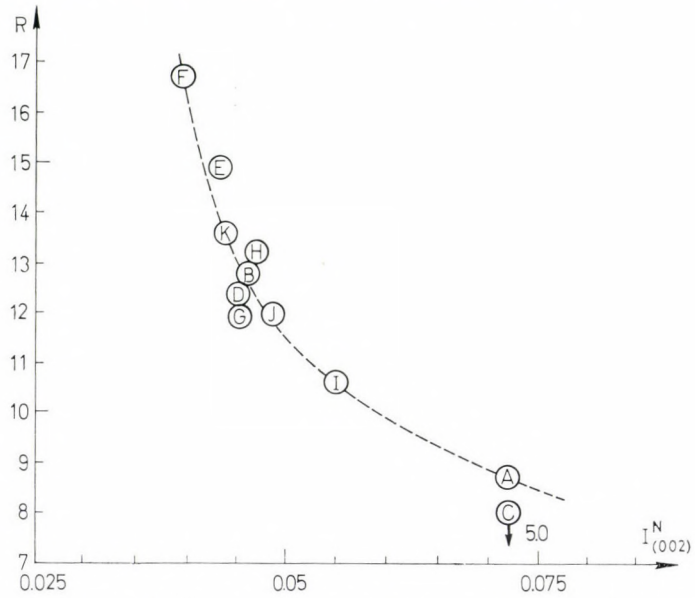

Fig. 2. Relation between the R sveltess ratio and the normalized intensity. The increase in reflection intensity of the basal plane calls forth the decrease of the R sveltess ratio

Fig. 2 alone does not offer a satisfactory answer to the question whether this or that case is met with at the bone examined. The relation of $I^N_{(hkl)}$ and $I^N_{(002)}$, on the other hand, does render a good selection possible (Fig. 3). The normalized intensities are with good approximation independent of the concentration and of chemical composition of the crystalline substance of the bone; thus, the different bones can be compared directly. Comparison of Figs 2 and 3 clearly shows that native canine corticalis (Table 1 F), the peripheric part of the transplanted canine corticalis (Table 1 G), the native guinea pig femur (Table 1 J) all be long to case "*a*", while the other bones belong to case "*b*". It is also clear from the Figures that the canine-bone has the most slender, and the calf corticalis has the stubbiest crystal shape.

The line profile broadening of the X-ray diffraction depends on the crystallite dimension, the inside order – degree of crystallinity – and the deformation of the crystal lattice. This latter can probably be neglected in a system having such a dynamic balance as bone. The Scherrer formula is valid directly and formally, respectively, for the former two factors (Guinier, 1956).

The crystallite dimension and the degree of crystallinity can be calculated from the line profile broadening of the diffraction only if the effect of the other factor is negligible. According to our observations the line-profile broadening depends on the order of reflection so much that the lines of the secondary reflection do not even emerge from the background radiation. In this case the inner disorder must have a greater effect than that of the decrease in size.

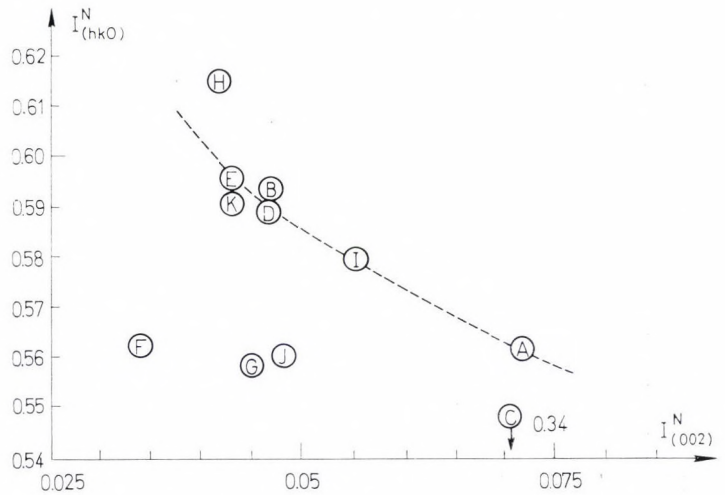

Fig. 3. Relation between the intensity of the reflection of apatite prism plane and the reflection intensity of the basal plane of the bone. In some bones the intensity of reflection of prism plane decreases with the increase of the reflection intensity of the basal plane

The β value of the Scherrer formula and, in general case, the integral angle broadening respectively let us conclude to the degree of crystallinity of the bone-apatite. In order to compare in the above sense crystals having practically the same composition and similar structure, it is not necessary to carry out complicated corrections based on uncertain data. Instead of the β value measured at different bones its modified form was used, which is independent of the concentration of apatite.

$$\beta' = \frac{\beta}{h}$$

h = the height of the measured line.

For the sake of a more clear representation $\frac{1}{\beta'_{(hk0)}}$ value was represented against $\frac{1}{\beta'_{(002)}}$ (Fig. 4).

Independently of the origin of the bone it can be stated that the points are situated on two convergent curves. The apatite of the bone is more ordered in the direction of the longitudinal axis in both cases; at the same time, the degree of crystallinity increasing in the direction of the *a* axis calls forth also the increase of the degree of crystallinity in the direction of the *c* axis. The relation is valid for

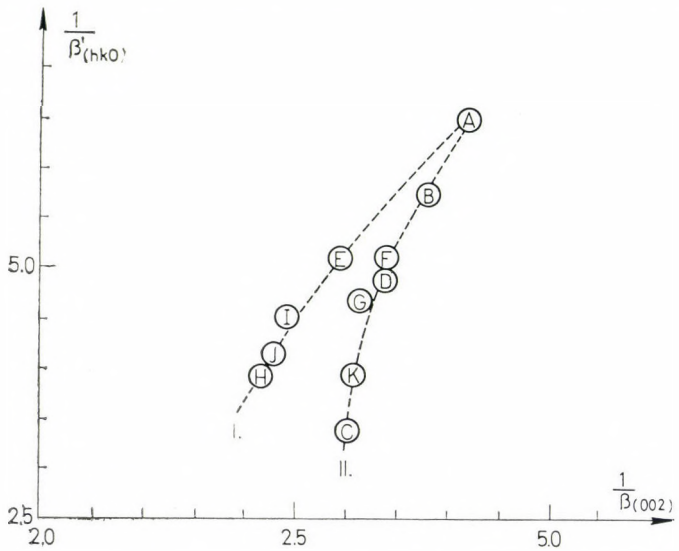

Fig. 4. Relation between the order degree in the directions of the *a* and *c* axes, respectively. The order degree of the bone crystals can be characterized by two different curves

both curves but, with bones representing curve II, the longitudinal degree of crystallinity increases more quickly.

The examination of the boundary surface of the transplanted and later removed bones (Table 1 C and G) with X-ray diffraction showed that the bone placed into the living organism and destructed there shows even more blurred reflections of X-ray diffraction (Fig. 5). On the superficial layer – according to our observations – decomposition happened first of all in the direction of the longitudinal axis, because the sveltess decreased, and the degree of order decreased too.

The rabbit bone, which is the most brittle, most fragile of the bones examined (Table 1 K) showed a weakly middle degree of crystallinity, almost complete isotropy, and crystals having a shape somewhat more elongated in the direction of the *c* axis.

It was conspicuous that the bovine desantigenized bone often used for transplantation showed mineralogically a relative great difference from the human bone, at the same time the native cortical canine bone greatly agreed with the human bone.

Table 1 summarizes the data obtained from the X-ray diffractometric analysis.

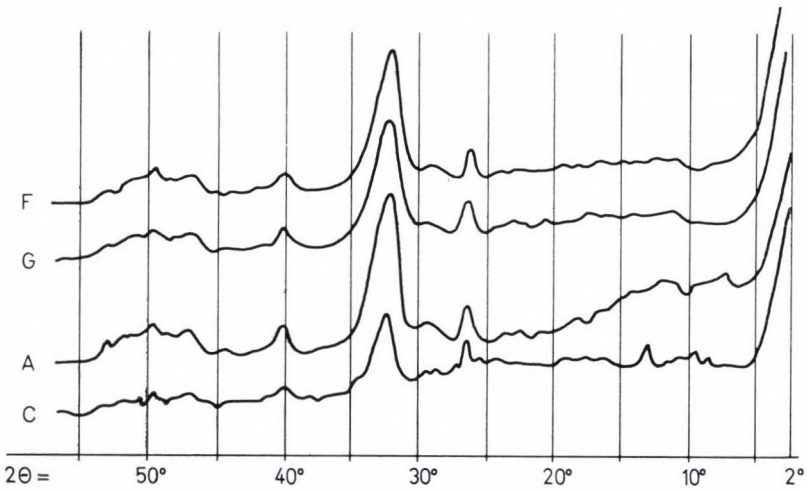

Fig. 5. X-ray diffraction pattern of the boundary surfaces of decomposed bone as compared with the diffraction pattern of intact one

Discussion

1. Formerly the mineral substance of the bone was examined with methods of X-ray diffraction chiefly to analyze its chemical composition and to find out what the size and shape of the crystals are like (Mosebach, 1965; Lénárt et al., 1968; Münzenberg, 1970).

If the mineral substance of different bones is analyzed comparatively, one can find a way for comparing the different kinds of bone and determining the crystallographic differences. For this purpose, beside the simple measurement technique, characteristic and sensitive index-numbers had to be developed. From the working curve constructed on the basis of these index-numbers it was possible to conclude to the different or identical crystallographic characteristics of various bones. The differences found – for instance, in the case of human and bovine bones – may explain the failure of certain transplantations.

2. At the present stage the procedure described above has to be considered as a working method. A great many experiments will be necessary to demonstrate constant relationships between the crystalline structure of the bone, on the one hand, and its physical and biological qualities as well as its transplantation compatibility, on the other. Considering the fact that the differences and agreements can be well shown by the method described there are, in principle, no obstacles of this kind of examinations. From a mineralogic point of view the problems of bone decomposition are completely unsolved.

Our results show that – in the examined cases – the crystals forming the bone undergo the greatest decomposition in the direction of their longitudinal axis. This conclusion offers a good starting point of a large-scale investigation of this important problem.

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Discrimination of Isotopes by Living Systems IV. Stimulus-threshold of Sartorius Muscles in ^{39}KCl and ^{41}KCl

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According to previous experiments frog hearts discriminate ^{39}K and ^{41}K; the present paper describes investigations whether sartorius muscles behave similarly. After determination of their threshold stimuli, the sartorii were soaked in "Ringer solution" lacking KCl, so their irritability gradually decreased (phase I). Having been so treated at about +2 °C for a few days the sartorii with an odd number were soaked in a Ringer solution containing 10, 20, 40, 60 mg ^{39}KCl[1] (and the equal amount of $CaCl_2$) per l, those with an even number in a similar solution made with ^{41}KCl (phase II). The thresholds of the electric stimuli decreased in both phases, but sartorii soaked in ^{41}KCl showed in general lower values indicating increased irritability in comparison to the controls treated with ^{39}KCl.

Introduction

In previous articles of this series (Ernst, 1970; 1970a; Niedetzky, Dalnoki, 1970) the problem was put forward whether living systems are able to discriminate isotopes. This more general question was specialized to the more limited one whether living systems discriminate two isotopes one of which contains *a surplus of neutrons*.

Being a common content of all living systems, the two inactive isotopes of potassium were investigated in the form of KCl (Ernst, 1970) with the result that frog hearts and pieces of sinuses are able to discriminate ^{39}KCl and ^{41}KCl, the latter containing ^{41}K artificially enriched and consequently being costly. Therefore similar investigations were planned with thallium and indium which, in the natural state, have the heavier isotopes enriched without any artificial intervention. These experiments are for the time being unsuccessful; i.e. we have not succeeded in overcoming the difficulties in experiments performed with TlCl and $TlNO_3$ on the one hand, and in acquiring corresponding compounds of In, on the other.

Thus the question of whether the sartorius muscles of the frog discriminate ^{39}KCl and ^{41}KCl was investigated with a positive result.

[1] In ^{39}KCl the ratio of $^{39}K : {}^{41}K$ = 93 per cent : 7 per cent, in ^{41}KCl this ratio = 4 per cent : 96 per cent.

Method*

Both sartorii of *Rana esculenta* were prepared and put in plexi glass containers of equal dimensions (30 mm long by 8 mm wide by 8 mm high). The muscles were soaked in a "Ringer solution" containing no KCl at +2° C for a few days, taken out of the bath twice a day and stimulated directly. In every case the threshold voltages were determined, and afterwards the soaking solution renewed. This procedure was repeated for a few days until the irritability of the muscles decreased to a low level signalling that it might cease the next day. In this first phase (I) of the experiments both sartorii of every frog were treated equally.

Four series of experiments were performed. All of them were similar in the 1^{st} phase; in the 2^{nd} phase (II) the sartorii of every frog were soaked in a "Ringer solution" containing

10 mg KCl and $CaCl_2$ in the 1^{st} series,
20 mg KCl and $CaCl_2$ in the 2^{nd} series,
40 mg KCl and $CaCl_2$ in the 3^{rd} series and
60 mg KCl and $CaCl_2$ in the 4^{th} series.

One sartorius of the n^{th} frog was numbered $2n$-1, and the other $2n$; the soaking solution of every sartorius with an odd number ($2n$-1) contained analytical grade KCl, the ratio of the isotopes $^{39}K : {}^{41}K$ being 93% : 7%, the KCl for sartorii with an even number ($2n$) contained ^{41}K artificially enriched to the ratio of $^{39}K : {}^{41}K = 4\% : 96\%$. The irritability of the muscles was measured similarly twice a day in phase II, and the thresholds were determined for a few days running.

The sartorii were stimulated with rectangular impulses of 1 and 10 ms duration at their lower thirds through electrodes made of platinum wire and being 4–5 mm apart from each other. The lowest value of four consecutive measurements (performed within 1 minute on the same muscle) was considered as the *threshold* of the stimulus at the time of this measurement.

The *evaluation* of the results was carried out in two ways, the first one being a semiquantitative method.

1. The differences of the threshold-values (V) for the two sartorii of the n^{th} frog

$$d_k^n = V_k^{2n-1} - V_k^{2n} \quad (k = 1, 2, 3, \ldots)$$

were computed and in the case of

$V_k^{2n-1} - V_k^{2n} > 0$ marked with +, in the case of

$V_k^{2n-1} - V_k^{2n} < 0$ marked with −, and in the case of

$V_k^{2n-1} = V_k^{2n}$ marked with 0.

In every series the *numbers* of the differences marked with +, −, or 0 be

$$N^+, N^- \text{ and } N^0, \text{ and } N = N^+ + N^- + N^0,$$

* Co-worker: Catherine Gábor.

when N is the number of all d-values in a certain series. On the basis of these numbers expressed in per cent

$$\frac{100\ N^+}{N}, \frac{100\ N^-}{N} \text{ and } \frac{100\ N^0}{N}$$

were computed for phases I and II and compared.

2. The simple Student's test was applied as the second method of evaluation of the results. For this purpose the differences of the threshold voltages

$$d_k^n = V_k^{2n-1} - V_k^{2n} \quad (k = 1, 2, 3, \ldots)$$

were summed up separately in the first and the second phase

$$\sum_1^k d_I^n \text{ and } \sum_1^k d_{II}^n .$$

The differences of these sums divided by k_I and k_{II}, respectively,

$$\Delta_n = \frac{\sum_1^k d_{kII}^n}{k_{II}} - \frac{\sum_1^k d_{kI}^n}{k_I}$$

were used as the x_i values, when the results were evaluated by Student's test in its reduced form of

$$t_{n-1} = \frac{\sum_{i=1}^n x_i}{\sqrt{\sum_1^n (x_i - \bar{x})^2}} .$$

Results

1. A decrease in irritability of muscles perfused with a "Ringer solution" lacking K was described in an earlier paper (Gábor, Varga-Mányi, 1969). In the experiments described in the present paper isolated sartorii were soaked for 6 days in a "Ringer solution" lacking K, and their loss in K-content was determined as shown in Table 1.

2. The results of the experiments were evaluated first by a semiquantitative method comparing the differences in stimulus threshold between the two sartorii of the same frog as shown in Table 2.

In the 1st series 30 sartorii of 15 frogs were stimulated 130 times in phase I and 119 times in phase II, indicating a smaller decrease of irritability, i.e. a little increase in irritability of the muscles soaked in a solution made with ^{41}KCl (compared with ^{39}KCl).

Table 1

K-loss and Na-gain of sartorii soaked in a K- and Ca-free Ringer solution

No	Number of sartorii	Weight				
		in g			in mg	
		fresh	dry	ashes	K	Na
17–18	20 of 10 frogs	2.15	0.220	0.0160	0.31	5.32

Table 2

The percental figures of the differences in the threshold values of sartorii with an odd and an even number (1 ms)

Sign* of the differences	Series							
	1		2		3		4	
	Phase							
	I	II	I	II	I	II	I	II
+	42	50	36	68	40	66	35	61
–	43	35	43	20	36	21	44	27
0	15	15	21	12	24	13	21	12

* If the stimulus threshold of a sartorius (with an odd number) is greater than that of the other sartorius (with an even number) of the same frog, the sign + is used, in the opposite case the sign —, and the sign 0 means that the threshold stimuli for both were equal.

In the 2^{nd} series 48 sartorii of 24 frogs were measured 224 times in phase I and 140 times in phase II, where the irritability of the muscles treated with ^{41}KCl was considerably greater than that of the sartorii treated with ^{39}KCl.

In the 3^{rd} series the threshold values of 40 sartorii (of 20 frogs) were measured 180 times in phase I and 132 times in phase II, the results are very similar to those of the 2^{nd} series.

In the 4^{th} series the values of the threshold stimuli determined for 40 sartorii (of 20 frogs) 190 times in phase I and 142 times in phase II display uncertainty.

All these results of 1257 measurements were obtained with impulses of the duration of 1 ms; the results of 1265 measurements with 10 ms impulses are similar. Summing up: the irritability of sartorii (I) soaked in a K-free so-

Table 3

The numerical values of the experiment on the 99th and 100th sartorii of the 50th frog ($n = 50$, the 3rd series) measured in the k^{th} state

Phase	$k =$	1	2	3	4	5	6	7	8	9	
I	V_k^{99}*	0.2	0.4	0.5	0.8	0.8	0.8	1.2	2.0	1.6	
	V_k^{100}	0.2	0.5	0,7	1.0	0.5	0.6	1.0	1.4	1.8	
	d_k^{50}	0	−0.1	−0.2	−0.2	+0.3	+0.2	+0.2	+0.6	−0.2	$\sum_1^9 d_I^{50} = +0.6$
II	V_k^{99}	1.4	1.4	1.4	1.8	1.8	2.0	2.5	2.5		
	V_k^{100}	0.7	1.0	1.2	0.9	1.2	1.4	1.4	1.4		
	d_k^{50}	+0.7	+0.4	+0.2	+0.9	+0.6	+0.6	+1.1	+1.1		$\sum_1^8 d_{II}^{50} = +5.6$
Δ_{50}	$\frac{\sum_1^8 d_{II}^{50}}{8} - \frac{\sum_1^9 d_I^{50}}{9} \sim 0.7-0.1 \sim 0.6$										

* I $V_1^{99} = 0.2$ V, II $V_4^{100} = 0.9$ V (duration = 0.1 ms).

lution, and therefore deprived of the greatest part of their K-content, decreased continuously; (II) after changing over to a solution made with ($CaCl_2$+) ^{39}KCl or ^{41}KCl, *the irritability of the sartorii treated with* ^{41}*KCl was greater than that of the muscles treated with* ^{39}*KCl.*

3. Owing to the importance of this statement a quantitative evaluation seemed to be advisable; the mathematical method applied to this task has been shortly described above. Applying the Student's test to all thresholds gained in 79 frogs for stimuli of the duration of 1 and 10 ms, – one example is shown in Table 3 – i.e. for 158 independent values, one could come to a misleading conclusion.

Table 4

Numerical values of the Student's test
a) *in tables and* b) *computed from the experimental results*

Value	Series			
	1	2	3	4
a) in tables	$P = 0.05$ $t_{14} = 2.15$	$P = 0.01$ $t_{23} = 2.81$	$P = 0.001$ $t_{19} = 3.88$	$P = 0.05$ $t_{19} = 2.09$
b) computed 1 ms	1.30	2.95	4.21	1.12
10 ms	<0	3.43	3.99	2.24

Viz., e.g. on the basis of $t_{157} = 1.98$, one could come to the *global* result, that the irritability of sartorii treated with ^{41}KCl is in general (at a level of $P < 0.05$) significantly greater than that in the case of ^{39}KCl.

In contrast to that the Student's test computed separately for all four series leads us to a quite different conclusion, as shown in Table 4. This indicates that the *difference in irritability of sartorii soaked in solutions made with* ^{41}KCl *and* ^{39}KCl *at concentrations of* 10 *mg/l is not significant, in the case of* 20 *mg/l the difference is significant at* $P < 0.01$ *level, for* 40 *mg/l there is significance even at* $P < 0.001$ *level, for* 60 *mg/l the significance is doubtful.*

Discussion

1. According to these experimental results the difference in irritability between sartorii treated with ^{39}KCl and ^{41}KCl depends on the amount of these compounds. These differences are significant even at a level of $P < 0.01$ or 0.001 when 20 or 40 mg/l was used, and not significant at 10 or 60 mg/l. These results concerning the *irritability* of sartorii are very similar to those in our earlier paper (Ernst, 1970) describing differences in *pacemaker activity of* frog hearts.[2]

[2] This remarkable similarity (Fig. 2 in Ernst, 1970) of the results could be seen in experiments performed in laboratories on two different floors of the institute by two different teams without knowing of each other's work.

2. The theoretical analysis of the fact that the difference in the activites of ^{41}KCl and ^{39}KCl depends on their amounts contained in the soaking solution should be postponed until more experiments performed of more points of view have produced a larger basis for explanation.

3. The importance of K and Ca for the irritability of striated muscles is very widely discussed in the literature and has been treated many times also by us (Gábor, Varga-Mányi, 1969). The present paper *gives evidence of the importance of K:* 1) the irritability of sartorii decreased due to the absence of K in the soaking solution (phase I) and afterwards improved in a renewed solution containing K and Ca (phase II); 2) in this phase II *irritability was greater in* ^{41}KCl *than in* ^{39}KCl *the solutions containing the same amount of all constituents (also of* $CaCl_2$*).*

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Effect of Antibiotics on the Ion-exchange of Bacteria

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The effect of different streptomycin and chloramphenicol concentrations on the ^{42}K release from E. coli B. cells was investigated. It was found that, in each case, the efflux of ^{42}K could be described by an exponential function represented by a straight line in a semilogarithmic coordinate system. The slope of the straight lines increases with increasing streptomycin concentration and decreases with increasing chloramphenicol concentration. The slope of the straight sections has been brought into relation with the frequency of events for ^{42}K release. In the case of a culture not treated with antibiotics the average frequency of events for ^{42}K release for one bacterium is about $2.2 \times 10^{-4}\ s^{-1}$. This value shows, upon the effect of streptomycin an about eightfold increase and, after chloramphenicol, a decrease to about fourfold of the control value.

On the basis of our considerations we think it proved that the frequency of ^{42}K efflux is modified by the regions of the membrane influenced by the antibiotics for about an hour after the addition of the antibiotics, independently of the fact whether the antibiotics penetrated into the interior of the bacteria or not.

In our previous investigations (Tamás, Szőgyi, 1966; 1968) it has been demonstrated that the ^{42}K release from sensitive E. coli B. cells treated with streptomycin is faster, and that of cells treated with chloramphenicol is slower than the release from untreated ones. In the present paper we report of how the ^{42}K release depends on the streptomycin or chloramphenicol concentration of the medium. An attempt will be made of interpreting and also describing this relationship with the aid of a mathematical formula.

Method

A sensitive E. coli B. strain was used in the investigations. The preparation of bacteria, their incubation with ^{42}K and the measurement of the decrease in their specific activity were described in our previous papers in detail (Tamás, Szőgyi, 1966; 1968). The order of magnitude of the bacterium concentration was 10^8/ml, and the concentration of streptomycin and chloramphenicol, respectively, added to the M9 medium was changed within a range of 20 μg/ml to 80 μg/ml. The experiments were performed at room temperature.

Results

Fig. 1 shows the decrease of the specific ^{42}K activity of microbes plotted against the time in a semilogarithmic coordinate system. The "untreated" curve was taken under normal conditions, i.e. the medium did not contain antibiotics. The activity of ^{42}K taken up by the microbes decreases according to an exponential function in each case except the initial period lasting a few minutes. If the

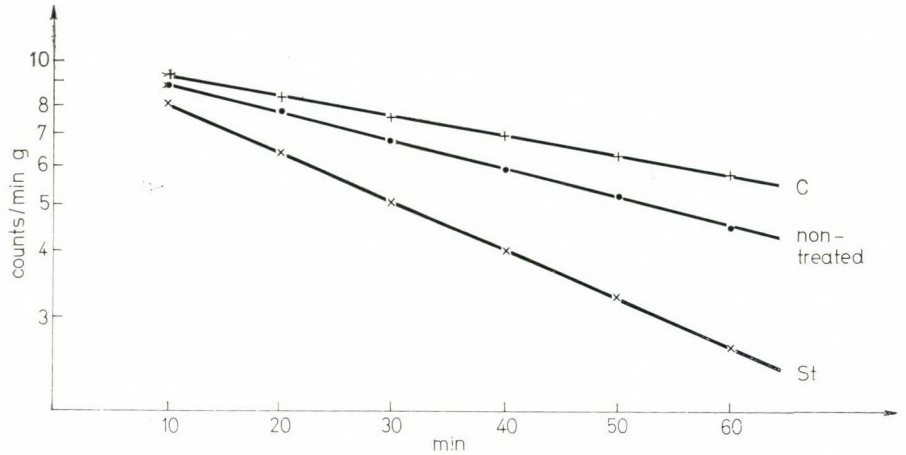

Fig. 1. ^{42}K release from E. coli B. cells plotted against the time in the case of "untreated" streptomycin-treated (St), and chloramphenicol-treated (C) bacteria

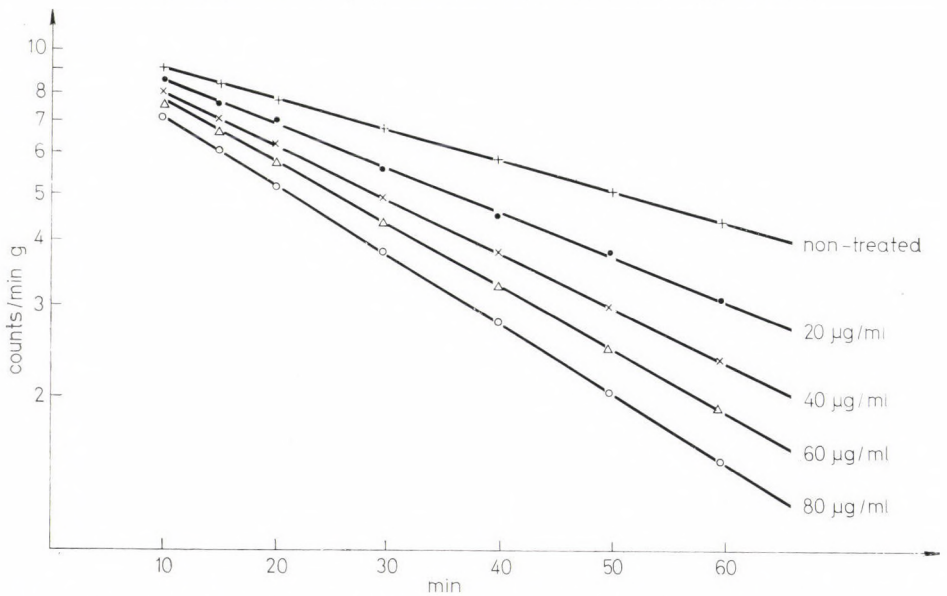

Fig. 2. ^{42}K release from E. coli B. cells plotted against the time in the case of normal conditions ("untreated" bacteria) and in the presence of different streptomycin concentrations

medium contains streptomycin the decrease of activity is faster (St curve); if it contains chloramphenicol it is slower (C curve) than in normal conditions.

Fig. 2 shows the ^{42}K release obtained in the case of a medium containing streptomycin in different concentrations plotted against time similarly in a semilogarithmic coordinate system. Fig. 3 shows the results obtained in the case of chloramphenicol. The slope of the straight sections increases with increasing concentration of streptomycin and decreases with increasing concentration of chloramphenicol.

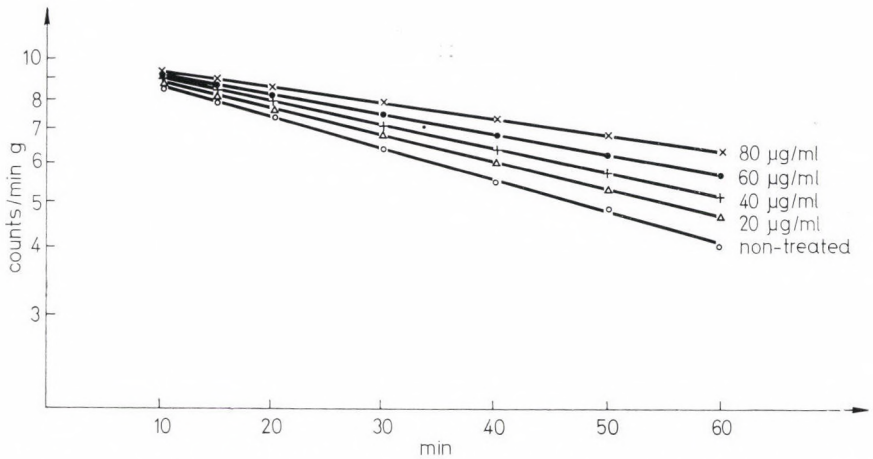

Fig. 3. ^{42}K release from E. coli B. cells plotted against time in the case of "untreated" bacteria and in the presence of different chloramphenicol concentrations

Discussion

1. First of all we deal with the case when the medium does not contain antibiotics, and the activity of ^{42}K in the bacteria decreases according to the "untreated" curves of Figs 1 to 3. This decrease – not considering the radioactive decay taking place meanwhile – is the result of the outflow of ^{42}K into the extracellular space, and can be described by the following correlation:

$$dn = -k\, n\, dt \qquad (1/a)$$

where n denotes the average number of ^{42}K atoms in one bacterium at a given point of time, and dn is the change in the number of these atoms during dt time. 1/a expresses the plausible assumption that the number of K atoms released from one bacterium during dt time is proportional to both the number of atoms inside the bacterium at the moment and the dt period of time. The k proportionality factor is the average frequency of events for ^{42}K release for one bacterium when the medium does not contain antibiotics. k shows the average fraction of the ^{42}K

atoms in one bacterium released into the extracellular space in unit of time. Instead of 1/a the following expression may also be written:

$$n = n_0 \exp(-k\,t), \tag{1/b}$$

where n_0 and n denote the average number of ^{42}K atoms in one bacterium at the beginning of the measurement, and after t time respectively. As the appropriate specific activities can be written instead of n and n_0, k can be determined from the slope of the "untreated" curves of Figs 1–3:

$$k \approx 2.2 \times 10^{-4}\,s^{-1}.$$

2. Now let us consider the cases when the medium contains streptomycin in different concentrations. The activity of ^{42}K inside the microbe decreases according to an exponential function in these cases also (Fig. 2). The absolute value of the slope increases with increasing concentration of streptomycin. This fact, according to the above-mentioned considerations, indicates that the frequency of events of the ^{42}K release increases with increasing concentration of streptomycin. We can interpret our experimental results simply, if we assume the frequency of ^{42}K efflux to increase only in those regions of the bacterium membrane, where a streptomycin molecule is adsorbed on the surface (Szőgyi et al., 1969). Let α denote the fraction which shows the portion of the bacterium surface where the frequency of ^{42}K efflux increases as a consequence of the adsorption of streptomycin. Evidently, $1-\alpha$ shows the fraction of the surface on which the ^{42}K release remains unchanged. The regions not influenced by streptomycin are further on characterized by the factor k, and the parts of the surface "covered" with streptomycin are characterized by the factor $k_S > k$. Hereafter – for a reason similar to that in section 1 – the following relation can be written:

$$dn = -k\,(1-\alpha)n\,dt - k_S\,\alpha\,n\,dt, \tag{2/a}$$

and

$$n = n_0 \exp\left[-\{k + (k_S - k)\,\alpha\}\,t\right] \tag{2/b}$$

Instead of n and n_0 the appropriate specific activities can be written again and then the t factor in the exponent of 2/b can be identified with the slope of the straight section of the curves obtained in the case of mediums containing streptomycin. Thus the absolute value of the slope is equal to the expression $[k + (k_S - k)\,\alpha]$ which means that the treatment with streptomycin increases the absolute value of the slopes by $(k_S - k)\,\alpha$ value.

As α denotes the fraction of the bacterium surface "covered" with streptomycin, α is proportional to the number of streptomycin molecules on the surface (N_S), i.e.:

$$(k_S - k)\,\alpha \sim N_S\,. \tag{3}$$

Expression (3) is really in accordance with empirical results, as it is shown by the data of Table 1. The data of the second column were calculated from the slopes of curves. The N_S given in the third column can be directly measured, but in this case its values were also calculated. It is made possible by a former statement of ours (3), according to which the binding of streptomycin on the bacterium surface can be described with the well-known law of adsorption

$$N_S = \frac{C_S N}{\kappa + C_S} \tag{4}$$

where N denotes the number of possible binding places of streptomycin on the surface of one bacterium, and N_S denotes the number of the actually occupied places in the case of an adsorption equilibrium. κ means the equilibrium constant belonging to the given conditions (temperature, medium). In our experimental conditions:

$$\kappa \approx 3.6 \times 10^{-4} \text{ gcm}^{-3},$$

$$N \approx 6 \times 10^5.$$

Our conclusions are supported by the data of the last column, according to which the quotients indicated here are approximately constant.

α – as mentioned above – denotes the fraction of the bacterium surface "covered" with streptomycin. If we accept that $\alpha = N_S/N$, then k_S can be calculated by the aid of the above relations, namely:

$$k_S \approx 1.7 \times 10^{-3} \text{ s}^{-1}.$$

This means that in the case when the possible binding places are all occupied by streptomycin molecules, an average of about 1/2000 part of the ^{42}K atoms in the bacterium would be released from one single bacterium into the extracellular space in one second. Thus, the frequency of the release in the places covered with streptomycin is about 8 times higher than in the intact regions.

3. The experimental results obtained in the case of chloramphenicol can be interpreted in a similar way. The only difference is that chloramphenicol, in con-

Table 1

C_S (μg/ml)	$10^5(k_S - k)\alpha$ (s^{-1})	$10^{-4}N_S$	$10^9(k_S - k)\alpha/N_S$ (s^{-1})
20	7.7	3.2	2.4
40	13.7	6.0	2.3
60	21.5	8.6	2.5
80	30.1	10.9	2.8

Table 2

C_C (μg/ml)	$10^5 \cdot (k - k_C)\alpha$ (s^{-1})	$10^{-5}N_C$	$10^{10}(k - k_C)\alpha/N_C$ (s^{-1})
20	3.3	1.2	2.8
40	5.9	2.0	2.9
60	7.5	2.6	2.9
80	9.0	3.0	3.0

trast to streptomycin does not increase but decreases the frequency of the ^{42}K efflux. Table 2 contains the most important data. Each quantity is denoted with the same letters as in Table 1, only the lower index S was changed into C. The last column of the Table shows that in Fig. 3, the change of the slope due to the treatment with chloramphenicol is proportional to the number of the adsorbed chloramphenicol molecules. The value of the factor $k_C < k$, characterizing the frequency of ^{42}K release is:

$$k_C \approx 5.6 \times 10^{-5} s^{-1}.$$

This means that if the possible binding places are all occupied by chloramphenicol, an average of about 1/50 000 part of the ^{42}K atoms in the bacterium would flow out into the extracellular space from one single bacterium in one second. Thus the frequency of the release is about 4 times lower in the places covered with chloramphenicol than in the intact regions.

4. In our previous examinations (Tamás, Szőgyi, 1966; 1968) the uptake of streptomycin and chloramphenicol, resp., in time was illustrated in a three-section curve. We would expect that the three-section process is reflected also in the ^{42}K efflux. According to our empirical results here also exists an initial period in which the slope of the curves differs from the later value characterizing the efflux; but the determination of the slopes in this initial period lasting for a few minutes presents technical difficulties. We want to stress especially that apart from this the slope remains unchanged even after half an hour. This means that the ^{42}K efflux is not influenced by the fact that the molecules of antibiotics penetrated into the interior of bacteria, i.e. it is the state of the membrane that is decisive for the ^{42}K efflux.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 6 (2), pp. 179–181 (1971)

Effect of Trace Elements (Cu, Co, Mn and Zn) on Excitatory Processes

(Preliminary Communication)

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(Received August 1, December 17, 1970)

More and more research workers deal with the biological role of the trace elements (Al, B, Cu, Mn, Ti, Zn, etc.) occurring in a very small quantity in the living organism (e.g. Lamb et al. 1958). The works performed in our institute (Ernst, 1963; Lakatos, 1962; Niedetzky, Hajnal-Papp, 1963) demonstrated the role of trace elements in the production and course of excitatory processes, and called attention to certain semi-conductor analogies. It is the aim of the present paper to examine the effect of different trace elements in the nerve-muscle excitatory process.

A Läwen-Trendelenburg's frog preparation (Rana esculenta) was perfused with normal Ringer's solution, and then with a Ringer's solution containing different quantities of Cu-, Co-, Mn- and Zn-chlorides, resp. The ischiadic plexus of frog was stimulated with square wave impulses of 0.3 ms duration and of threshold amplitude, and the action potentials were recorded from the middle section of the M. gastrocnemius. The action potential was recorded with one of the beams of a cathode-ray oscilloscope; a square wave impulse of 100 c/s was led to the other beam. The sensitivity of the oscilloscope was 0.2 mV/cm; the voltage of stimulation could be changed by steps of 0.001 V.

First the frog preparation was perfused with a normal Ringer's solution; then, in the further part of the experiment, with a Ringer's solution containing 10^{-6} to 10^{-4} gmol/ litre of Cu-, Co-, Mn-, and Zn-chloride, resp. The lowest stimulus voltage eliciting an action potential was determined with the aid of the perfused frog preparation. Of our investigations 85 experiments were performed with Cu as trace element, 45 with Co, 15 with Mn, and 10 with Zn. (The composition of the normal Ringer's solution was: 6.6 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$ sicc., 0.2 g $NaHCO_3$ in 1000 ml bidistilled water.)

As an example Table 1 shows the results of experiments performed with one of the four trace elements. The Table contains the lowest values of stimulus voltage which could yet elicit an action potential from the M. gastrocnemius. The perfusion with normal Ringer's solution was performed for about 25 to 30 minutes prior to the perfusion with the Ringer's solution containing trace elements. The frog preparation was perfused with Ringer's solution containing trace element as long as the decrease of excitability occurring after a period of initial increase in irritability set in.

According to our results (Table 1) the stimulus voltage shows a slow increase when the preparation is perfused with normal Ringer's solution. But when the preparation after the perfusion with normal Ringer's solution is perfused with Ringer's solution containing trace elements the value of the stimulus voltage decreases for about the first hour. Namely, a 10 to 35 per cent decrease in the value

Table 1

Perfusion of Läwen-Trendelenburg's frog preparation with normal Ringer's solution (1); with normal Ringer's solution and then with Ringer's solution containing Cu-, Co-, Mn- and Zn-chlorides, resp., in a concentration of 10^{-6} gmol/litre

Number	Trace element	Stimulus threshold in mV								
		Initial value	Duration of perfusion with solutions containing trace element							
			10'	20'	30'	40'	50'	60'	70'	80'
1.	—	44	43	67	73	79	82	83	86	86
2.	Cu	88	66	66	64	62	62	61	58	63
3.	Co	153	138	133	115	115	115	112	108	112
4.	Mn	83	78	76	76	75	75	71	76	—
5.	Zn	89	90	85	82	78	75	82	83	89

of the stimulus voltage is seen when a Ringer's solution containing 10^{-6} gmol of trace element/litre is used.

With a concentration of 10^{-5} gmol of trace element/litre muscle fibrillation was observed in many cases, and some spontaneous action potentials occurred too. With the 10^{-4} gmol/litre concentration a series of spontaneous action potentials appeared some minutes after beginning the perfusion (Fig. 1).

A swelling of the muscle was also experienced in the experiments; it was not significant in the case of Co and Mn; with Cu and Zn a 15 to 20 per cent increase of weight was obtained.

According to our experiments trace elements increase the excitability of the muscle; above a certain concentration also a series of spontaneous action potentials occurs. The appearance of spontaneous action potential and the increase of excitability were observed only when perfusing the muscle with a Ringer's solution containing trace elements, but it was not observed when the muscle was perfused with normal Ringer's solution.

These results, according to which the excitability of the nerve-muscle preparation increases upon the effect of a Ringer's solution containing trace elements, are in accordance with the results of Edman and Grieve (1961) as well as with those of Isaacson and Sandow (1963); accordingly, the trace element Zn increases the

duration of the active state of the muscle. It seems that trace elements might play a part in the formation and course of excitation processes.

In further experiments we should like to determine the change of trace element content of the perfused nerve-muscle preparations as compared with the

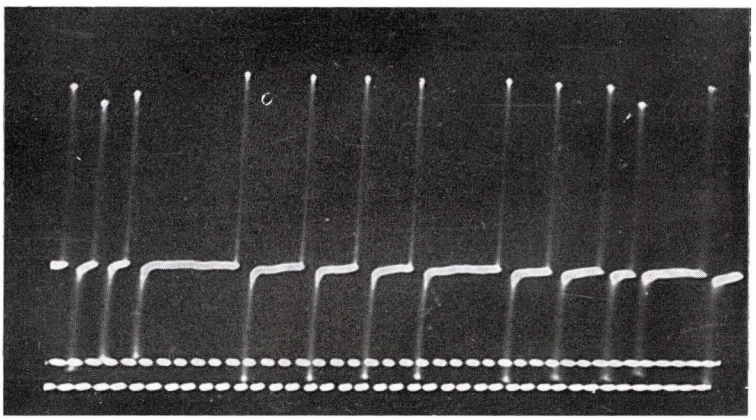

Fig. 1. Series of spontaneous action potentials (upper beam) appearing during perfusion of a Läwen-Trendelenburg's frog preparation with a Ringer's solution containing 10^{-4} gmol of $CoCl_2$/litre; the lower beam is 10 ms.

control, as well as the exact site of action of the above trace elements in the nerve and muscle.

The author thanks Prof. E. Ernst for his raising the theme.

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Book Reviews

The Actinomycetales. (The Jena International Symposium on Taxonomy.) Edited by Helmut Prauser, Jena. VEB Gustav Fischer, Jena 1970; 439 pages with 134 figures and 95 tables. Price: 90,— MDN

The interest in Actinomycetales is mainly due to their use in the production of antibiotics, vitamins, industrial enzymes and in the microbial transformation of chemical compounds.

Since their possession and the right to use them industrially has become a patentable matter, their identification is of utmost importance.

The papers in this volume deal mainly with new techniques employed in the classification and differentiation of Actinomycetales. The authors emphasize the taxonomic value of lipids, antibiotics, pigments, DNA homology and composition, sensitivity to actinophages and antibiotics. Serology, infrared spectrophotometry, rapid enzymatic methods are also recommended in the study of Actinomycetales.

Though the authors appreciate the necessity of modern instrumental methods, they emphasize that results obtained by these methods — aside from some of their problematic features — supplement but do not substitute the data obtained from microscopic examination and cultural studies.

L. PATTHY

Fortschritte der experimentellen und theoretischen Biophysik. Heft 12. Ed. Prof. Dr. W. Beier, Leipzig. *Biophysik des Muskels.* Prof. Dr. E. Ernst. VEB Georg Thieme, Leipzig, 1970. pp. 71, Abb. 38

This new broad-based review by Prof. E. Ernst of the knowledge about the structure and work of the striated muscle is the third one in his series of monographs published during the last fifteen years. His statements — even if we do not agree with them in every respect — are always remarkable, they often put the problem under examination in a surprisingly new light, and they always inspire exact experimental work. In such a sense, although the author consistently calls also his present work a report, it is considerably more than that: it is an attitude of a much experienced biophysician, expert in experiments and having a thorough theoretical grounding, in connection with a classical and still modern problem in biophysics.

The review is divided into five chapters: Introduction, The Structure of Muscle, The Muscle-machine, Retrospect, Prospect.

In the introduction the author calls attention to the fact that the chief problem of muscle research has always been the mechanism of contraction, and investigations performed in any fields must aim at clarifying this problem.

The 2nd chapter is grouped around two great problems: the problem of the micro- and submicrostructure, and the problem of the localization of the constituents of the fibril. Instead of the examination of fibre structure often dealt with formerly — also in his own previous (1958) work — the manysided examination of the structure of the fibril comes into prominence. The author lays stress on the study of submicrostructure because — as it is pointed out also in the introduction — the problem is in the foreground of experiments nowadays, and it

undoubtedly bears a close connection with the main problem. His discussion about the longitudinal and transversal structural elements of the fibril is worth attention. Within this part of the work he gives a detailed treatment of the longitudinal filamental system, the thick and thin filaments, and of their changes in size as well. He points out the changes in the characteristics of the A-substance during an active and a passive stress ("crystallization" — solubility, moving away, etc.). In connection with the transversal structure — chiefly on the basis of his own experiments — he brings up the possibility of the net structure (it is a pity that the 13th picture cannot be properly evaluated in order to help to decide the problem because of its poor quality; and that the series of experiments summarized in Fig. 14 would probably result in the same pictures even if the experiments were done in a filamental system not having a transversal structure but having a certain elasticity, e.g. a bunch of thin threads of rubber. But Figs 15, 16, 17, 18 and 19 really bring up the possibility of the existence of a transversal structural element), then he raises the question of what the real structure of the fibril is like. He demonstrates on model substances and in experiments that the formation of the structure seen in the electron microscopic pictures can be traced back to several factors. Therefore further experimental work is necessary also in this field. Perhaps it is worth mentioning that the study of the structure of building-stones and of the structures that can be brought about artificially from them is indispensable in order to form a complete picture. I mean first of all proteins, about which the author has a rather devastating opinion, but this opinion eventually stimulates further experiments, as the next chapter will convince us of it.

In connection with the localization of the constituents of the fibril the author deals with the position of proteins, water, K, Na, Ca, P in the structure.

The chief problem of Prof. Ernst in connection with the proteins is that there are too many of them to let the picture be easy to survey, and he presents wittily that if a single protein of 10^6 molecular weight were dissected into pieces of 10^4 and 10^5 molecular weight respectively with "skilful" manipulations, it would become possible to "produce" nearly 10^4 kinds of molecules. This is true, but it is also true that during the 15 years that passed since the quotation from Waugh (1954) referred to by him [1]... (Adv. in Protein Chem. *9* 325, 1954) a lot of things happened and were clarified in the field of the physics and chemistry of proteins (and within this field the muscle proteins), e.g. the problem of protein synthesis, and we obtained a picture of myosin that can be said to be very useable (see e.g. Bálint M. et al. J. Mol. Biol. *37* 330, 1968). Similarly, on the basis of electron microscopic results of 1948 (Fig. 25: electron microscopic image of dried Na-desoxyribonucleate and actomyosin suspension) we cannot state, that there is no difference between the submicroscopic structure of myosine and nucleic acids. It seems that the author simplifies this question too much. Recent informations from different areas of biology convince me of the fact that biological processes, especially the highly differentiated and thus precise ones are highly organized, regulated, and the number of molecular components which are qualitatively different from each other, distinguishable, and can be precisely defined, is rather large.

Naturally these components form an organic working unit, and one of the central problems of the present biological researches is how the components are able to form a regularly working unit. Thus it is necessary to examine the characteristics of both the individual components and the whole working unit. I think this question to be worth raising because the author devotes some pages to the same problem at the end of the chapter under the title "Interlude". He cites a quotation from Masarovic's work (1968): [2]..., which is placed in the centre of his discussion. It is a statement giving food for thought, and it throws light on the fact how necessary a comprehensive and critical work like that of Prof. Ernst is in raising and enlightening a basic problem — such as the problem of muscle contraction. For the science of our times is from the one side characterized undoubtedly by the multiplying minor works but, on the other hand, it is

also unquestionable that biology advances in a synthetizing way, with large steps by making use of the precise "minor work"...

The author stresses in particular the significant role of water in developing the structure and work of muscle, and — chiefly on the basis of his own experimental work — he demonstrates that a significant part of the water in the muscle is closely connected to the formed elements of the muscle. He expresses his hope that further examinations of bound water will significantly help in understanding the chief problem (i.e. muscle contraction).

While dealing with the localization of further inorganic constituents, the author stresses their variability of localization connected with the work and physiological state of muscle.

In the introduction of the 3rd Chapter (Muscle Machine) he emphasizes the economical, social and individual significance of muscle activity and he calls attention to the fact that this significance unfortunately is still not reflected by concrete investigations.

In this chapter he is dealing with the mechanics, elasticity, unstrained changes in shape, expenditure of energy and energetics of the muscle. All these were focuses of biophysical investigations already at the time of muscle investigations which are now considered classical. In this chapter the review of Prof. Ernst reveals the basic connections of the mechanism of contraction taking for basis also his own investigations of considerable number. It is doubtless that one who wants to interpret muscle activity, cannot contradict the facts supplied in several parts of the chapter also in quantitative relations. This statement concerns especially the changes taking place reversibly under physiological conditions.

The author touches also upon the problem of ATP in the passage treating muscle-energy. It is unquestionable that the role of ATP in muscle activity is not clarified at all, but on the basis of our knowledge of other biological processes Prof. Ernst can be said to exaggerate his negative point of view and his reconciliation of "lactic acid era" and "ATP era" in explaining muscle contraction.

In this chapter the author deals briefly also with the theories of muscle contraction, and he analyses especially the contradictions of the so-called "sliding"-hypothesis, widely accepted nowadays, as reflected by the data in the special literature.

The last chapter of the review — on one and a half page altogether — discusses the future of muscle research based on the past. The author warns the research workers dealing with muscle problems of the dangers of wrong tracks with the words of Empedokles, and then he stresses the fields of research considered important by him (and their unforeseeable difficulties): the investigation of the localization of substance to molecular or even atomic sizes in the field of structure, the specification of the myosin-ATP notion in the field of muscle chemistry, the further study of the mechanism of contraction (the author means here first of all the clarification of the mechanism of the change in shape and volume) and, lastly, the clarification of problems of energetics, especially as to the more precise measurement and "place" of the thermogenesis during activity.

The whole work is woven through with the author's striving for solving the questions considered important by him with the aid of his own experimental works, and so this summary really lets us obtain an insight into the inner dynamism of the workshop of a scientist.

The work gives us an opportunity for getting acquainted with the problems in which Prof. Ernst is interested in connection with muscle activity; and we can get acquainted with the experimental proofs he accepts and considers appropriate in solving the problems, and also with the ones he does not.

We warmly recommend this review to experts in muscle problems and to biologists interested in basic problems.

F. GUBA

[1] "... the proteins ... have not yet been characterized sufficiently. Such systems include notably the muscle protein systems."

[2] "The extreme abundance of partial investigations of an analytical character threatens to produce chaos in the investigator's consciousness and to inject complete helplessness as far as understanding the main theme for which he strives."

Kompendium der Physik. By Dr. Günther Schmidt. VEB Gustav Fischer Verlag, Jena 1971. 332 pages, Price: 23,— MDN

According to the author's intention this book has been written for students studying medicine, biology or chemistry. The book has got the programme to make its readers acquainted with the methods of description of physical processes; it gives the definition of the most important quantities and terms outlining their essential relationships. The author does not regard as his duty to confer about practical application of physical principles, however, a few of them is presented in an independent section.

The book — according to its character — is confined to use mathematics in unavoidable cases only; and an elementary review of a few functions and operations — including the differential and integral calculus — is given in an extent of 10 pages. The construction of the subject-matter is generally similar to that of the usual introductory courses of physics. In the section entitled "The Motion of Bodies (Mechanics)", the mechanics of mass point, of systems of mass point, and of rigid bodies is dealt with, as well as the motion of ideal fluids and gases. The next section (Electrostatic and Electromagnetic Interaction) is divided into parts, as follows: electrostatics, current and magnetic field, electromagnetic induction; the last one has been concluded by writing down Maxwell's equations. The section entitled "Linear Building Elements and Simple Systems" deals with problems of modelling and it draws a comparison between electrical and mechanical systems. The longest section of the book is "The State of Bodies (Thermodynamics)", in which the theses and methods of the classic thermodynamics (thermostatics) are performed in detail. A part of the solid state physics, the surface physics, and the study of wave motion, as well, belongs to this section. The "Atomic Physics" begins with the kinetic theory which is followed by the treatment of motion of charged particles, and by making known the basic knowledge about the experimental background and introductory theory of quantum physics. No effort is made to give a visual picture of atomic structure (less than one page is written about the Rutherford—Bohr model), the author preferred to explain the methods how to describe the phenomena in a quantitative way. The theory of chemical binding, the spectra of molecules, solid state physics viewed by a quantum-mechanical standpoint, and the nuclear physics take up only 20 pages, and radiation physics is neglected almost completely. A supplementary section deals with gravitation, the fundamental principles of energy transformation, the physical basis of transformation, transmission, and storage of information. At last the way of operation of several optical instruments is explained (otherwise no independent chapter can be found about optics, the most important terms are included by the part studying waves).

The book has up to date conceptions, the only exception is the treatment of thermodynamics, it completely avoids the ideas of the irreversible (nonequilibrium) thermodynamics, which plays a great role in biology at the time being.

The author provides a list of all the symbols used in the book, hereby making easier the reading, a detailed subject index (of 13 pages) renders the quick orientation possible, which is supported by the decimal system of chapters. 165 figures and 7 tables complete the book.

Technical execution is excellent, the composition of type area helps to accentuate what the author wants to say. This work can enrich the teaching of physics in the training of medical students and those of biology with some very useful and new point of view.

T. LAKATOS

Hoppe-Seyler's Zeitschrift für Physiologische Chemie

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FEBS–BIOCHEM 1972

The 8th Annual Meeting of the Federation of European Biochemical Societies (FEBS) will be held in the International Congress Centre RAI, Amsterdam from August 20–25, 1972.

The Dutch capital expects to welcome 2,500 biochemists from the 24 member-countries of the FEBS.

The Dutch Biochemical Society will act as host at the 8th Annual Meeting. The organization of the meeting has been entrusted to "Stichting 8th FEBS-meeting", established for this purpose. The secretariat of "Stichting 8th FEBS-meeting" is seated in Amsterdam, postbox 7205, telephone 020-422595, telex 13499 (RAICO) and cable address ORBU Amsterdam.

The scientific program of the 8th FEBS-meeting shows nine symposia on specific biochemical subjects and 80 experts from any part of the world will be invited to address the meeting. Apart from this, a large number of lectures has been planned for group meetings, the lectures being free in their choice of subject. English is the language to be employed.

The international exhibition of biochemical laboratory equipment, called FEBS-BIOCHEM 72, will be annexed to the meeting.

Information: FEBS-BIOCHEM 72, RAI Gebouw N. V., Europaplein 8, Amsterdam.

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Degradation of Rat Liver Polysomes upon Treatment with Various Salts

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The effect of several alkali salts on rat liver polysomes was studied. The salts were found to degrade polysomes. The particles obtained by treating polysomes with 0.8 M LiCl were studied in detail. These particles sediment somewhat slower than ribosomal monomers, they can be dissociated into subunits by EDTA, contain intact 18*S* and 28*S* ribosomal RNA's and show higher sensitivity towards pancreatic RNase than control ribosomes.

Introduction

The effect of various salts on ribosomes of different origin has been the subject of widespread investigations. Increase in salt concentration may result in the dissociation of ribosomes (Suzuka, Kaji, 1968; Martin et al., 1969), in splitting off of proteins from ribosomal particles (Meselson et al., 1964; Lerman, 1968) as well as in the total disruption of the native structure (Spitnik-Elson, 1965; Hamilton, Ruth, 1967) of both bacterial and animal ribosomes.

The effect of salts on polysomes, which represent the majority of ribosomal preparations from animals, has not yet been studied. Therefore, it is unknown whether the degradation of polysomes to monoribosomes precedes any alteration(s) in the structure of ribosomes.

In this paper it will be shown that rat liver polysomes can be disrupted by various salts in the presence of 5 mM Mg^{2+} and the resulting particles resemble monomers, but they sediment somewhat slower than the monomers originally present in ribosome preparations, and their sensitivity towards pancreatic RNase is higher than that of the control ribosomes.

Materials and methods

Materials. Diethyl pyrocarbonate was the product of Bayer Werke. Acrylamide and methylene bisacrylamide were purchased from BDH, tetramethylethylenediamine from Fluka and coomassie brillant blue R-250 from Serva. All other chemicals were the products of Reanal (Budapest).

Abbreviations used: EDTA, ethylenediaminetetraacetate; RNP, ribonucleoprotein; tRNA, transfer RNA; RNase, ribonuclease; SDS, sodium dodecylsulfate.

Methods. Preparation of rat liver ribosomes: animals of both sexes weighing between 150–250 g were killed by decapitation and their livers were removed and immediately immersed into ice-cold physiological saline solution. Fat and connective tissue were removed and the livers were homogenized in a teflon-glass Potter–Elvehjem type tissue homogenizer with two volumes of TKM solution (50 mM trisHCl, pH 7.6; 25 mM KCl; 5 mM magnesium acetate) containing 8.5% sucrose. Nuclei, mitochondria and cell debris were removed by centrifugation at 10 000 *g* for 15 min, and 4.5% sodium deoxycholate was added to the supernatant solution to give a final concentration of 0.5%. Ribosomes were sedimented either in rotor No. 50.1 or No. 40 at maximum speed for 90 min in a Spinco ultracentrifuge L2-65B or L2-65K. The ribosome pellets were suspended in TKM, clarified by centrifugation at 10 000 *g* for 10 min and sedimented again. The washed pellets were suspended in TKM solution and clarified as described above. 60–80% of this ribosome preparation sedimented more rapidly than monomers did.

Treatment of ribosomes with salts: ribosomes were mixed with salts at 0 °C. The composition of the mixture was the following: ribosomes 0.5 to 2 A_{260}/ml, 50 mM tris-HCl, pH 7.6, 25 mM KCl, 5 mM magnesium acetate and salts in the indicated concentrations. The pH of NH_4Cl was adjusted to 7.6 by addition of concentrated NH_4OH.

After mixing ribosomes with the indicated salt, samples were immediately layered onto 10 to 30% (w/w) linear sucrose density gradient containing TKM and the salt in a concentration corresponding to the sample. The centrifugations were performed as described in the legends to figures.

In the experiments in which the products of salt treatment were further analyzed the concentration of ribosomes during treatment with salt was 20 A_{260}/ml and the ribosomes were layered immediately or 14 hours after the addition of salt on 3 ml of 1 M sucrose solution containing TKM and centrifuged at 64 000 rpm in rotor No. 65 of Spinco ultracentrifuge L2-65B for 3 hours. The pellet was suspended in TKM solution, clarified by centrifugation at 10 000 *g* for 10 min and analyzed by sucrose density gradient centrifugation as described in the legends to figures. All operations were carried out between 0 and 4 °C.

Incubation with RNase: ribosomes and salt-treated ribosomes purified from salt as described above at 34 OD_{260} units/ml concentration in TKM solution were incubated with 0.1 μg/ml of pancreatic RNase at 0 °C for 10 min. To destroy RNase activity diethyl pyrocarbonate was added to the reaction mixture at the end of incubation, as described by Hüvös et al. (1970a).

Liberation and sedimentation analysis of RNA from the particles were performed as described earlier (Hüvös et al., 1970a).

All gradients were monitored by the ISCO model UA-2 density gradient analyzer.

Polyacrylamide-gel electrophoresis of ribosomal proteins: Ribosomal proteins were isolated by the modified method of Spitnik-Elson (1965), which uses 3 M LiCl and 5 M urea to dissociate ribosomal proteins and nucleic acids. After standing for about 16 hours at 4 °C the precipitated RNA was removed by centrif-

ugation and the proteins were dialysed overnight against 6 M urea containing 0.06 M acetate buffer, pH 6.8, and 10% sucrose at 4 °C. About 200 μg of protein was subjected to acrylamid-gel electrophoresis at pH 4.3, according to the modified method of Reisfeld et al. (1962), essentially as described by Leboy et al. (1964). However, more concentrated gels which contained 18% acrylamide and 0.1% methylene bisacrylamide were used in a discontinuous buffer system in the presence of 6 M urea with 3 mM per tube in the first 30 min, then 5 mA per tube for a further 3 hours at 4 °C. After electrophoresis the proteins were fixed by soaking the gel rods in 20% sulfosalicylic acid solution overnight, stained with 0.01% coomessie brilliant blue R-250 in 12.5% trichloroacetic acid (Chrambach et al., 1967) and destained with 7% acetic acid.

Results

Breakdown of polysomes by salts

Since the concentration of magnesium ion strongly influences the structure of ribosomes, its concentration was kept at 5 mM throughout the experiments described here. Figs 1 and 2 show the polysome content of ribosomes treated with various salts (NaCl, KCl, NH_4Cl, LiCl) in either 0.4 or 0.8 M concentration. It can be seen that all salts applied effected the degradation of polysomes with the concomitant increase of material in the region of monomers. This degradation depends upon the concentration of salts: whereas 0.4 M breaks down only a portion of polysomes, in 0.8 M solutions breakdown is almost complete.

To decide whether the degradation of polysomes depended on the nature of the anion, alkali salts containing bromide and sulfate ions (LiBr, Li_2SO_4, K_2SO_4) were also added to ribosome preparations. In Fig. 3 it can be seen that these salts, in the concentration of 0.8 M, also disrupted polysome structure, which indicates that chloride ion, in itself, cannot be responsible for the degradation of polysomes. It has to be mentioned that although there are some differences among the sedimentation patterns, the breakdown of polysomes is an effect common to all salts.

The fact that salts in 0.4 M concentration degrade only a portion of polysomes suggests that this action may be continuous. Fig. 2 demonstrates that LiCl in 0.075 and 0.15 M concentrations dissociates some polysomes and the amount of monosomes increases parallel with LiCl concentration. Thus it may be concluded that the disruption of polysomes begins at a very low concentration of LiCl and becomes practically complete at 0.8 M.

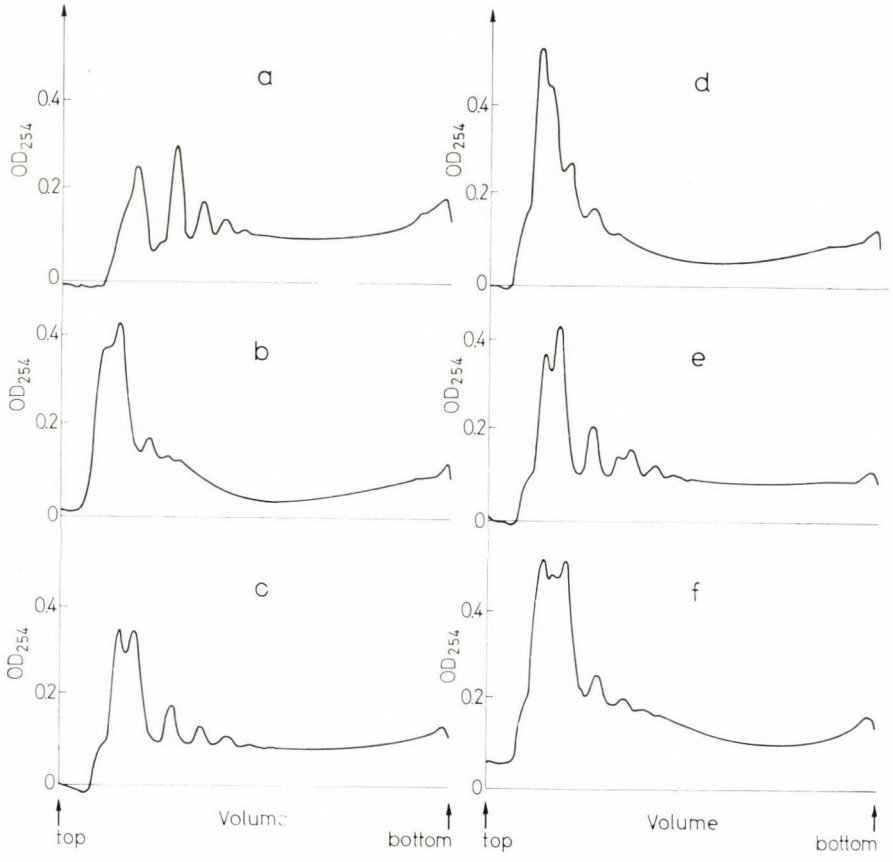

Fig. 1. Effect of alkali chlorides on rat liver polysomes in the presence of 5 mM Mg^{2+} ions. Rat liver ribosomes were treated with a) – ; b) 0.8 M NaCl; c) 0.4 M KCl; d) 0.8 M KCl; e) 0.4 M NH_4Cl; f) 0.8 M NH_4Cl, and immediately layered onto a 10 to 30% (w/w) linear sucrose density gradient containing TKM and the indicated salt in the concentration corresponding to the sample and centrifuged at 41 000 rpm in rotor No. SW-41 at 4 °C for 40 min. The gradients were evaluated by the ISCO model UA-2 density gradient analyzer using a range of 0.5

Some properties of the particles obtained by treating ribosomes with 0.8 M LiCl

To study the particles formed by treating ribosomes with LiCl, ribosomes (final concentration 20 A_{260}/ml) were mixed with LiCl (final concentration 0.8 M) and sedimented through 1 M sucrose as described in Methods.

Figs 4 and 5 show that incubation of ribosomes with 0.8 M LiCl at 4 °C for 14 hours does not result in further alterations in the sedimentation pattern

of the material obtained from ribosomes treated with LiCl, thus the degradation of polysomes takes place very rapidly. The resulting material seems to be homogeneous on the basis of its sedimentation properties and sediments a little slower than monomers.

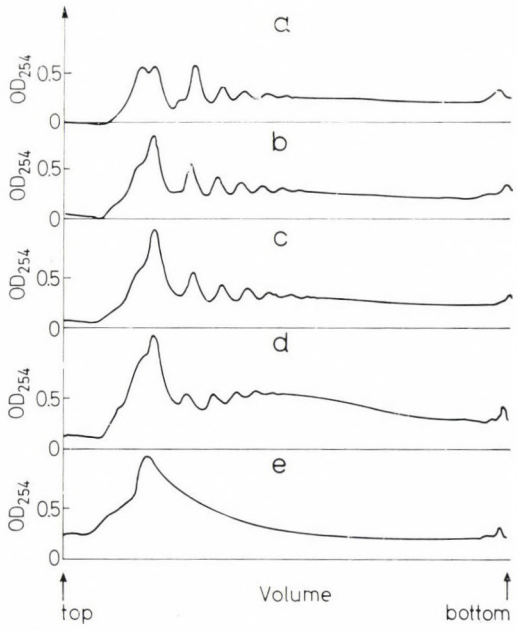

Fig. 2. Effect of LiCl on rat liver polysomes in the presence of 5 mM Mg^{2+} ions. Rat liver ribosomes were treated with a) –; b) 0.075, M c) 0.155 M; d) 0.45 M and e) 0.8 M LiCl, and immediately layered onto 10 to 30% (w/w) linear sucrose density gradient containing TKM and LiCl in the concentration corresponding to the sample and centrifuged at 24 000 rpm in rotor No. SW-25.1 at 4 °C for 120 min. The gradients were evaluated by the ISCO Model UA-2 density gradient analyzer using a range of 2.5

To decide which RNAs are present in the particles resulting from the degradation of polysomes, the RNA liberated from the particles was analyzed by sucrose density gradient centrifugation. As shown in Fig. 6 the particles contained both 18*S* and 28*S* ribosomal RNA in the intact form.

From the experiments described above it follows that disruption of polysomes with LiCl results in particles which contain 18*S* and 28*S* ribosomal RNAs. This fact suggests that both ribosomal subunits are present in these particles. By the addition of EDTA the two subunits can be separated, the smaller subunit sediments with the same velocity as that obtained from control ribosomes, while the larger subunit seems to sediment somewhat slower than that of control ribosomes (Fig. 7). The decrease in sedimentation velocity may be attributed to the loss of certain ribosomal proteins or to other structural alterations in the particles.

The proteins from salt-treated ribosomes were analyzed by polyacrylamide-gel electrophoresis. As shown in Fig. 8 no difference could be found by this method between the protein patterns of control and LiCl- or KCl-treated ribosomes; however, the release of a few ribosomal proteins cannot be excluded on the basis

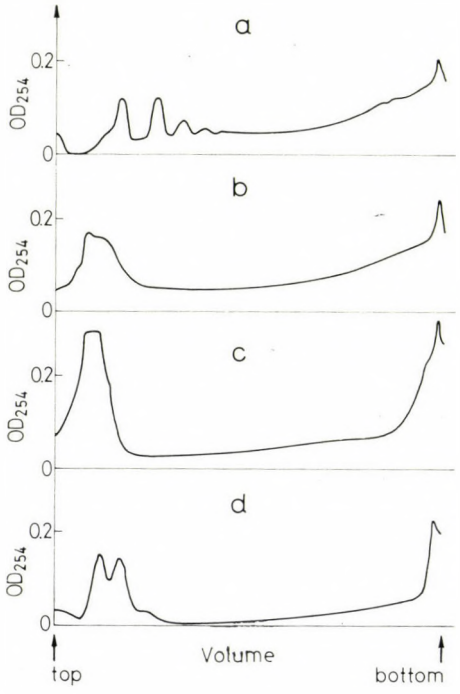

Fig. 3. Effect of salts on rat liver polysomes in the presence of 5 mM Mg^{2+} ions. Rat liver ribosomes were treated with a) —; b) 0.8 M LiBr; c) 0.4 M Li_2SO_4; d) 0.4 M K_2SO_4, and analyzed as described in Fig. 1

of this experiment. That structural alterations take place in the particles formed by treating ribosomes with 0.8 M LiCl is suggested by the fact that the sensitivities of ribosomal RNAs in the LiCl-treated and control ribosomes towards pancreatic RNase differ significantly (Fig. 9).

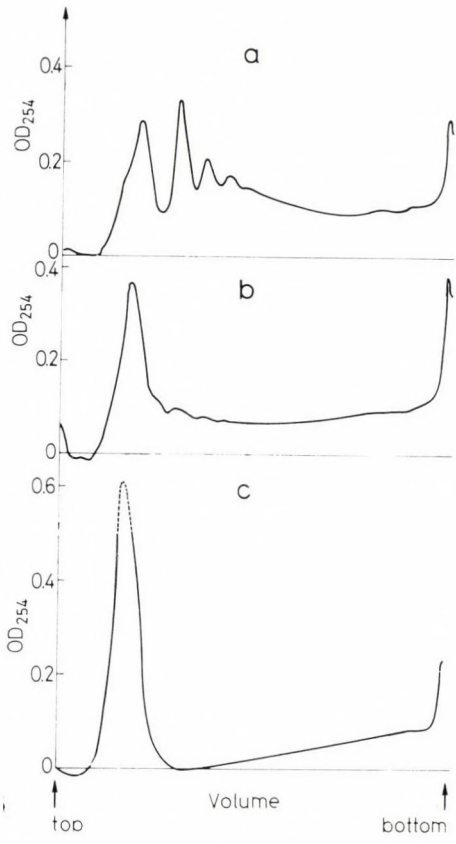

Fig. 4. Time course of degradation of polysomes upon LiCl treatment. Ribosomes were treated with 0.8 M LiCl in the presence of 5 mM Mg^{2+} ions and sedimented through 1 M sucrose, b) immediately after the addition of LiCl, c) after incubation at 4 °C for 14 hours as described in Methods. a) Untreated control which was stored at 4 °C for 14 hours before sedimenting through 1 M sucrose layer. The pellets were suspended in TKM and layered onto 10 to 30% (w/w) linear sucrose density gradient containing TKM and centrifuged at 41 000 rpm in rotor No. SW-41 at 4 °C for 40 min. The gradients were evaluated by the ISCO model UA-2 density gradient analyzer using a range of 0.5 (_____) or 2.5 (. . . . .). In the latter case the results obtained were magnified fivefold.

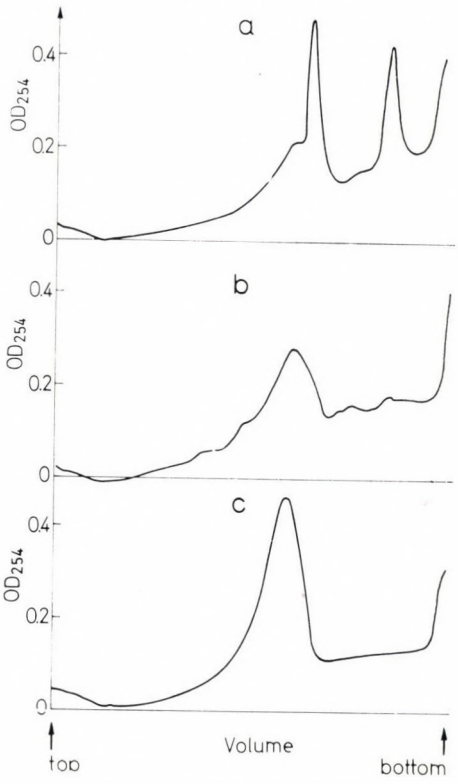

Fig. 5. Sedimentation patterns of particles derived from ribosomes treated with 0.8 M LiCl. Aliquots of the particles analyzed for polysome content in Fig. 4 were layered onto 10 to 30% (w/w) linear sucrose density gradient containing TKM and centrifuged at 41 000 rpm in rotor No. SW-41 at 4 °C for 3 hours. Letters *a*, *b* and *c* indicate the same preparations as in Fig. 4. The gradients were evaluated by the ISCO model UA-2 density gradient analyzer using a range of 0.5

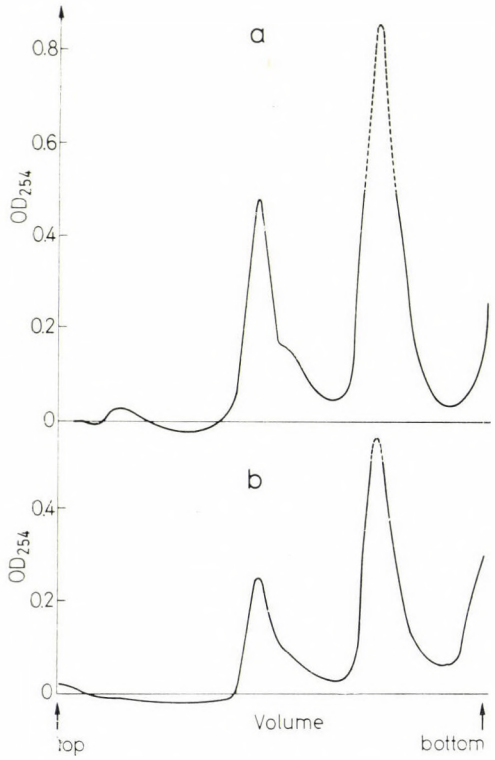

Fig. 6. Sucrose density gradient analysis of RNA from particles obtained by treating ribosomes with LiCl. Rat liver ribosomes were treated with 0.8 M LiCl and sedimented through 1 M sucrose as described in Methods. To the samples taken for RNA analysis diethylpyrocarbonate was added to prevent RNase action. The RNA was liberated by SDS as described earlier (Hüvös et al., 1970a) and layered onto 5 to 20% (w/w) linear sucrose density gradient and centrifuged at 24 000 rpm in rotor No. SW-25.1 at 4 °C for 15 hours. a) RNA from untreated control; b) RNA from 0.8 M LiCl-treated ribosomes. The gradients were evaluated by the ISCO model UA-2 density gradient analyzer using a range of 0.5 (_____) or 2.5 (.....). In the latter case the results obtained were magnified fivefold

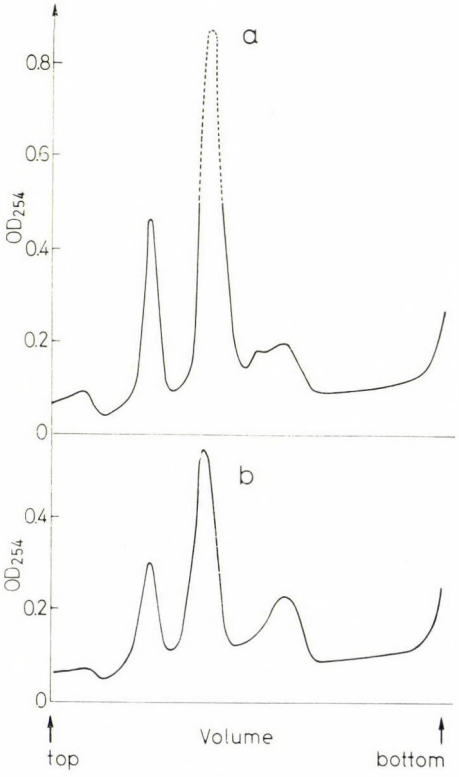

Fig. 7. Effect of EDTA on particles derived from ribosomes treated with 0.8 M LiCl. Ribosomes were treated with a); – b) 0.8 M LiCl, sedimented through 1 M sucrose and the pellets were suspended in TKM solution as described in Methods. EDTA was added in 1 mM final concentration and the samples were layered onto 10 to 30% (w/w) linear sucrose density gradient containing 50 mM tris-HCl, pH 7.6, 25 mM KCl and 1 mM EDTA, and centrifuged at 41 000 rpm in rotor No. SW-41 at 4 °C for 3 hours. The gradients were evaluated by the ISCO model UA-2 density gradient analyzer using a range of 0.5 (_____) or 2.5 (.....). In the latter case the results obtained were magnified fivefold

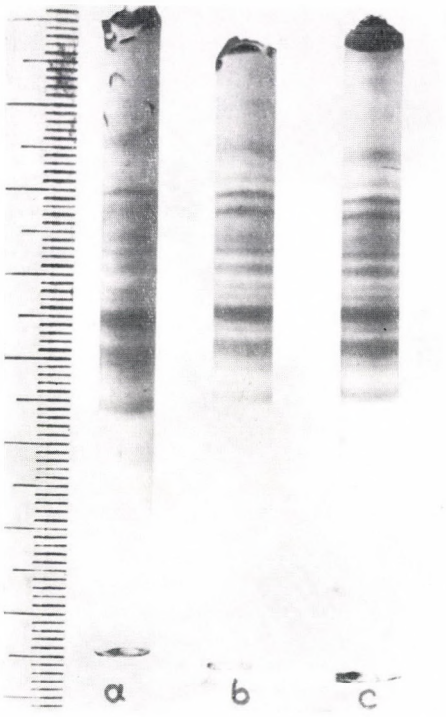

Fig. 8. Polyacrylamide-gel electropherogram of proteins from LiCl- and KCl-treated ribosomes. Ribosomes were treated with 0.8 M LiCl or 0.8 M KCl in the presence of 5 mM Mg^{2+} ions, sedimented through 1 M sucrose. The proteins were isolated from the pellets and subjected to polyacrylamide-gel electrophoresis as described in Methods. The proteins are from untreated control ribosomes (a); from particles obtained by treating ribosomes with 0.8 M LiCl (b); with 0.8 M KCl (c)

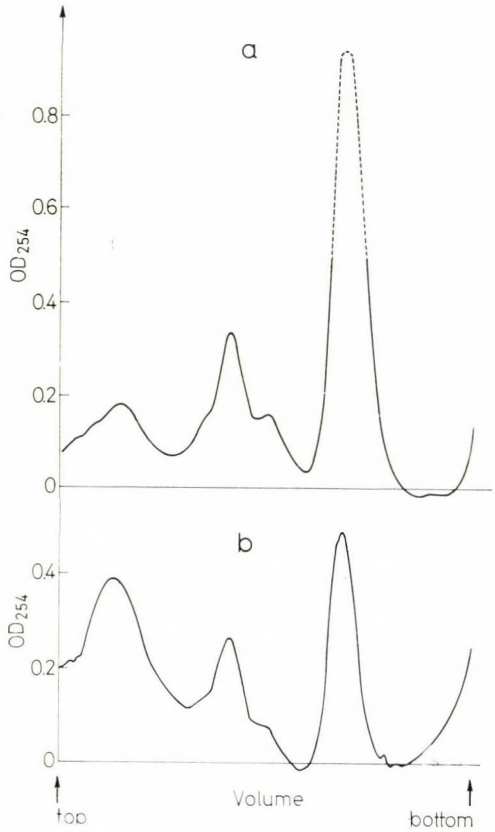

Fig. 9. RNase sensitivity of ribosomes treated with 0.8 M LiCl. Ribosomes were treated with 0.8 M LiCl and sedimented through 1 M sucrose as described in Methods. The pellets were suspended in TKM and incubated with pancreatic RNase at 0 °C for 10 min. (Final concentration of ribosomes: 34 OD_{260} units/ml; final concentration of pancreatic RNase: 0.1 μg/ml in the reaction mixture.) The reaction was stopped by the addition of diethyl pyrocarbonate and the RNAs were liberated by SDS and analyzed as described in Fig. 6. a) RNA from control ribosomes incubated with RNase; b) RNA from LiCl-treated ribosomes incubated with RNase. The gradients were evaluated by the ISCO model UA-2 density gradient analyzer using a range of 0.5 (_____) and 2.5 (. . . . .). In the latter case the results obtained were magnified fivefold

Discussion

In the present work we studied the effect of various salts on rat liver polysomes and we observed the degradation of polysomes in the presence of 5 mM magnesium acetate upon addition of salts (LiCl, NaCl, KCl, NH_4Cl, LiBr, Li_2SO_4) in 0.075 to 0.8 M concentration. Among the sedimentation patterns of ribosomes treated with different salts some minor differences can be found, and these differences may be attributed to the specific action of individual salts. However, an effect common to all salts was clearly demonstrated: the degradation of polysomes. The products of the degradation caused by LiCl were subjected to more detailed analysis.

The extent of degradation of polysomes is a function of the concentration of the salt applied. LiCl added in a final concentration of 0.075 M resulted in a slight degradation of polysomes and the degradative effect increased with increasing salt concentration. Thus the degradation of polysomes seems to be a continuous rather than a stepwise process.

The degradation of polysomes upon salt treatment may be attributed either to the disruption of messenger RNA by endogeneous RNase, or to the release of messenger RNA or messenger RNP (Lissitzky et al., 1970).

The first possibility seems to be unlikely since both the 18*S* and 28*S* RNA's were intact in particles obtained by treating ribosomes with 0.8 M LiCl and it was previously found that the sensitivity of messenger and ribosomal RNA's did not differ significantly towards pancreatic RNase in eucariotic polysomes (Hüvös et al., 1970b). The fact that incubation of ribosomes with 0.8 M LiCl for 14 hours did not alter the sedimentation properties of the particles, and that the results were reproducible, also suggest that degradation of polysomes may not be due to nucleolytic action.

The second possibility, i.e. the release of messenger RNA or messenger RNP from polysomes, may be visualized as a direct effect of salts on the binding forces between ribosomes and messenger RNA, or it may be a result of the effect of salts on ribosome structure. The direct effect of salts on the binding forces cannot be verified experimentally since the nature of the binding forces between ribosomes and messenger RNA is not known.

On the other hand, the possibility of changes in ribosome structure is supported by certain observations. The particles obtained upon treatment of ribosomes with 0.8 M LiCl contained both 18*S* and 28*S* ribosomal RNA's in intact form but sedimented a little slower than monomers. Similarly, the larger subparticle of the LiCl-treated ribosomes also seemed to sediment slower than that from untreated control ribosomes. The slight decrease observed in the sedimentation velocity of the LiCl-treated ribosomes and the higher sensitivity of these particles towards pancreatic RNase strongly suggest that these particles possess an altered structure. From the results described above it cannot be decided whether the degradation of polysomes is due to the structural alteration of ribosomal particles or this alteration is a secondary effect of LiCl following polysome disruption.

Our results show that degradation of polysomes into particles resembling monomers is not necessarily accompanied by dissociation into subunits, i.e. these processes are independent of each other, since the particles obtained by treating ribosomes with LiCl can be dissociated into subparticles by EDTA. Martin et al. (1969) reported that KCl in concentrations higher than 0.5 M brought about the degradation of rat muscle polysomes into particles of 90 and 105*S*, when the treatment was carried out at 4 °C, but the treatment of ribosomes with 0.8 M KCl at 28 °C resulted in the formation of subunits.

In preliminary experiments we also found that 0.8 M LiCl at 28 °C caused the dissociation of polysomes into subunits. These results suggest that temperature plays a critical role in the dissociation process.

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Chemical Modification of Various Phosphorylases in the Presence of Allosteric Effectors

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The inhibitory effect of glucose-6-phosphate on the enzyme activity, conversion to phosphorylase-*a* and tryptic digestion of phosphorylase-*b* prepared from rabbit, bovine and pig skeletal muscle have been studied. In the presence of glucose-6-phosphate the three phosphorylases of different origin exhibited not only different enzymic activities but also differed in their ability of being converted to phosphorylase-*a* and in their tryptic digestibility. Rabbit phosphorylase-*b* was inhibited to the greatest extent by glucose-6-phosphate in respect of all the three above abilities. The inhibitory effect of glucose-6-phosphate was less pronounced in the three tests with bovine phosphorylase, whereas no inhibition was found in case of pig phosphorylase-*b* in any of the three tests. The results seem to support the earlier assumption according to which the inhibition by glucose-6-phosphate on the conversion reaction of phosphorylases is caused by an effect on phosphorylase-*b* used as substrate, rather than on phosphorylase kinase.

Introduction

According to recent data in the literature the chemical modification, i.e. phosphorylation of the enzyme phosphorylase by phosphorylase kinase (in the following kinase) and its dephosphorylation by phosphorylase phosphatase (in the following phosphatase), may be regarded as a new regulatory mechanism (Holzer 1969). Whereas allosteric effectors induce changes in the tertiary and quaternary structure of enzyme proteins, the chemical modifications alter the primary structure by the formation or rupture of covalent bonds, which may also result in conformational changes.

It is known that the activity of rabbit skeletal muscle phosphorylase-*b* (E. C. 2.4.1.1) can be inhibited by glucose-6-phosphate (Morgan, Parmeggiani, 1964; Madsen, Shechosky, 1967; Silanova et al., 1969). In our previous investigations we have shown (Bot et al., 1970) that glucose-6-phosphate decreases not only the activity of phosphorylase-*b* but also affects the conversion of the enzyme, i.e. it reduces the rate of phosphorylation catalyzed by the kinase. We have shown that AMP is capable of suppressing this inhibition. We assumed that glucose-6-phosphate and AMP exerted their effects not on the transforming enzyme, kinase, but rather affected kinase reaction in an indirect way by altering the conformation of phosphorylase-*b* used as substrate.

Recent experiments have shown (Davis et al., 1966; Vereb, Csornai, 1970 Hanabusa, Kohno, 1969; Will et al., 1970) that glucose-6-phosphate inhibits the

activities of various phosphorylase-*b*'s to different extents. On this basis it could be assumed that the different behaviour of phosphorylases will also manifest itself in the kinase reaction and that kinase reaction will be susceptible to inhibition by glucose-6-phosphate only if phosphorylase-*b* used as substrate is also sensitive to this substance, i.e. its enzymic activity can be inhibited by this effector.

In the present work we compared the inhibition of glucose-6-phosphate on enzymic activity and on the conversion of phosphorylases from rabbit, bovine and pig muscles. It was shown that the tryptic digestion of phosphorylases which can be inhibited by glucose-6-phosphate was also influenced by this effector, which indicates that conformational changes took place in these phosphorylases.

Our experiments provide further evidence for the assumption that the effectors influencing kinase reaction exert their effects not on the kinase but rather on phosphorylase-*b* used as substrate.

Materials and methods

Preparation of phosphorylase-b

Rabbit muscle phosphorylase-*b* was prepared according to the methods of Fischer and Krebs (1962). The first crystallization was made from 0.05 M glycerophosphate–0.05 M mercaptoethanol buffer,pH 6.8, containing 0.1 M Mg-acetate and 0.01 M AMP. The second crystallization was performed from the same buffer but without Mg-acetate and AMP. The nucleotides were removed from the preparation by treatment with Norit-cellulose (Fischer, Krebs, 1958). The absorption ratio E_{260}/E_{280} was 0.59, which fell within the range of values found in the literature (DeVincenzi, Hedrick, 1967; Bresler, Firsov, 1968). Further treatment with Norit-cellulose did not alter the ratio.

Bovine muscle phosphorylase-*b* was also prepared by the above procedure. This phosphorylase-*b* crystallized readily both in the presence and absence of AMP and magnesium acetate.

The first part of the isolation of pig muscle phosphorylase-*b* was also conducted according to the method of Fischer and Krebs (1962). However, as pig phosphorylase-*b* could not be crystallized in the presence of AMP and magnesium, the preparation was further purified by DEAE-cellulose column chromatography.

Pig phosphorylase-*b* (about 5 000 units) was loaded on a DEAE-cellulose column previously equilibrated with 0.5 mM glycerophosphate–1 mM EDTA–15 mM mercaptoethanol buffer, pH 6.8. Column size: 2.5 × 50 cm. The phosphorylase dissolved in 10 ml of the equilibrating buffer was applied to the column, then the column was washed with the same buffer until contaminating proteins were removed. The washing required about 800 ml of buffer at 5° and a flow rate of 40 ml per hour. The protein content in the eluate was monitored by the absorption at 280 nm. The adsorbed and washed phosphorylase was then eluted with 1 mM glycerophosphate–1 mM EDTA–15 mM mercaptoethanol buffer, pH 6.8, at a flow rate of 40 ml per hour. Fractions of 20 ml were collected and phosphorylase content

was determined by activity assay. The fractions containing phosphorylase were poled and dialysed against saturated ammonium sulphate solution. The precipitated phosphorylase was centrifuged, the pellet was dissolved in 1 ml of 20 mM glycerophosphate–1 mM EDTA–15 mM mercaptoethanol buffer, pH 6.8, at 5°, then the solution was dialysed against the same buffer until the complete removal of ammonium sulphate.

Pig phosphorylase-*b* purified in this way had a specific activity of 25 units per mg of protein. It proved to be homogeneous on gel-filtration on a Sephadex G-200 column (0.9 × 70 cm). After the removal of nucleotides by treatment with Norit-cellulose the ratio E_{260}/E_{280} was 0.64. This preparation could be completely converted to phosphorylase-*a*.

Preparation of phosphorylase-b kinase

Kinase was prepared from rabbit muscle according to Krebs et al. (1964). The purified enzyme was converted to the active form in the presence of Ca^{2+} ions (Krebs, Fischer, 1962). The product in 0.05 M glycerophosphate–0.002 M EDTA buffer, pH 7.0, could be stored in the frozen state for several weeks without loss of activity.

Assay of the kinase reaction

The rate of kinase reaction was measured by a slightly modified method of Krebs et al. (1964). The incubation mixture contained: phosphorylase-*b* 130 to 150 units per ml; kinase in an amount that does not transform more than 10% of phosphorylase-*b* in 5 minutes; ATP 0.003 M; Mg^{2+} 0.01 M, in 0.02 M glycerophosphate–0.02 M Tris–0.02 M mercaptoethanol buffer, pH 6.8.

The reaction was started by the addition of ATP and Mg^{2+}. The incubation was carried out for 5 minutes at 30°. After incubation the reaction was stopped by the addition of 0.04 M glycerophosphate–0.002 M EDTA buffer, pH 6.8. The amount of phosphorylase-*a* produced was determined by the method of Illingworth and Cori (1953) by measuring the quantity of inorganic phosphate (P_i) liberated from glucose-1-phosphate. P_i was determined by the method of Taussky and Shorr (1953).

The amount of phosphorylase-*b* and phosphorylase-*a* is expressed in units; one unit of enzyme liberates 1 μmole of P_i in one minute. The activity of phosphorylase-*a* was measured without AMP, that of phosphorylase-*b* in the presence of 1 mM AMP.

Determination of tryptic digestion

The rate of tryptic digestion was determined by following the decrease of activity of phosphorylase-*b* measured in the presence of 1 mM AMP. The incubation mixture contained: phosphorylase-*b* 20 to 23 units per ml; trypsin 40

μg/ml, in 0.04 M glycerophosphate–0.01 M mercaptoethanol–0.002 M EDTA buffer, pH 6.8; the phosphorylase : trypsin weight ratio was 10 : 1. Incubation was performed at 30°. At appropriate time intervals samples were taken and diluted 30fold with 0.04 M glycerophosphate–0.01 M mercaptoethanol–0.002 M EDTA, pH 6.8, containing also soy-bean trypsin inhibitor in a fivefold molar excess. The activity of phosphorylase-*b* was assayed according to the method of Illingworth and Cori (1953) in the presence of 1 mM of AMP.

Results and discussion

Inhibition by glucose-6-phosphate of the activity of various phosphorylase-b preparations

First the inhibition by glucose-6-phosphate of the enzyme activity of various phosphorylase-*b* preparations was compared. Table 1 shows the inhibitory effect of glucose-6-phosphate on the activity of rabbit, bovine and pig muscle phosphorylases at two different AMP concentrations.

It can be seen in Table 1 that the activity of rabbit phosphorylase is inhibited to the greatest extent by glucose-6-phosphate at both AMP concentrations. Bovine phosphorylase is inhibited to a lesser extent, whereas pig phosphorylase-*b* is only slightly, if at all, inhibited by 1 or 2 mM glucose-6-phosphate in the presence of 0.1 and 1 mM AMP.

Table 1

Effect of glucose-6-phosphate on the activity of various phosphorylases

The reaction mixture contained: 16 mM glucose-1-phosphate, 1% glycogen in 0.02 M glycerophosphate–0.01 M mercaptoethanol-0.001 M EDTA buffer, pH 6.8, and phosphorylase-*b* in amounts to convert not more than 15% of glucose-1-phosphate in 10 minutes at 30°. The values are given in percentage of the activity measured without glucose-6-phosphate.

Glucose-6-phosphate	AMP	Activity		
mM	mM	rabbit	bovine	pig
		phosphorylase		
0	0.1	100	100	100
1	0.1	30	64	86
2	0.1	13	43	71
0	1	100	100	100
1	1	88	97	100
2	1	77	93	100
20	1	22	70	95

Inhibition by glucose-6-phosphate on the conversion of various phosphorylase-b's from different species to phosphorylase-a

As the above results have shown that the activity of various phosphorylases can be inhibited by glucose-6-phosphate to different extents, it was reasonable to assume that glucose-6-phosphate might exert different effects on the conversion of these enzymes to phosphorylase-*a*. The inhibitory effect of glucose-6-phosphate on the kinase reaction is shown in Fig. 1 with rabbit, bovine and pig phosphorylase-*b*'s used as substrate.

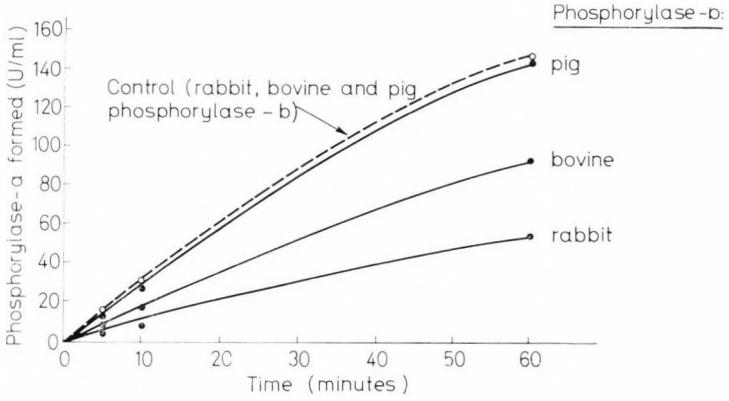

Fig. 1. Effect of glucose-6-phosphate on the conversion of phosphorylase-*b* to phosphorylase-*a*. ●——●: kinase reaction in the presence of 20 mM glucose-6-phosphate; ○ — — ○: kinase reaction in the absence of glucose-6-phosphate. The assay of kinase reaction and that of phosphorylase-*a* activity is described in Methods

It is seen in Fig. 1 that the three types of phosphorylase-*b* are converted to phosphorylase-*a* at the same rate in the absence of glucose-6-phosphate. If 20 mM glucose-6-phosphate was added the conversion of rabbit phosphorylase-*b* was inhibited in the highest degree, whereas glucose-6-phosphate had no effect on the conversion of pig phosphorylase.

Inhibition by glucose-6-phosphate on the tryptic digestion of various phosphorylases

It is known that the enzymic activity of phosphorylase-*b* is lost during the course of limited tryptic digestion. Muszbek et al. (1968) and Muszbek and Sümegi (1970) have shown that the tryptic digestion of phosphorylase-*b* can be inhibited by glucose-6-phosphate. The inhibition can be attributed to the conformational change of phosphorylase-*b*.

The differential sensitivities of rabbit, bovine and pig phosphorylases towards glucose-6-phosphate with respect to enzymic activity and conversion to phosphor-

ylase-*a* called the attention to the possibility that this metabolite might also affect the tryptic digestibility of these proteins in different manners. Fig. 2 shows the tryptic digestion of the three types of phosphorylase-*b* in the presence and absence of glucose-6-phosphate.

It is apparent from Fig. 2 that in the absence of glucose-6-phosphate the three phosphorylase-*b*'s are digested by trypsin at the same rate, i.e. their activity measured in the presence of AMP is lost with the same rate.

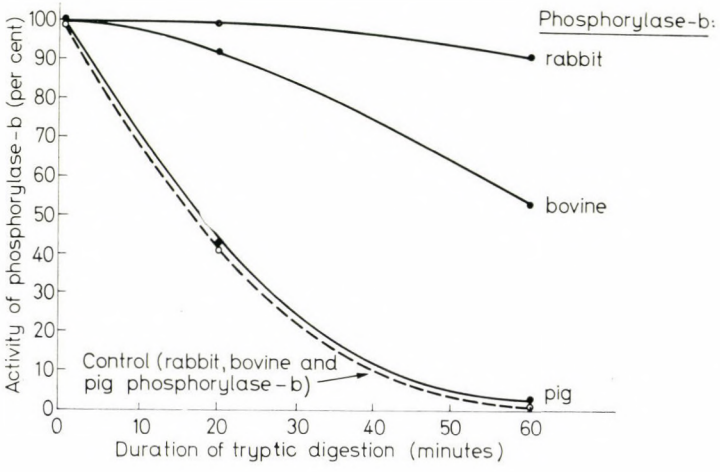

Fig. 2. Effect of glucose-6-phosphate of the tryptic digestion of various phosphorylases. ●———●: limited tryptic digestion in the presence of 20 mM glucose-6-phosphate; ○— — —○ limited tryptic digestion in the absence of glucose-6-phosphate. Activities are expressed in per cent of the untreated controls

However, in the presence of glucose-6-phosphate the digestibilities of the three phosphorylase-*b*'s become markedly different. It can be seen that 20 mM glucose-6-phosphate almost completely inhibits the digestion of rabbit phosphorylase-*b*, whereas the digestion of the bovine phosphorylase-*b* is only partly inhibited and the digestion of pig phosphorylase-*b* is not affected at all.

Our results indicate that there is a close relationship between the inhibitions by glucose-6-phosphate on the enzymic activity, conversion to phosphorylase-*a* and tryptic digestion of the different phosphorylase-*b* preparations. Table 2 summarizes the inhibition by glucose-6-phosphate of the enzymic activity, kinase reaction and tryptic digestion of the different phosphorylases.

It is to be seen in Table 2 that glucose-6-phosphate has parallel effects in the three tests. Rabbit phosphorylase is inhibited by glucose-6-phosphate to the greatest extent in all the three tests. With bovine phosphorylase the metabolite gives only partial inhibition, whereas pig phosphorylase is entirely resistant to the effect of glucose-6-phosphate in all of the three tests.

Table 2

Inhibitory effect of glucose-6-phosphate on the activity, kinase reaction and tryptic digestion of various phosphorylases

The values shown in the table were calculated from the data of Table 1 and Figs 1 and 2

Phosphorylase	Specific activity U/mg protein			Kinase reaction phosphorylase-*a* produced U/ml/10 min			Tryptic digestion decrease of phosphorylase-*b* activity, U/ml/60 min		
	rabbit	bovine	pig	rabbit	bovine	pig	rabbit	bovine	pig
Glucose-6-phosphate 0 mM	50.0	21.0	25.0	32	30	31	20	18	21.5
Glucose-6-phosphate 20 mM	10.6	14.8	23.8	9	19	29	2	10	21.5
Inhibition by glucose-6-phosphate, per cent	78	30	5	72	37	6	90	45	0

Conclusions

It is well established that the conversion of phosphorylase-*b* to phosphorylase-*a* is affected by several circumstances, such as the activated or non-activated state of kinase, the pH of the incubation medium, the ratio of Mg^{2+} to ATP. However, there are still few data available about the fact that phosphorylase-*b* itself, i.e. its conformational state, may also influence the rate of conversion (Bot et al., 1970).

Phosphorylase-*b* preparations from rabbit, bovine and pig muscle proved to be heteroenzymes that display different sensitivities towards the inhibitor glucose-6-phosphate. The different sensitivity manifests itself not only in the activity of the enzyme but also in its conversion to phosphorylase-*a*. We have shown that glucose-6-phosphate inhibited kinase reaction only if the phosphorylase-*b* used as substrate was sensitive towards glucose-6-phosphate, i.e. its enzymic activity too could be inhibited by the metabolite (rabbit and bovine phosphorylase-*b*).

Based upon the observation that the tryptic digestions of rabbit and bovine phosphorylases could also be inhibited by glucose-6-phosphate, it can be assumed that the metabolite causes conformational changes in these proteins. Accordingly, the inhibition by glucose-6-phosphate of the conversion catalyzed by kinase can be ascribed to the altered conformation of phosphorylase-*b* used as substrate.

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The Effect of Polyethyleneglycol on the Iodine-Sensitivity of Penicillinase from B. cereus

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It has been shown that polyethyleneglycol, depending on its molecular weight, can influence the iodine-sensitivity of penicillinase. In the presence of a few μg per ml of polyethyleneglycol the "pH resistant" reaction of the iodine-induced inactivation of penicillinase rapidly takes place even in an otherwise unfavourable, mildly acidic (pH 6) medium.

Introduction

We have previously described that the inactivation of penicillinase with iodine can be resolved into two independent reactions. One of the reactions, designated by us as "pH sensitive", is completed within 1 min at pH 9 and 0 °C in a medium containing 10^{-3} N iodine. As a result of this reaction 30 to 35% of the enzymatic activity is lost, and one molecule diiodotyrosine per mol can be detected in the modified enzyme. The other reaction, designated by us as "pH resistant", proceeds only at pH's higher than 9, or at lower pH but at higher temperature (Csányi et al., 1970; Ferencz et al., 1971; Mile et al., 1970b).

As shown in our earlier investigations a short incubation in concentrated urea or in a strongly alkaline medium results in conformational changes which promote both iodine-induced inactivation reactions (Hajdu et al., 1970; Mile et al., 1970a).

On oxidation in mildly alkaline (pH 9) medium one of the tryptophyl side chains of penicillinase is oxidized. In this way the enzyme conformation formed in the alkaline medium becomes stabilized, which renders possible that the "pH sensitive" reaction readily takes place even in acidic (pH 6) medium (Csányi et al., 1971).

It has also been shown that the competitive inhibitor of penicillinase, methicillin, specifically influences the "pH resistant" reaction. Methicillin enables the reaction to proceed also in the acidic pH region (Ferencz et al., 1971).

In the present paper we describe our experiments on the effect of low concentrations of polyethyleneglycol on the iodine-induced inactivation reaction.

Materials and methods

The exopenicillinase produced by B. cereus strain 569/H has been used. The purification of the enzyme was described earlier (Csányi et al., 1970).

Polyethyleneglycols of different molecular weights were purchased from Fluka AG, the potassium salt of penicillin-G was prepared by Biogal. (If it is not mentioned otherwise, polyethyleneglycol of 4000 molecular weight was used.)

The conditions of iodine treatment of penicillinase have been earlier discussed in detail (Csányi et al., 1970). The enzymatic assay of penicillinase has also been described (Csányi, 1961). Polyethyleneglycol up to 20% concentration does not affect the enzymatic activity of penicillinase.

In discussing the results the symbols introduced earlier are used: A_{R6} denotes the enzymatic activity remaining after iodine treatment at pH 6 in the percentage of the activity of the untreated control; A_{R9} denotes the enzymatic activity remaining after iodine treatment at pH 9 in the percentage of the untreated control. As described earlier, A_{R6} and A_{R9} represent the two steps of the inactivation of penicillinase (Mile et al., 1970a).

Results

The substances that affect the iodine-sensitivity of penicillinase can be divided into two groups. To the first group belong urea and guanidine. These substances are effective only at high concentration and can be regarded as aspecific from the point of view of enzyme function. Substances belonging to the other group,

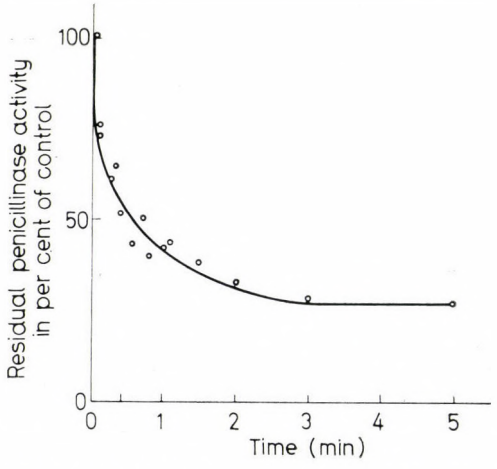

Fig. 1. Time course of iodine-induced inactivation of penicillinase in the presence of polyethyleneglycol. 1000 units of penicillinase was iodinated in 5 ml of 0.05 M phosphate buffer, pH 6, in the presence of 7×10^{-3} N iodine and 20 μg/ml PEG at 0 °C for different time periods. After removing excess iodine the enzymatic activity of the samples was measured and expressed as the percentage of the activity of the untreated control

(methicillin, cephalosporin) exert their effect already in very low concentrations and, as substrate analogues, they may be regarded as strictly specific with respect to enzyme function (Citri et al., 1958, 1960, 1966).

By systematic search eventually we found a substance which influences the iodine sensitivity of penicillinase even at very low concentration, but from the point of view of enzyme function, at least according to our present knowledge, it cannot be regarded specific. This substance is polyethyleneglycol (PEG).

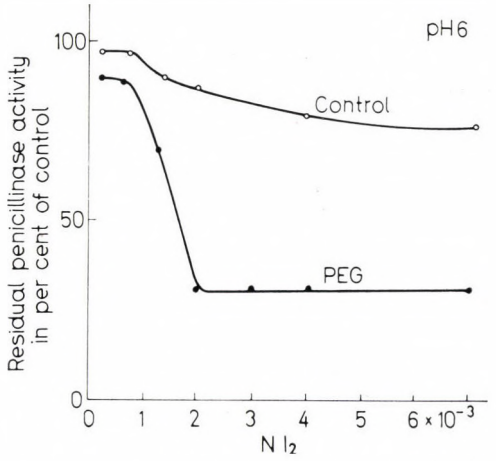

Fig. 2. Effect of iodine concentration on the inactivation of penicillinase at pH 6 in the presence of PEG. 1000 units of penicillinase was iodinated in 5 ml of 0.05 M phosphate buffer, pH 6, in the presence of 20 μg/ml of PEG and various concentrations of iodine at 0 °C for 3 min.

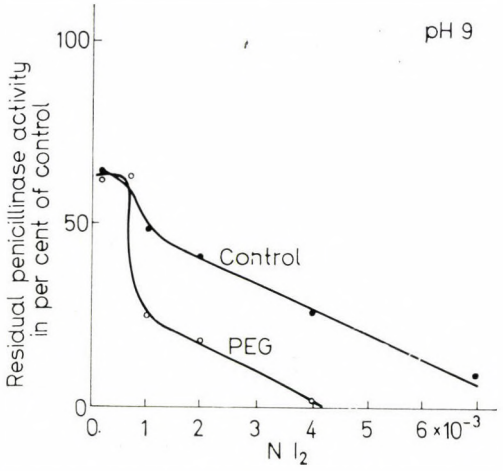

Fig. 3. Effect of iodine concentration on the inactivation of penicillinase at pH 9 in the presence of PEG. 1000 units of penicillinase was iodinated in 5 ml of 0.05 M phosphate buffer, pH 9, in the presence of 20 μg/ml of PEG and various concentrations of iodine at 0 °C for 3 min.

Fig. 1 shows the effect of a low concentration (20 μg/ml) of 4000 molecular weight PEG on the time course of inactivation of penicillinase by 7×10^{-3} N iodine at pH 6. Maximal inactivation is reached in 2.5–3 min, and even after prolonged incubation 30–35% of the initial enzymatic activity is present.

Figs 2 and 3 show the effect of iodine concentration on the extent of inactivation at pH 6 and 9, respectively.

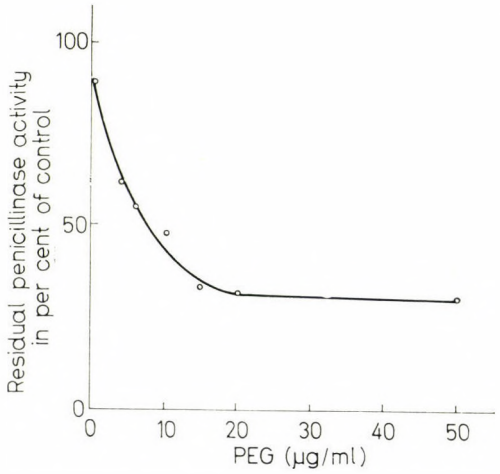

Fig. 4. Effect of PEG concentration of the iodine-induced inactivation of penicillinase at pH 6. 1000 units of penicillinase was iodinated at 7×10^{-3} N iodine concentration at pH 6 and 0 °C for 3 min in 5 ml of 0.05 M phosphate buffer at various PEG concentrations

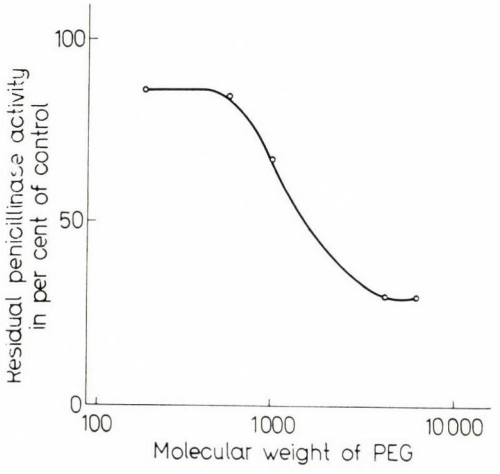

Fig. 5. Effect of the molecular weight of PEG on the iodine-induced inactivation of penicillinase. 200 units of penicillinase was iodinated at pH 6 in the presence of 20 μg/ml of PEG of different molecular weights, with 7×10^{-3} N iodine at 0 °C for 3 min in 5 ml of 0.05 M phosphate buffer

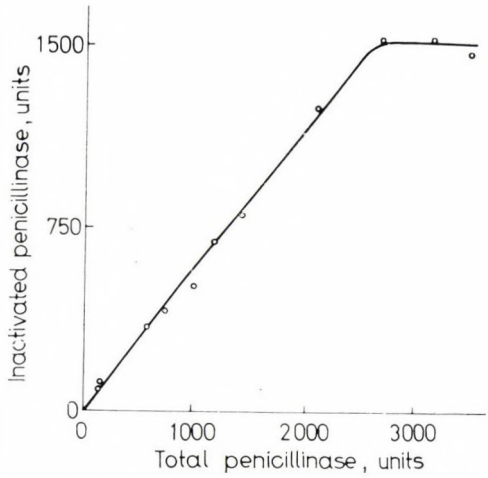

Fig. 6. Effect of penicillinase concentration on the iodine-induced inactivation in the presence of PEG. The amount of penicillinase indicated on the abscissa was incubated in 5 ml of 0.05 M phosphate buffer in the presence of 10 μg/ml of PEG with 7×10^{-3} N iodine for 3 min. In the figure the amount of inactivated enzyme is plotted as a function of initial amount of enzyme

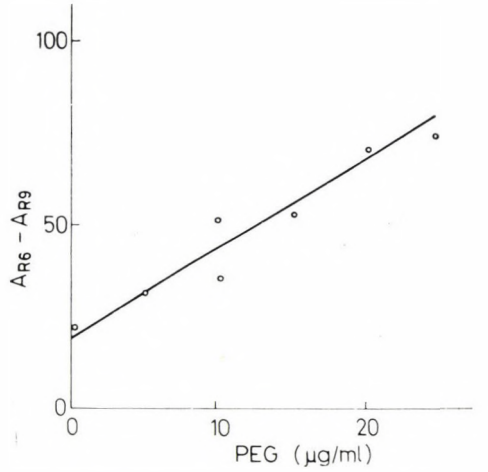

Fig. 7. Effect of iodination carried out at pH 6 in the presence of PEG on the A_{R6} and A_{R9} activities of the enzyme. Penicillinase was iodinated with 7×10^{-3} N iodine for 3 min in 5 ml of 0.05 M phosphate buffer, pH 6, at 0 °C with PEG concentrations indicated in the figure. After iodination excess iodine was removed with thiosulfate and the solution was diluted 100-fold; then iodination was performed again with 10^{-3} N iodine at 0 °C for 1 min in phosphate buffers, pH 6 and 9. A_{R6} and A_{R9} activities were calculated from the residual activities and their differences were plotted as a function of PEG concentration applied in the first iodine treatment

Fig. 4 shows the effect of PEG concentration on the extent of inactivation. It can be seen that PEG increases the extent of the inactivation of the enzyme to a certain limit.

The experiments indicate that at low PEG concentration the iodine-induced inactivation of penicillinase follows the pattern found in the presence of methicillin (Ferencz et al., 1971). PEG increases the iodine sensitivity of the enzyme, especially at pH 6. The inactivation-enhancing effect, just as in the case of methicillin, can be observed only above 10^{-3} N iodine concentration.

Fig. 5 demonstrates the effect of the molecular weight of PEG on the extent of inactivation. As it is apparent from the figure, up to a molecular weight of 600 there is practically no effect, between 600 and 4000 molecular weights the iodine-sensitivity enhances with increasing molecular weight. Above 4000 the effect is constant.

In the next experiment we examined whether the concentration of PEG or the PEG/enzyme ratio was decisive in the enhancement of iodine sensitivity. Various amounts and concentrations of penicillinase were iodinated at constant PEG concentration and amount. As it is clearly seen in Fig. 6, the enhancement of iodine sensitivity is proportional to the enzyme/PEG ratio, not to PEG concentration.

Since, as with methicillin, also in the presence of PEG iodine-induced inactivation at pH 6 exhibits the greatest difference as compared with the control, it seemed probable that PEG, too, exerted its effect on the "pH resistant" iodine-induced inactivation reaction (Ferencz et al., 1971).

To prove this hypothesis the following experiment was performed. Penicillinase was iodinated with 7×10^{-3} N iodine at pH 6 in the presence of increasing amounts of PEG. After removing iodine the samples were again iodinated after appropriate dilution at pH 6 and in another series at pH 9, i.e. the A_{R6} and A_{R9} activities of samples iodinated in the presence of PEG were measured at a PEG concentration that already did not affect iodine sensitivity. If our assumption was true, i.e. PEG exerted its enhancing effect on the "pH resistant" reaction, then the enzymatic activity remaining after iodination in the presence of increasing amounts of PEG would show an ever greater iodine sensitivity at pH 9 (pH 9 is favourable to the other, "pH sensitive", reaction at 10^{-3} N iodine concentration). As seen in Fig. 7, our assumption proved to be valid.

Discussion

Citri and his coworkers have described that by the measurement of iodine sensitivity of penicillinase the conformational changes of the enzyme can be detected (Citri et al., 1958, 1960). We have shown that iodine-induced inactivation takes place in two steps which are independent of each other. Urea, strong alkaline medium and competitive inhibitors cause a selective or combined enhancement of these iodine-induced inactivation reactions (Csányi et al., 1970; Ferencz et al., 1971; Hajdu et al., 1970; Mile et al., 1970a).

We have shown above that another substance, PEG, also increases the iodine sensitivity of penicillinase at low concentration. Maximal enhancing effect can be achieved already at 25 : 1 PEG : penicillinase molar ratio. It is obvious that here, in contrast to the effect of urea or strong alkaline medium, some kind of a tight, structurally specific binding between penicillinase and PEG is to be assumed.

Oru results indicate that PEG influences only one of the iodine-induced inactivation reactions, the one designated by us as "pH resistant". On the effect of PEG this reaction proceeds even in a mildly acidic medium (pH 6), which is otherwise unfavourable to it. In this respect the effect of PEG is very similar to that of methicillin, where the structural complementarity of the protein molecule and of the agent that influences iodine-sensitivity is obvious. In case of PEG the direct changes which cause the enhancement of iodine-sensitivity remain to be clarified.

The authors express their sincere thanks to Professor F. B. Straub for the valuable discussions and suggestions, and Mr I. Balázs for his devoted and precise technical assistance.

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The Factors Affecting the Stability of the Penicillinase Messenger RNA of B. cereus 569

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It has been shown by treatment with alkali and chloramphenicol that the lag-phase of penicillinase synthesis is influenced by two protein-like factors presumably through the stabilization of messenger RNA. One of these is the already known penicillin-binding factor, the other is a protein participating in the stabilization of messenger RNA. The results are interpreted in terms of the earlier described "firmator" model.

Introduction

In the induction of penicillinase a central role is played by penicillin irreversibly bound to the cells (Pollock, 1950). The inducing penicillin is bound to a substance intimately associated with the cell membrane, designated as penicillin-binding factor, which is very probably a protein (Duerksen, 1964).

The role of penicillin-binding factor in the induction of penicillinase has been earlier studied in detail (Csányi et al., 1967). It has been shown that if the cells are treated in a slightly alkaline medium prior to induction with penicillin, then the lag-phase of synthesis following induction becomes considerably longer, in direct proportion to the duration of alkaline incubation. We assumed that the alkali treatment applied before induction damaged a protein-like substance participating in the stabilization of penicillinase messenger RNA. In the present paper further data are provided which support the above assumption.

Materials and methods

The culturing of B. cereus 569 and the composition of culture medium, as well as the method of alkaline incubation, have previously been described (Csányi, 1966; Csányi et al., 1967).

Penicillinase activity was assayed by means of iodometric titration (Csányi, 1961).

The dry-weight of cultures was determined photometrically.

Results

It was found that prolongation of the lag-phase following induction, i.e. the delayed formation of stable messenger, could be achieved not only by alkaline incubation prior to induction but also by the temporary inhibition of protein synthesis.

Two series of experiments were performed. In the first one the cells were incubated with chloramphenicol for various time periods, then the antibiotic

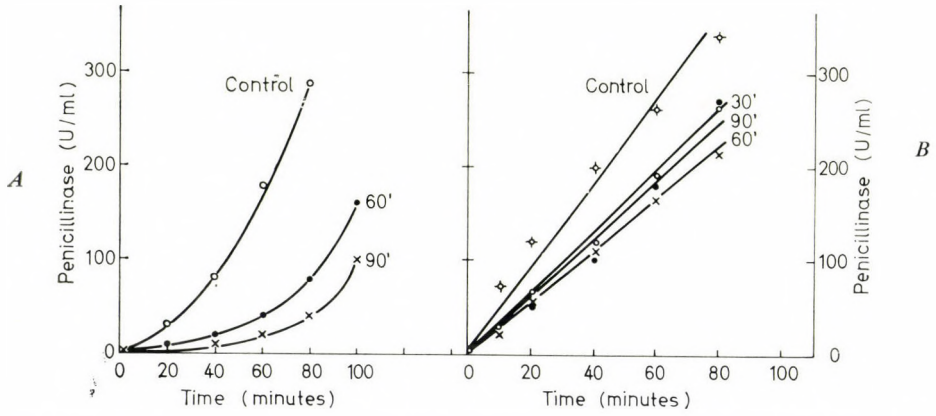

Fig. 1. Effect of chloramphenicol on penicillinase synthesis. *A*. Cells in the logarithmic phase were incubated with 20 μg/ml chloramphenicol for the time periods indicated in the figure, then chloramphenicol was removed by washing the cells repeatedly. Finally, the cells were suspended in fresh medium and were induced with 10 U/ml of penicillin. *B*. Cells in the logarithmic phase were induced with 10 U/ml of penicillin. After 40 min 20 μg/ml chloramphenicol was added to the culture, which was, after different time periods, removed by repeated washing. The cells were suspended in fresh medium and the amount of penicillinase produced was measured

was removed and the synthesis of penicillinase was measured after induction in a fresh culture medium (Fig. 1A). It can be seen that chloramphenicol treatment applied before induction increases the lag-phase, similarly to alkali treatment. After the removal of chloramphenicol the cells grew in the normal way, like the control. In the second series of experiments the cells induced with penicillinase were treated with chloramphenicol for various time periods 40 min after induction, that is in the active phase of penicillinase synthesis. As shown in Fig. 1B, chloramphenicol has no effect on the already functioning penicillinase-synthetizing system.

In our earlier experiments, when the lag-phase was prolonged by means of alkali treatment, we assumed that the alkali affected a protein-like factor, though no direct evidence of this assumption was available at that time (Csányi et al., 1967). By the use of chloramphenicol we also made an attempt to clarify this question.

We examined the effect of the inhibition of protein synthesis and the absence or presence of inducing penicillin on the "regeneration" following alkali treatment. The cells were treated in 0.1 M Tris-buffer, pH 9.5, for 30 min, which increased the lag-phase to about 40 min. After alkaline incubation the cells were suspended

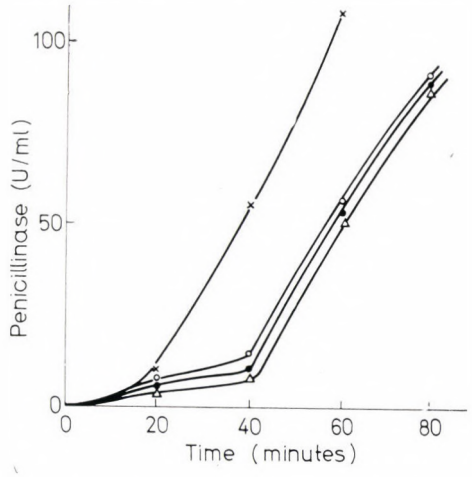

Fig. 2. Effect of chloramphenicol on the regeneration following alkali treatment. Cells grown at 30 °C were subjected to alkali treatment (0.1 M Tris-buffer, pH 9.5, 35 °C, 30 min), then the cell suspension was divided into three portions and centrifuged. The first portion was taken up in culture medium and was induced with 10 U/ml of penicillin (○—○). The second portion was suspended in a medium containing 20 μg/ml of chloramphenicol; the cells were induced with 10 U/ml of penicillin, shaken at 30 °C for 60 min, then centrifuged and suspended in fresh, chloramphenicol-free culture medium (●—●). The third portion was treated essentially in the same way as the second: it was shaken in the presence of chloramphenicol for 60 min, but induction with penicillin was applied only after the cells were transferred into fresh, chloramphenicol-free medium (△—△). The enzyme production of control cells, which did not receive either alkali or chloramphenicol treatment, is also shown (×—×). The dry weight production of all the four cultures was the same in the period examined

in a medium containing chloramphenicol to inhibit protein synthesis possibly required for regeneration. The duration of chloramphenicol treatment was 60 min. One part of the cells was induced with penicillin in the first minute, another part in the last minute of chloramphenicol treatment. The cells were then collected by centrifugation, suspended in fresh medium and the amount of penicillinase was measured. As shown in Fig. 2, the control and chloramphenicol-treated cultures (either induction was performed in the first or last minute of chloramphenicol treatment) began to produce penicillinase in an identical way, i.e. about 40 min after the transfer into fresh medium. It follows that the damage brought about by alkali treatment failed to regenerate under the effect of chloramphenicol, the effects of alkali and chloramphenicol treatments were not superimposed.

Discussion

We have previously described the so-called "firmator" model for the interpretation of the control of penicillinase synthesis, according to which the messenger RNA for penicillinase is being constantly synthetized at a low rate in the inductive cells, but this messenger is metabolically labile. In case of induction with penicillin, the inducing penicillin is bound to a penicillin-binding factor, denoted by us as firmator, and the penicillin-firmator complex would then stabilize the messenger RNA. The lag-phase of penicillinase synthesis is, in turn, the time period during which the firmator-penicillin complexes of the cell become "saturated" with messenger (Csányi et al., 1967). According to our original hypothesis the penicillin-binding factor was identical with the firmator, the substance that stabilizes the messenger.

The present experimental results suggest that the stabilization of the messenger and the binding of penicillin are brought about by two different protein-like substances. We have shown that the lag-phase can be prolonged not only by treatment with alkali, but also by inhibition with chloramphenicol (applied prior to induction). Regeneration after alkali treatment is prevented by chloramphenicol, which indicates that the substance damaged during alkali treatment is a protein.

It is still a question, whether this protein-like substance is identical with the penicillin-binding factor, or it is a different entity. However, since regeneration following alkali treatment is equally abolished whether the inducing penicillin was given at the beginning or at the end of incubation with chloramphenicol (Fig. 2), it seems rather improbable that the penicillin-binding factor and the substance damaged during alkali treatment were identical; indeed, penicillin is strongly bound to the cells, and for the regeneration of the factor influencing the lag-phase protein synthesis is needed. The possibility that penicillin is transferred from a damaged penicillin-binding factor to a newly synthetized one seems not to have any support.

On the basis of available data it appears to be a sound assumption that two different substances take part in the stabilization of messenger RNA. One is the penicillin-binding factor, whereas the other is a substance that directly participates in the stabilization of messenger RNA. The complex of these two may also be called "firmator", since the stabilizing effect of the latter manifests itself only if the former binds penicillin.

We presume that the substance stabilizing the messenger is metabolically labile. In the presence of chloramphenicol (Fig. 1) the synthesis of the stabilizing substance is blocked, which soon results in a decrease of the concentration of the stabilizing substance; this is why there is a prolonged lag-phase in the induction of penicillinase after chloramphenicol treatment.

Our assumptions are supported by the results of Richmond (1966, 1967, 1968), who studying by genetic methods the synthesis of penicillinase in Staphylococcus has described two regulatory proteins: one of them is related to the binding of the inducer, the other to the commencement of messenger synthesis.

In our opinion this modified firmator model is valid not only for B. cereus 569, but also for the inductive penicillinase synthesis of Staphylococcus.

The authors are indebted to Professor F. B. Straub for his continued interest and valuable suggestions.

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The Effect of Induced Withdrawal on the Penicillinase Synthesis Induced Aspecifically with Phosphate in B. cereus

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The functioning of penicillinase-synthetizing system aspecifically induced with phosphate has been studied by gradually withdrawing the inducer. The decrease of penicillinase synthesis followed inducer withdrawal only after a prolonged time period and in direct proportion to cell growth. The results suggest that the messenger RNA participating in the synthesis of penicillinase is stable.

Introduction

We have previously described that penicillinase in B. cereus 569 can be induced with high concentrations of electrolytes, especially phosphate, and in this case the inducer should be permanently present to maintain continuous synthesis (Csányi et al., 1967). Phosphate as inducer is not irreversibly bound to the cells as penicillin, therefore it is suitable to study the behaviour of induced cells after inducer depletion. In case of β-galactosidase, from the effect of inducer depletion conclusions could be drawn as to the rapid turnover of the messenger RNA (Kepes, 1963). For the life span of penicillinase messenger several contradictory values have already been presented (Pollock, 1963; Davies, 1969). It seemed therefore desirable to examine this question by the withdrawal of the phosphate aspecific inducer.

Materials and methods

The culturing of B. cereus 569, the composition of culture medium and the induction with high concentrations of electrolytes have previously been described (Csányi, 1966; Csányi et al., 1967).

Penicillinase activity was assayed by means of iodometric titration (Csányi, 1961).

The dry-weight of cultures was determined photometrically.

Results

We examined the relationship between the concentration of inducing phosphate and the penicillinase production of cells grown for prolonged time periods at different phosphate concentrations. Cells were inoculated into culture media

of different phosphate concentrations and after 10 to 12 hours of growth (in the logarithmic phase) the cells were transferred into a fresh medium of the same phosphate concentration; the changes of dry weight and enzyme production were measured. The change of medium rendered it possible to have identical cell con-

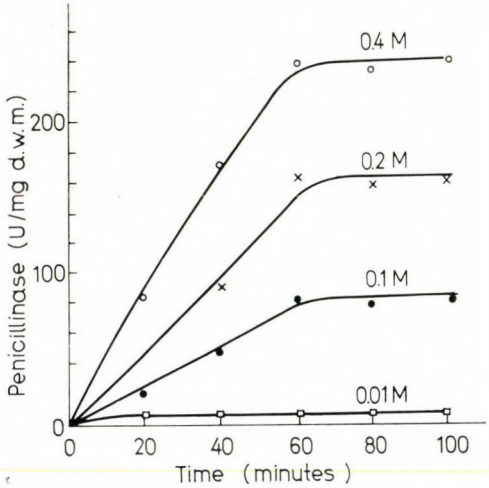

Fig. 1. Effect of phosphate concentration on penicillinase synthesis after prolonged culturing. Cells were inoculated into media containing various concentrations of phosphate at 39 °C and the cultures were shaken. When the logarithmic phase was reached the cells were suspended in fresh medium at 0.1 mg/ml dry weight concentration and the production of penicillinase and dry weight was followed. No significant differences were observed in dry weight production at different inducer concentrations

centrations at the beginning of measurements. If the data obtained are expressed as a ratio of produced enzyme/dry weight and this value is plotted as a function of time, then after attaining equilibrium the height of the plateaus of the individual curves will be proportional to the synthetizing capacity evoked by the inducer. (The various plots and the information obtainable therefrom have been earlier dealt with in detail; Csányi et al., 1967.)

Fig. 1 shows one of the above experiments. It can be seen that as a result of higher inducer concentrations the amount (U/mg) of enzyme produced increases, i.e. the rate of synthesis is enhanced. (To demonstrate the rate of synthesis the differential method of presentation, the plot enzyme produced vs. dry weight, would be more suitable. However, in this case the time course of the process could not be visualized, which would exclude any conclusions to be drawn as to the life span of the messenger.) No significant differences were found in dry weight production at different inducer concentrations. This is due to the fact that the cells were inoculated into media containing the inducer at high concentration and the measurements were done after reaching the logarithmic phase of growth, when the cells had already adapted to high electrolyte concentration and their growth was undisturbed. We have shown in an earlier work (Csányi

et al., 1967) that high electrolyte concentrations affect (inhibit) the growth of the notoriously "salt-resistant" B. cereus only in the early phase of growth, but in the logarithmic phase the rate of growth at high electrolyte concentrations only slightly differs from the control.

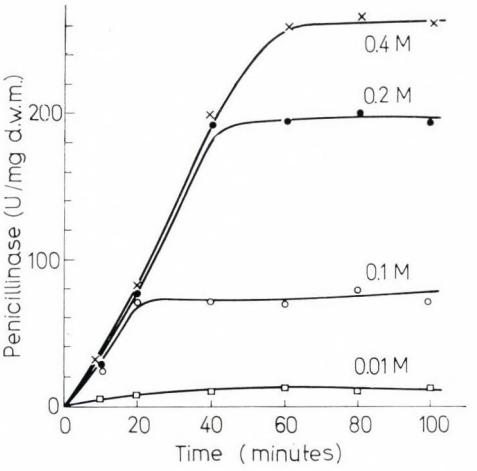

Fig. 2. Changes in penicillinase synthesis upon lowering phosphate concentration. Cells were inoculated into media complemented with 0.4 M phosphate at 39 °C. In the logarithmic phase of growth the cells were centrifuged and suspended in fresh medium of different inducer concentrations (dry weight 0.8 mg/ml) and the production of penicillinase and dry weight was measured

We examined the effect of transfer from media containing the highest inducer concentration used (0.4 M) into media containing lower inducer concentrations. Cells grown in media containing 0.4 M phosphate and being in the logarithmic phase of growth were transferred into media containing lower concentrations of phosphate and the changes in enzyme and dry weight production were registered. As shown in Fig. 2, the decrease in inducer concentration alters the rate of synthesis only after a prolonged time period. It is noteworthy that at 0.1 to 0.4 M inducer concentrations the rate of synthesis runs parallel with that of the control containing high phosphate concentration until it reaches the U/mg value characteristic of the lower inducer concentration and then it becomes constant.

The enzyme production of cells transferred into media containing very low inducer concentrations was studied in more detail. As shown in the experiment in Fig. 2, returning to the normal medium containing 0.01 M phosphate immediately stops enzyme synthesis. However, more refined measurements revealed that the effect is not as simple as that. Fig. 3 demonstrates an experiment similar to that in Fig. 2, but where lower inducer concentrations were studied. It can be

seen that in the 0.01 – 0.025 M range synthesis comes to a standstill very soon. To attain the constant U/mg value requires about 10 min. For the continuous maintenance of synthesis induced with 0.4 M phosphate at least 0.05 – 0.1 M phosphate is needed.

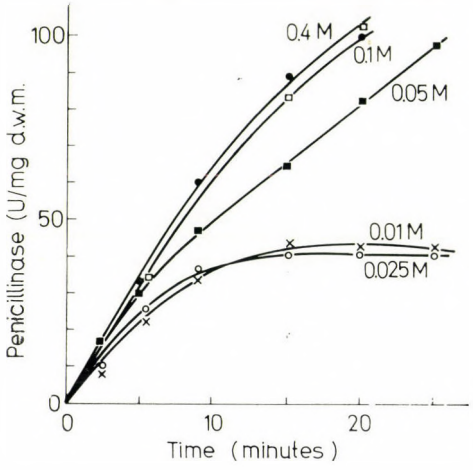

Fig. 3. Determination of minimal phosphate concentration required for the maintenance of penicillinase synthesis induced with phosphate. Cells were inoculated into media complemented with 0.4 M phosphate at 39 °C. In the logarithmic phase of growth the cells were centrifuged and suspended in fresh media of different inducer concentrations. Dry weight content: 0.8 mg/ml. The production of penicillinase and increase in dry weight were measured

Discussion

One of the key substances in the regulation of the synthesis of β-galactosidase, the inductive enzyme studied so far in most detail, is the rapidly decomposing, labile messenger RNA (Jacob, Monod, 1961). The existence of labile messenger has been demonstrated, among others, by investigations in which the cells induced with high inducer concentration were transferred into media containing low inducer concentration and the changes in enzyme synthesis were registered (Boezi, Cowie, 1961). It was shown that in case of β-galactosidase the rate of synthesis was altered within a few minutes after dilution and attained the value corresponding to the new inducer concentration. The underlying mechanism was shown to be the rapid turnover, the marked metabolic lability of the messenger RNA.

In the present experiments with aspecific phosphate inducer we observed that the penicillinase-synthetizing capacity was proportional to inducer concentration (Fig. 1), after prolonged culturing a constant rate of enzyme synthesis was attained characteristic of a given inducer concentration. However, in contrast to β-galactosidase, if the inducer concentration was lowered, the rate of synthesis

was altered only after a prolonged time period (Fig. 2). Enzyme production ran parallel with that of the control, which contained high inducer concentration, until it reached the U/mg value corresponding to the lower phosphate concentration, where it became constant. In 0.1 M phosphate enzyme synthesis ran parallel with the control (0.4 M) for 20 min, whereas in 0.2 M phosphate for 40 min.

From the above data it follows that the messenger RNA formed on the effect of aspecific phosphate inducer remains stable even after the lowering of inducer concentration. At low inducer concentration enzyme synthesis comes to a standstill because the cells multiply and in the new cell-mass penicillinase messenger is formed at the level corresponding to the low inducer concentration. From the experiments with very low inducer concentration it appears that the messenger RNA is decomposed in a biphasic manner. The complete withdrawal of inducer results in an exponentially decreasing synthesis and after about 10 min the messenger RNA formed on the effect of high inducer concentration is already not functioning. However, at inducer concentrations higher than 0.05 M the messenger formed will be functioning until it becomes diluted out by cell growth.

It seems probable that the complete withdrawal of inducer prevents the initiation of new chains and only penicillinase molecules whose synthesis is already under way will be accomplished. This process takes about 10 min. After this stage the decomposition of messenger RNA is probably commenced which, however, escaped detection so far. Above 0.05 M inducer concentration the amount of phosphate is sufficient to chain initiation, therefore synthesis is not ceased, only the messenger RNA becomes diluted out as a result of cell growth.

We hope that by applying the technique of inducer withdrawal data can be obtained about the decomposition and exact life span of messenger RNA.

Thanks are due to Professor F. B. Straub and Dr I. Horváth for their valuable advices.

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Enzymatic Hydrolysis of Glycopeptides from Bovine Fibrinogen with β-Galactosidase and β-N-acetyl-glucosaminidase

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Glycopeptides from bovine fibrinogen were desialysed by mild acid hydrolysis. β-Galactosidase liberates all of the galactose residues from the sialic acid-free glycopeptides. From the intact glycopeptides, however, only a part of the galactose molecules were set free by the same enzyme. From the galactose-free product N-acetylglucosamine was liberated by β-N-acetylglucosaminidase. These observations suggest that a part of the galactose residues are at the nonreducing termini, the other part, however, in penultimate position protected by sialic acid against the action of the enzyme. N-acetylglucosamine groups are the next in the sequence of the oligosaccharide molecule. The galactosyl- and N-acetylglucosamyl residues are linked through β-glycosidic bonds.

Several glycopeptides have been isolated from the proteolytic digest of fibrinogen and their amino acid sequences have also been determined (Haschemeyer et al., 1966; Mester et al., 1967; Mészáros, 1968). The composition of the covalently bound carbohydrate moiety is known; it consists of galactose, mannose, N-acetyl-glycosamine and also some sialic acid (Mester, 1963). The nature of the linkage between carbohydrate and peptide has been already reasonably well established by isolation of 2-acetamido-1-β-(L-β-aspartamido)-1,2-dideoxy-D-glucose (Mester et al., 1967). Mester was able to isolate lactosamine and another disaccharide consisting of two molecules of D-mannose (Mester et al., 1967). From these findings and from the results of periodate oxidation he concluded that sialic acid residues form the nonreducing termini of the carbohydrate chains and are linked to galactose residues.

The aim of this investigation was to get information about the nature of the glycosidic linkages coupling galactose to glucosamine and glucosamine to mannose. Although the chemical methods give valuable information about the structure of polysaccharides, the use of glycosidases (in this case: β-D-galactosidase and β-N-acetyl-glucosaminidase) enables the determination of the structure of oligosaccharides from small quantities of the substance.

Materials and methods

β-D-galactosidase was prepared from pinto beans (Phaseolus vulgaris) by the method of Bahl (Agrawal, Bahl, 1968). It was tested for proteolytic activity and other glycosidase activities with azocoll or the appropriate p-nitrophenyl glycosides as substrates. It was free from proteases, β-N-acetyl-glucosaminidase and α-mannosidase.

β-N-acetyl-glucosaminidase was prepared from pinto beans according to Bahl (1968). The enzyme was free from α-mannosidase and β-galactosidase.

Preparation of glycopeptides has been described in the previous communication (Mészáros, 1968).

Preparation of desialysed glycopeptides. The glycopeptide (100 mg) was treated with 10 ml of 0.1 N sulphuric acid at 80 °C for 75 minutes. After cooling it was fractionated on a Sephadex G-25 (25 × 1000 mm) column. The fractions containing carbohydrate were eluted with water, combined and freeze-dried to yield 65 mg glycopeptide mixture.

Analytical techniques. The neutral sugars were detected by the orcinol-sulphuric acid method (Weimer, Moshin, 1952), galactose and mannose were determined by gas liquid chromatography, either after methanolysis as trimethylsilylethers (Sawardeker, Sloneker, 1965), or after reduction with sodium borohydride as alditol acetates (Sawardeker, Sloneker, 1965). Reducing sugars were detected with aniline-hydrogenphthalate (Wilson, 1959), or with triphenyl-tetrazolium chloride (Fischer, Dörfel, 1954). Sialic acid was detected by the direct Ehrlich method (Böhm et al., 1954), and determined by the thiobarbituric acid method (Warren, 1959). Gas liquid chromatography was performed using a Willy Giede GCHR 18 – 3 analytical gaschromatograph with flame ionisation detector. Separations were made on stainless steel columns (3 × 2000 mm) filled with 10% Carbowax 20 M on Celite 100/120 mesh at 150 °C or a 3% ECNSS-M on Gaschrom Q 100/120 mesh at 190 °C.

Digestion with β-galactosidase. Sialic acid-free glycopeptide mixture (50 mg) was added to the solution of β-galactosidase (21 units) in 0.05 M sodium citrate buffer, pH 4.6 (10 ml). It was thermostated at 30 °C and during enzymatic hydrolysis the digest was checked for β-galactosidase activity with p-nitrophenyl-β-D-galactopyranoside from time to time. Aliquots (100 μl) were taken at different time intervals to follow the reaction. The samples were heated in boiling water bath for 3 minutes, the galactose released was determined by the Nelson – Somogyi method and identified by paper chromatography. Samples for paper chromatography were desalted by passing an aliquot of the digest through a mixed-bed resin with a charcoal layer. After concentration they were chromatographed on Macherey – Nagel 214 paper with galactose and mannose as standards in ethyl-acetate–pyridin–water 4 : 11 : 6.

The digest was purified on a Sephadex G-25 column, after treating it with trichloroacetic acid and the freeze-dried product (30 mg) was analyzed for neutral sugars by gas liquid chromatography.

Enzymatic hydrolysis with β-N-acetyl glucosaminidase. The above compound (10 mg galactose-free glycopeptide) was incubated at 30 °C with 5 units of pinto bean β-N-acetyl glucosaminidase in 3 ml of 0.05 M sodium citrate buffer, pH 4.4. Aliquots were taken at different time intervals to follow the reaction. The liberated N-acetyl glucosamine was estimated by the method of Reissig (1955) and the digest was checked for enzyme activity. After 40 hours the reaction was stopped and the mixture was worked up in the same way as described in the preceding paragraph.

Results and discussion

The gaschromatographic analysis gave the same galactose-mannose ratio for both the sialic acid-free and the intact glycopeptides, which indicated that all of the sialic acid, but none of the galactose or mannose residues were removed by mild acid hydrolysis.

After removal of sialic acid the galactose residues were hydrolysed with Phaseolus vulg.-galactosidase. The positive reaction indicates that the galactose molecules are on the nonreducing termini and are β-glycosidically linked to the backbone. The rate of the release of galactose was monitored by the Somogyi–Nelson method of Bahl (1968b) and the completion of hydrolysis with β-galactosidase was checked by determining the sugar content of the isolated galactose-free product after acid hydrolysis, reduction and acetylation. The resulting mixture of alditol acetates was analyzed by gas liquid chromatography. The gaschromatographic analysis indicated that the hydrolysis of the galactose residues was nearly complete.

In experiments with sialic acid-containing glycopeptides as substrate, only a part of the galactose molecules was set free by β-galactosidase, which indicated that the other part was protected against enzymatic hydrolysis. This is in good agreement with the sialic acid content and suggests that galactose molecules are located at two different positions in the molecule. Those split off were on the nonreducing termini, the resistant ones were in the penultimate position (Table 1).

Table 1

Sugar content of the glycopeptide mixtures
The quantities are expressed in per cent of the glycopeptides

	Galactose	Mannose
Intact glycopeptide mixture	11.6	15.3
After treatment with β-galactosidase	5.7	16.2
Neuraminic acid-free glycopeptide mixture	12.5	16.8
After treatment with β-galactosidase	1.8	19.0

Since galactose residues might be linked to the N-acetyl glucosamine units an attempt was made to remove them from the galactose-free compound by β-N-acetyl glucosaminidase. After 40-hour digestion the level of liberated N-acetyl glucosamine ceased to increase. It follows from this observation that part of the N-acetyl glucosamine residues are β-glycosidically linked.

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Extraction of a Secretory Protein from the Tissues of the Seminal Vesicle of the Rat

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Proteins of the secretion of rat seminal vesicles and those of a hot saline extract prepared from the ether-dried powder of the vesicular tissues were characterized by means of polyacrylamide disc gel electrophoresis. The secretory proteins were resolved into two main components which corresponded, on the basis of their electrophoretic mobility at pH 4.3, to a strongly basic, clottable protein and a neutral protein described by Mányai. Electrophoresis of the proteins of the vesicular tissue extracts resulted in the separation of two major protein bands. On the basis of electrophoretic mobility in gels with different pore size and of the rapid labelling with ^{14}C-valine during incubation of vesicular mince, one of these proteins was identified as the clottable protein or its precursor. The validity of this identification was confirmed by the fact that this protein component was absent from the vesicular extracts of castrated rats, but it could be found again following testosterone treatment of the castrates. Moreover, hot saline extracts prepared from an ether-dried powder of vesicular total secretion were shown to contain the clottable but not the neutral protein, which indicated the preferential extraction of clottable protein with the procedure employed. The other protein of the vesicular tissue extract migrated even slower than the neutral protein of the secretion. It was scarcely labelled upon incubation of the mince of the vesicles with ^{14}C-valine, and was present in the vesicular tissue extracts whether normal or castrated animals or testosterone-treated castrates were the sources of the vesicles.

Introduction

It has been reported (Mányai, 1964) that surprisingly large amounts of a protein-like material can be extracted with concentrated hot sodium chloride solutions from the ether-dried powder of the seminal vesicles of adult rats. The same author has demonstrated, by both in vivo and in vitro experiments, a rapid uptake of labelled amino acids into this material, which had been referred to (Mányai, 1964; 1964a) as the APHSE protein (Acid Precipitate Hot Saline Extract). These and other data suggested that the vesicular secretory proteins,[2] in particular

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[2] There are two major protein components in the vesicular secretion of the rat (Mányai, 1964a). The isoelectric points of these proteins are about at pH 7 and pH 10, respectively. The basic protein has been shown to be responsible for the clotting of the secretion, therefore it may simply be denoted as the clottable protein. The physiological function of the other protein is so far unknown and it will be referred to as the neutral protein.

a strongly basic clottable protein, or their precursors were extracted in the denatured form into this fraction (Mányai, 1964a, 1965; Mányai et al., 1965; Tóth, Mányai, 1968). With respect to the rate of synthesis, the APHSE protein of the castrated animals distinguished itself from the other proteins of the vesicle by a later but much greater response to testosterone (Tóth, Mányai, 1968), a finding which appeared to confirm the above suggestion.

In the experiments presented in this paper the proteins of the vesicular hot saline extract (APHSE protein) were characterized by means of polyacrylamide gel electrophoresis. We have found that only one fraction of the total extracted proteins can be rapidly labelled with ^{14}C-valine, and this protein component is electrophoretically indistinguishable from the strongly basic, clottable protein of the vesicular secretion.

Materials and methods

Materials

Testosterone phenylpropionate (Retandrol) was a product of Richter (Budapest). Acrylamide and methylene-bisacrylamide were obtained from BDH, England. TEMED (N,N,N,N-tetramethyl-ethylenediamine) and β-alanine were the products of FLUKA (Switzerland). Ribonucleic acid was obtained from EGA Chemie KG (West Germany). All the other chemicals were purchased from Reanal (Budapest). (1-^{14}C)-D,L-valine (specific activity: 9.9 mCi/mmole) was obtained from the Isotope Institute (Budapest).

Animals and their treatment

Adult white Wistar rats of 200–300 g body weight were used in the experiments. They were fed on a normal laboratory diet and received food and water ad libitum. Castration was performed via the scrotal route under ether anesthesia. Testosterone treatment of castrated rats consisted of intraperitoneal injections of 2.5 mg testosterone phenylpropionate administered daily for the time periods indicated.

Collection of vesicular secretion

The animals were stunned by a blow on the head, they were then bled to death by cutting through the neck arteries with scissors. The secretion was collected by tapping the seminal vesicles by means of a syringe equipped with a thick needle. The secretion was diluted first with distilled water, then with a solution containing 6 M urea and 10 M lithium chloride to attain a final concentration of 25 mg/ml for protein, 3 M for urea and 5 M for LiCl. The secretion was stored at -20 °C and diluted further with a 3 M urea–5 M LiCl solution just before electrophoresis.

Preparation of tissue extract

The seminal glands were excised and the secretion was removed by pressing. The vesicles were rinsed with ice-cold physiological saline solution, separated from the coagulatory glands, blotted, weighed, chopped with scissors and finally homogenized in ice-cold 0.4 N perchloric acid. The acid precipitate was washed successively with perchloric acid, ethanol and ether as described previously (Mányai, 1964; Tóth, Mányai, 1968) and the ether-dried powder was extracted at 100 °C with a 10% sodium chloride solution for 30 min. Proteins and nucleic acids in this extract were precipitated with 2–3 volumes of 96% ethanol at pH 5.0 and −20 °C. The precipitate was collected, dissolved in water and subsequently reprecipitated with ethanol. The final precipitate was dissolved in a solution containing 3 M urea and 5 M LiCl and stored then at −20 °C. After a few days the insoluble residue was removed by centrifugation.

Electrophoretic procedures

For electrophoresis in discontinuous polyacrylamide gels the procedure of Reisfeld et al. (1962) was used with the modifications described for the resolution of ribosomal proteins (Leboy et al., 1966; Traut, 1966). Electrophoretic runs were carried out at 4 °C with a tenfold dilution of the β-alanine-acetate buffer, pH 4.3, described by Reisfeld et al. (1962) and at a constant current of 5 mA per gel column. The gel columns were 7.5 cm long and 0.5 cm wide. Separating gels were polymerized either from 18% (w/v) acrylamide and 0.1% (w/v) bisacrylamide or from 8% (w/v) acrylamide and 0.2% (w/v) bisacrylamide (unless stated otherwise the former composition was used). Samples (100–250 μg protein) dissolved in 0.025 ml 3 M urea–5 M LiCl solution were applied between two layers of spacer gel. Gels and the tray buffers contained 6 M urea. Protein disks were made visible by staining the gels in a solution of 1% Amidoblack in methanol–water–acetic acid (5 : 5 : 1). The same solvent, without Amidoblack, was used to remove the background stain.

Labelling of APHSE protein in vitro

In order to label the APHSE protein, the mince of the seminal vesicles was incubated in Krebs-phosphate buffer (Krebs, 1950) in oxygen atmosphere with ^{14}C-D,L-valine under continuous shaking at 37 °C for 30 min.

Measurement of radioactivity

The labelled APHSE protein was analyzed by gel electrophoresis. The gels were stained, differentiated and cut into about 2 mm thick slices. The gel segments were homogenized in distilled water in a glass homogenizer. The homogenates were layered on aluminium planchets and dried under an infrared lamp. Radioactivity was measured in a Frieseke–Hoepfner methane gas flow counter.

Chemical determinations

Protein concentration was determined with Lowry's method (Lowry et al., 1951), and horse serum albumin served as a standard. DNA was determined from tissue portions pooled for this purpose and homogenized in 0.4 N perchloric acid. Separation of RNA from DNA was accomplished by the procedure of Schmidt and Tannhauser (cf. Leslie, 1955) and the DNA content was estimated according to Dische (1955).

Results

Gel electrophoresis of vesicular secretion

Disc gel electrophoresis of the vesicular secretion revealed two major protein bands (Figs 1 and 2). These were identified on the basis of their rate of migration at pH 4.3 as the clottable protein and the neutral protein, respectively. In "short time" runs pyronine was applied as tracking dye and electrophoresis was finished when the dye was about 0.5 cm from the outlet of the tube. This required about 4 hours. By this time the clottable protein was about half way along the separating gel, while the neutral protein had migrated only a few mm's in this gel (Fig. 1). The relative rate of migration of the two major secretory proteins could be better estimated when pyronine was allowed to migrate into the cathodic buffer and the electrophoresis was continued for an additional period of three hours (Fig. 2).

Gel electrophoresis of vesicular tissue extracts

Fig. 1 shows the disc electrophoresis of five different hot saline extracts prepared from the vesicles of normal rats. Each tissue extract contains a basic protein fraction which corresponds to the clottable protein of the secretion (for comparison, a sample of secretion was electrophoresed concomitantly). Ahead of and behind this major protein band there are further but less conspicuous bands. However, these are absent from the electropherogram of the secretion. On the other hand, the hot saline extract does not contain a fraction corresponding in electrophoretic properties to the neutral protein (Fig. 2). When electrophoresis of the saline extracts was run longer than 12 hours, a new band appeared in the separating gel. This second major protein component of the vesicular extract migrated even slower than the neutral protein of the secretion under the conditions employed.

Electrophoresis at low acrylamide concentration in the separating gel

As shown in Fig. 3, the increase of pore size of the separating gel, by using gel slabs polymerized from a solution containing 8% (w/v) acrylamide, 0.2% (w/v) bisacrylamide and 0.5% (w/v) TEMED, resulted in patterns which indicated

again that the electrophoretic mobilities of the clottable protein and the aforementioned basic protein of the hot saline extract were much the same. These proteins, however, migrated considerably faster in this gel than in the gels polymerized from 18% acrylamide,[3] even faster than the tracking dye. Moreover,

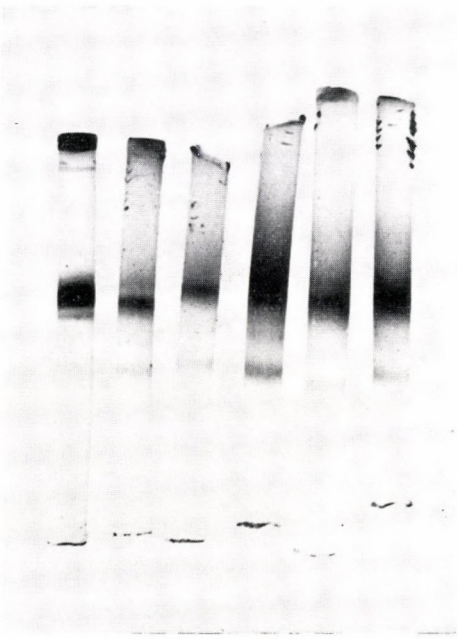

Fig. 1. Electropherograms of proteins of the vesicular secretion (left) and of various hot saline extracts prepared from the seminal vesicles of normal rats. 100—250 μg of protein was applied on top of polyacrylamide gels. Electrophoresis was from top (anode) to bottom (cathode) at pH 4.3 for 5 hours

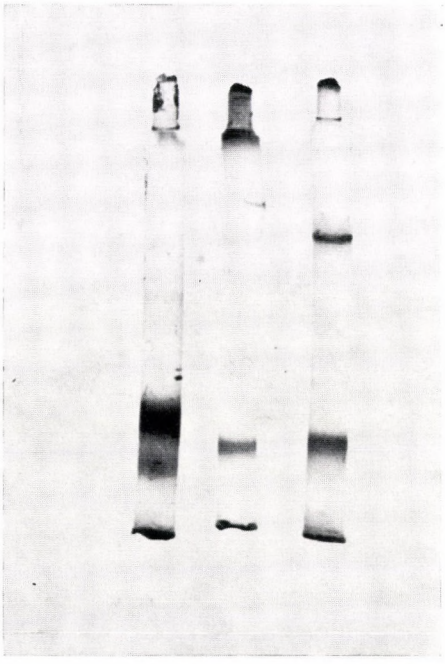

Fig. 2. Electrophoretic patterns after 8-hour runs toward the cathode (bottom). From left to right: two vesicular tissue extracts (230 and 100 μg of protein, respectively) and a vesicular secretion (130 μg protein)

the strongly basic protein of the tissue extract appeared to be resolved into two components. Our attempts to electrophorese the secretory proteins, as well as the APHSE protein, at pH 8.5 in a system similar to that of Davies (1964) but containing 6 M urea have failed.

In vitro labelling of the proteins of the vesicular tissue extract

A mince of the seminal vesicles of adult rats was incubated in Krebs phosphate medium (500 mg in 6 ml) with 6 μCi of ^{14}C-D,L-valine and the hot saline extracts were prepared afterwards as described in Materials and methods. Appro-

[3] This finding may account for the tendency of this protein to be dispersed in a relatively long gel section during electrophoresis (see Fig. 3).

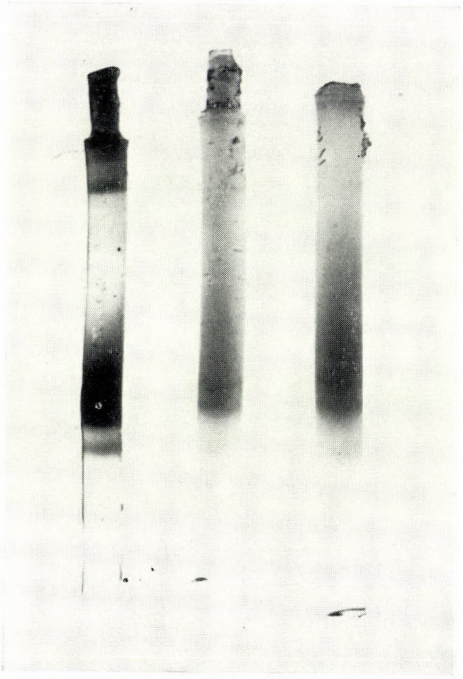

Fig. 3. Electrophoretic patterns obtained in 8 % acrylamide gels. From left to right the electropherograms of vesicular secretion (100 μg protein) and two different APHSE protein fractions (180 and 210 μg protein, respectively) can be seen. Duration of run: two hours, cathode at bottom

priate amounts from these extracts were analyzed by gel electrophoresis. The gels were cut into about 2 mm thick slices and the radioactivity of each slice was determined. The results are demonstrated in Fig. 4. It can be seen that a considerable amount of the label was present in the gel segments containing the basic protein, whereas the protein disc which appeared in the separating gel after 12 hours of migration contained only low levels of radioactivity. Since β-radiation is remarkably absorbed by the gel, it was impossible to calculate the percentage recovery of the total applied counts.

Effects of castration and replacement of testosterone

Following orchiectomy the production of secretory proteins by the seminal vesicle is ceased and the secretory fluid gradually disappears from the gland. Obviously, if the vesicular tissue extracts really contain some derivatives of the clottable protein, they must be devoid of these derivatives when prepared from castrated rats. Analysis of four different tissue extracts prepared from the vesicles of castrates (killed 12 to 14 days after castration) verified our expectation: the band characteristic of the clottable protein could not be demonstrated in these preparations.

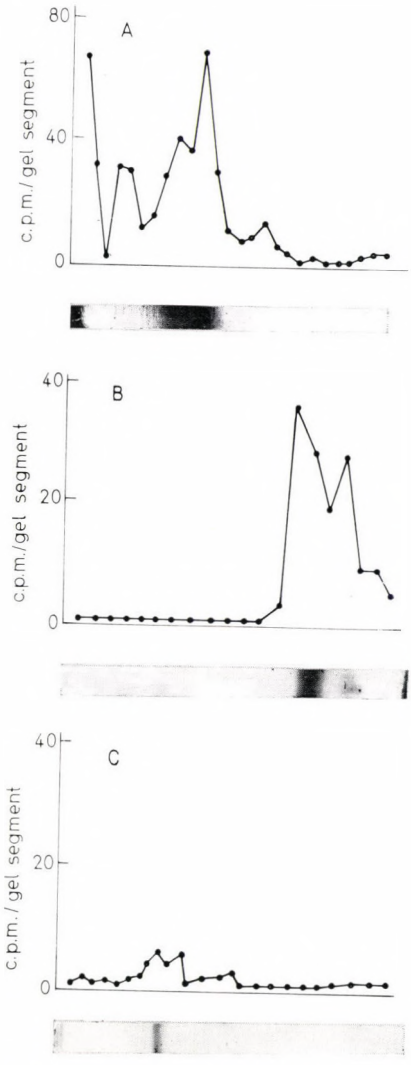

Fig. 4. Distribution of radioactivity among the protein components of the vesicular tissue extract after in vitro incubation of the vesicular mince with ^{14}C-valine. 500 mg mince of the seminal vesicles of 5 normal rats was incubated with 6 μCi ^{14}C-D,L-valine in 6 ml Krebs-phosphate medium for 30 min at 37 °C. Vesicular tissue extracts were prepared by the standard procedure. Electrophoresis was carried out for 4 hours (Fig. A), 8 hours (Fig. B) and 12 hours (Fig. C) in polyacrylamide gels. Migration was from left (anode) to right (cathode). The gels were stained, cut into slices, which were homogenized in distilled water. Samples of the homogenates were dried on aluminium planchets and measured for radioactivity

Fig. 5. Electropherograms of tissue extracts prepared from the seminal vesicles of castrated rats and of castrates treated with testosterone for various periods. Migration was from top (anode) to bottom (cathode) for 4.5 hours. The patterns are from left to right: vesicular secretion (60 μg protein), a preparation from normal rats (180 μg protein), three extracts prepared from the seminal vesicles of castrated rats treated with testosterone for 4, 2 and 1 days, respectively (160—180 μg protein), and a tissue extract from the vesicles of castrated animals (200 μg protein)

In the next experiment the induction of synthesis of the clottable protein after replacement of testosterone was studied by the electrophoretic technique. 30 adult rats were castrated a fortnight prior to the experiment and divided into four groups. Animals in three groups received testosterone phenylpropionate in the form of intraperitoneal injections for 1, 2 and 4 days, respectively, before killing. Rats in the fourth group were not treated with testosterone. The castrated animals and additional 5 normal rats were killed at the same time and from the pooled seminal vesicles of each group hot saline extracts were prepared according to the standard procedure. Comparable amounts of protein from these extracts were analyzed with polyacrylamide gel electrophoresis. The results are demonstrated in Figs 5 and 6. Protein bands corresponding to the clottable protein could be found in the preparations made from castrates treated with testosterone for 2 or 4 days and a similar but scarcely discernible band was observed when castrated rats received testosterone only one day prior to killing. The hot saline extract prepared from the vesicles of castrated animals was devoid of this protein component. By contrast, a similar vesicular extract prepared from normal rats was also shown to contain this protein.

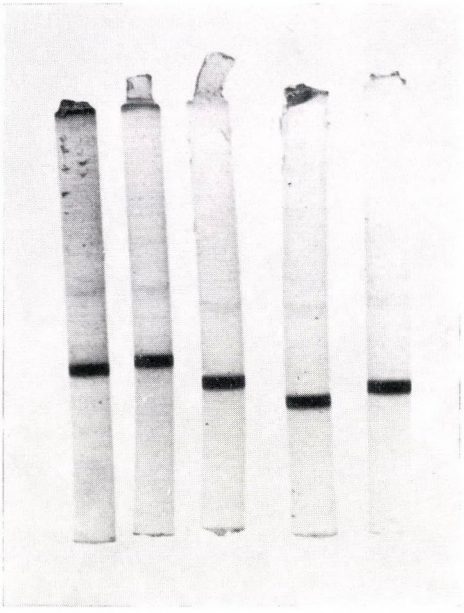

Fig. 6. Electropherograms of the vesicular preparations described in Fig. 5 after migration in polyacrylamide gels for 18 hours toward the cathode (from top to bottom). The sequence of the electropherograms from left to right is the same as in Fig. 5

Table 1

Amounts of protein extracted with 10% NaCl solution at 100 °C from the seminal vesicles of normal and castrated rats and of castrates treated with testosterone for various periods

30 rats castrated a fortnight earlier and 5 intact litter mates were used for the experiment. Three groups of 5—8 rats each had received daily injections of testosterone phenylpropionate for 1, 2 and 4 days, respectively, prior to killing. The vesicular tissue extracts were prepared by the standard procedure

Number of animals	Androgen state of the animals	Amount of extracted protein	
		mg/g wet tissue	mg/mg DNA
10	Castrated, untreated	6.3	1.13
8	Castrates, treated for 1 day	5.1	1.25
7	Castrates, treated for 2 days	4.0	1.25
5	Castrates, treated for 4 days	3.2	0.99
5	Normal, adult rats	3.2	1.39

The amount of the extracted protein was determined and the results are presented in Table 1. In spite of the substantial differences demonstrated by the electropherograms in the basic protein content of these extracts, the amounts of extracted protein (as related to unit quantity of DNA) were in the same range.

The cause of this apparent contradiction became obvious when the samples were electrophoresed for 18 hours. Patterns obtained after such prolonged electrophoresis are seen in Fig. 6. These patterns are uniform, that is, the protein component which migrates slowly under these conditions toward the cathode is equally present in all the five vesicular extracts.[4] When two different preparations from these five were mixed and analyzed by gel electrophoresis, these components were not separated from one another, but each one could be separated from the neutral protein of the secretion.

Extraction of clottable protein from the vesicular secretion

In order to clarify whether the extractability of the clottable protein by the above procedure depends on the formation of some protein–RNA complex, the vesicular secretion was incubated at room temperature for 30 min with and without sodium-ribonucleate (ribonucleic acid was dissolved in distilled water and the pH was adjusted to 6.6 with NaOH). Following incubation proteins and nucleic acids were precipitated with perchloric acid and the precipitates were washed, dried and extracted with a 10% NaCl solution as described for the preparation of tissue extract. It was shown by gel electrophoresis that the clottable

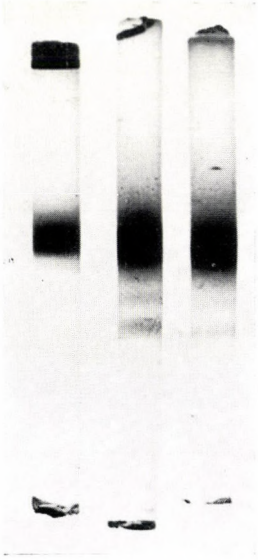

Fig. 7. (From left to right) Electrophoretic patterns of vesicular secretion, a hot saline extract from vesicular tissue and a similar extract from vesicular total secretion. Migration was from top (anode) to bottom (cathode) for 5 hours. Amounts of applied protein in the above order: 80 μg, 150 μg, 80 μg, respectively

[4] It is presumed that in direct proportion to the time period of testosterone administration an increasing part of the APHSE protein becomes a derivative of the clottable protein or its precursor.

protein, but not the neutral one, could be extracted in this way and its extractability was independent of the presence or absence of ribonucleate. When electrophoresis was performed for 5 hours (Fig. 7) the electropherograms obtained with secretion extracts were strikingly similar to those obtained with vesicular tissue extracts.

Discussion

Treatment of the ether-dried powder of various mammalian tissues with concentrated hot sodium chloride solution was originally employed for the extraction of RNA (Davidson, Smellie, 1952). The experiments presented in this paper strongly indicate that in the case of the seminal vesicle of the rat, in addition to RNA, one of the major vesicular secretory proteins, the "clottable" protein (Mányai, 1964a; Mányai et al., 1965) is also extracted from the vesicular tissues by this procedure. The presence of one or two secretory proteins in the vesicular tissue extracts of this type has already been suggested on a more or less tentative basis (Mányai, 1964; Tóth, Mányai, 1968) and attempts have been made to characterize the protein-like material extracted in this way (designated as APHSE protein) by ion exchange chromatography and paper electrophoresis (Mányai, 1964), on the basis of its amino acid composition (Mányai, Beney, 1961) and rate of labelling with various radioactive amino acids (Tóth, Mányai, 1968). Our experiments demonstrate that polyacrylamide disc electrophoresis, as applied for the analysis of the ribosomal proteins, is especially useful for the characterization of the APHSE protein. This is not surprising at all if one takes into account that high urea concentration may be needed to keep in solution the denatured protein substances obtained with the drastic hot saline extraction.

The APHSE protein can be resolved by this procedure into two major protein components. There are three lines of evidence suggesting that one of these components corresponds to the "clottable" protein of the vesicular secretion or to its precursor. First, they migrate similarly to each other in both 18% and 8% acrylamide gels. This finding suggests similarity not only in their specific charge but in their molecular size as well. Second, this protein component can be rapidly labelled by incubating the vesicular mince with ^{14}C-valine. This may reflect a relatively rapid synthetic rate which is thought to be characteristic for a protein produced for secretion. Finally, this protein substance is absent from the vesicular extracts of animals castrated a fortnight earlier but it gradually appears after testosterone administration to the castrated animals. This is in agreement with the dependence of the secretory activity of the seminal vesicles upon the androgenic hormones. The other protein component seems to be some structural protein derivative, which is not influenced by androgens, has a slow turnover rate and its electrophoretic mobility is much smaller under the conditions used than that of the secretory proteins.

It is somewhat surprising that the electrophoretic pattern of the vesicular tissue extract prepared from normal rats is fairly reproducible with the various

preparations. Moreover, when the ether-dried powder of the vesicular total secretion is extracted in a similar way with hot sodium chloride solution, the resulting preparation, after a five-hour run, has an electrophoretic pattern virtually indistinguishable from that obtained with the extracts from vesicular tissues. This finding suggests that the hot saline treatment can specifically solubilize a part of the dried clottable protein. The product extracted obviously represents some denatured forms of the clottable protein, but the size and the net charge of the extracted substance is essentially identical with that of the natural protein.

As to the origin of the clottable protein extracted from the vesicular tissues, the problem arises whether it represents a clottable protein (or its precursor) stored in the vesicular cells or this material originates merely from the secretion which remained, owing to the imperfect removal, in the vesicular tissues. Although the latter alternative cannot be excluded, there are two points which suggest that at least a portion of these polypeptides comes from the secretory cells of the seminal vesicles. First, this component was shown to be labelled rapidly with a radioactive amino acid and, second, its appearance in the extract following testosterone administration to castrated rats preceded the onset of the discharge of vesicular secretory fluid.

It has not escaped our attention that the results presented in this paper may serve as a basis, for the first time, to the study of the synthesis of a well-defined, testosterone-regulated protein. In this context it would be desirable to test the usefulness of our procedure for the study of the synthesis of the clottable protein in a soluble protein synthesizing system obtained from vesicular tissues.

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Comparative Investigations on Bone and Callus Enzymes

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Five bone enzymes and 6 callus enzymes were subjected to quantitative investigations. When comparing the enzyme activities in the bones of four animal species markedly high activities were observed: 1. for acid phenylphosphatase in guinea-pig bones; 2. for acid β-glycerophosphatase in guinea-pig and rabbit bones; 3. for alkaline phosphatase in rabbit bones; 4. for cathepsin in mouse and rabbit bones; 5. for lactate dehydrogenase in guinea-pig bones. In rats no outstandingly high enzyme activities were found. Investigation of the enzyme content of the callus as a function of time elapsed after the fracture has shown that the activity of acid phosphatase increased, while the activities of acid β-glycerophosphatase, alkaline phosphatase and cathepsin decreased, the activities of lactate dehydrogenase and β-glucuronidase first increased then dropped during the 28 days of the examination period.

Introduction

The first important discoveries related to the enzymes of the bone date back to the 1920's (Robison, 1923; Kay, Robison, 1924; Kay, 1926). Attention then was focused on the role of acid and alkaline phosphatases in ossification.

The current studies of bone enzymes have three main trends:

1. The majority of investigations are of histochemical character. This procedure has among others the advantage of allowing an insight into the enzymological conditions of individual cells and of enabling a differentiated study of the enzymes of various cells. It has, however, the drawback of offering practically no possibility for quantitative measurements and of having been elaborated so far only for a relatively small number of enzymes.

2. The utilization of tissue cultures for enzyme tests is still at its initial stage. Tissue cultures have been used up to now primarily for the detection of collagenase.

3. Investigations on the enzymes of bone tissue homogenates have as yet been rarely encountered in the literature (Vaes et al., 1965). The advantages and drawbacks of this method will be discussed later, as our present investigations follow this trend.

Materials and methods

Enzyme activity measurements were performed on the following animals:

1. Rat, "R" Amsterdam, inbred, 4 months old, body weight about 150 g.
2. White mouse, BALB/c, 2 months old, body weight 18 to 20 g.
3. Rabbit, 4 months old, body weight about 2500 g.
4. Guinea-pig, 4 months old, body weight about 500 g.

The animals were kept on standard diet (compressed food, water, greens). The rabbits and guinea-pigs were killed by air embolism, the rats and mice by decapitation.

For the bone enzyme tests the diaphysis of the right femur of the animal was used. The bone was removed immediately after death, cut and cleaned of blood and soft parts.

The role of fracture as a stimulus affecting the whole organism has already been studied from the biological aspect (Urist, 1952; Glauber, 1958). We have studied the question by inducing a fracture on the right femur of rats and after 14 days we compared the enzyme content of the left femur with that of the fractured one from which, however, the callus had first been removed.

The enzyme content of the callus was studied on rats. To obtain a callus the right tibia of narcotized rats was fractured. The fractured bone was not fixed, the animals were killed on the 7th, 14th, 21st and 28th day following the fracture. The callus was excised immediately after death and care was taken that no bone or soft part should be included in the specimen. The bone and callus specimens were kept at 0 °C during all operations up to the start of the enzyme reactions.

Disintegration was carried out as follows. The bone and callus were put into ten volumes of 0.25 M sucrose solution (volume/weight). The bones were first minced in an MSE blendor at 14 000 rpm for 1–2 minutes; this gave an adequate disintegration.

The callus was homogenized in a Potter and Elvehjem (1936) type glass homogenizer at 200–300 rpm for 5–10 minutes.

In both cases the material was subsequently treated with ultrasonic oscillation. Ultrasonic disintegration was performed in an MSE ultrasonic oscillator at 20 Kc/sec frequency for 1 minute with not more than 5 ml solution.

The disintegrated specimens were centrifuged in a refrigerated MSE centrifuge at 10 000 *g* for 15 minutes. The supernatant was used for the enzyme assays either directly or after dilution with 0.25 M sucrose solution. Enzyme activities were related to 1 mg total protein. The protein content was determined by Lowry's method (1951).

The following enzymes of the bone were studied: acid and alkaline phosphatases, acid β-glycerophosphatase, lactate dehydrogenase and cathepsin. In the callus β-glucuronidase was also measured.

The measurements were carried out at substrate saturation. The substrate concentrations used in the experiments, together with the true and apparent (for acid phenylphosphatase) values of the Michaelis constants are shown in Table 1.

Table 1

Substrate concentrations used in the enzyme assays and the Michaelis constants

Enzyme	K_M (mM)	Substrate concentration (mM)
Acid phenylphosphatase	0.5—1.0	28
β-Glycerophosphatase	5	50
Cathepsin	0.093	0.46
β-Glucuronidase	0.046	0.28
Alkaline phosphatase	2	65
Lactate dehydrogenase	—	0.3

Lactate dehydrogenase was assayed at 25 °C, the other enzymes at 37 °C. Three parallel measurements were made with each specimen for all enzyme reactions. In all cases a blank was used in which the enzyme reaction was stopped at 0 minutes. When longer reaction times were needed the reaction mixture also contained Na-merthiolate to prevent infection.

1. Assay of acid phosphatase

A. Assay of acid phenylphosphatase. The reaction mixture contained 28 mM phenylphosphate, 67 mM citric acid and 10 mM 4-aminoantipyrine in a final volume of 3 ml. The pH of the reaction mixture was 4.9 and the reaction time 30 minutes (Hansen, 1966).

The enzyme reaction was stopped with a solution which gave at the same time a colour reaction with the liberated phenol. This solution contained 6.7% NaOH, 1.4% $NaHCO_3$ and 2.4% $K_3(Fe(CN)_6)$. After one hour standing the optical density of the solution was measured at 500 nm.

One unit of enzyme activity corresponds to the liberation of 10 μg of phenol in 30 minutes under the reaction conditions described.

B. Assay of acid β-glycerophosphatase. The reaction mixture contained 50 mM β-glycerophosphate and 0.1 M acetate buffer, pH 5, in a final volume of 0.5 ml (Vaes, 1965). The reaction times varied between 16 and 18 hours. The enzyme reaction was stopped with trichloroacetic acid, and the inorganic phosphate content was determined from the filtrate. The blue colour which developed on the addition of ascorbic acid and Na_2MoO_4 was measured photometrically at 700 nm after 15 minutes standing.

One unit of enzyme liberated 1 μmole of phosphate in one hour.

2. Assay of alkaline phosphatase

The reaction mixture contained 65 mM phenylphosphate, 20 mM $NaHCO_3$, 30 mM Na_2CO_3, 10 mM 4-aminoantipyrine, pH 10.0 in a final volume of 3 ml. The reaction time was 15 minutes (Hansen, 1966).

The enzyme reaction was stopped with a solution of the following composition: 30 mM Na_2CO_3, 20 mM $NaHCO_3$ and 4.8% $K_3(Fe(CN)_6)$. The intensity of the red colour was measured photometrically at 500 nm after 15 minutes standing. One unit of enzyme liberated 10 μg of phenol in 15 minutes.

3. Assay of cathepsin

Cathepsin activity was quantitatively determined from the amount of aromatic degradation products formed in haemoglobin digestion. The reaction mixture contained 0.46 mM haemoglobin and 0.3 M acetate buffer, pH 3.6 in a final volume of 1.2 ml. The reaction time was 16 to 18 hours. The enzyme reaction was stopped with trichloroacetic acid and the aromatic degradation products were measured in the filtrate by means of the Folin–Ciocalteu reagent (Anson, 1937). The intensity of the blue colour was measured photometrically at 730 nm. One unit of enzyme liberated the amount of aromatic degradation products equivalent to 1 μmole of tyrosine in one hour.

4. Assay of lactate dehydrogenase

This enzyme catalyzes the following reaction: Lactate + $DPN^+ \rightleftarrows$ pyruvate + DPNH + H^+ (Bergmeyer, 1963).

The activity of lactate dehydrogenase was measured from the pyruvate + reduced diphosphopyridine nucleotide side. The decrease in optical density which is the measure of DPNH oxidation was determined spectrophotometrically at 340 nm. The reaction mixture contained 50 mM phosphate buffer, pH 7.5, 0.3 mM Na-pyruvate and 0.13 mM DPNH in a final volume of 3 ml. The enzyme solution was diluted so as the optical density decrease per minute should not exceed 0.050.

According to Wróblewski and LaDue (1955) one unit is that amount of enzyme which reduced optical density at 340 nm by 0.001 in one minute.

The measurements were continued for 5 minutes. During this time optical density readings were taken every 30 seconds.

5. Assay of β-glucuronidase

The reaction mixture contained 0.28 mM phenolphthalein glucuronate and 0.038 mM acetate buffer, pH 5.2, in a final volume of 3.6 ml. Reaction time was 16 to 18 hours. The reaction was stopped with 3.0 ml of 0.1 M glycine buffer, pH 11, and the red colour was measured photometrically at 540 nm. One unit of enzyme liberated 0.1 μmole of phenolphthalein in 1 hour.

Results

The first part of Table 2 shows the activities of 5 enzymes measured in the bone samples.

Table 2

Enzyme activities of the bone and callus

The enzyme specific activities are expressed in U/mg total protein, the mean errors and the number (in brackets) of the measurement are also shown

Tissue investigated	Experimental animal	Measured enzymes: Acid phenyl-phosphatase	Acid β-glycero-phosphatase	Alkaline phosphatase	Cathepsin	Lactate dehydrogenase	
Bone	Mouse	9.52 ± 2.5 (13)	0.162 ± 0.022 (13)	171 ± 18 (13)	0.338 ± 0.050 (13)	941 ± 67 (13)	
	Guinea-pig	31.4 ± 6.4 (8)	0.522 ± 0.090 (8)	233 ± 29 (8)	0.153 ± 0.017 (8)	1660 ± 160 (8)	
	Rabbit	11.1 ± 1.6 (13)	0.641 ± 0.097 (13)	773 ± 120 (13)	0.354 ± 0.026 (13)	1108 ± 78 (13)	
	Fractured rat bone	16.4 ± 4.0 (8)	0.220 ± 0.042 (8)	140 ± 22 (8)	0.182 ± 0.009 (8)	655 ± 51 (8)	
	Rat control	12.0 ± 1.8 (8)	0.245 ± 0.028 (8)	328 ± 100 (8)	0.141 ± 0.011 (8)	827 ± 69 (8)	

Tissue investigated	Days after fracture	Acid phenyl-phosphatase	Acid β-glycero-phosphatase	Alkaline phosphatase	Cathepsin	Lactate dehydrogenase	β-Glucuronidase
Callus	7	21 ± 3.2 (5)	0.522 ± 0.049 (5)	1410 ± 138 (5)	0.287 ± 0.034 (5)	983 ± 85 (5)	0.72 ± 0.03 (5)
	14	22 ± 3.8 (5)	0.380 ± 0.034 (5)	1040 ± 65 (5)	0.188 ± 0.020 (5)	1375 ± 182 (5)	1.07 ± 0.14 (5)
	21	29.9 ± 4.3 (5)	0.349 ± 0.030 (5)	875 ± 64 (5)	0.131 ± 0.032 (5)	221 ± 27 (5)	0.99 ± 0.09 (5)
	28	38.2 ± 3.7 (5)	0.337 ± 0.025 (5)	626 ± 76 (5)	0.109 ± 0.007 (5)	146 ± 23 (5)	0.82 ± 0.07 (5)

The second part of Table 2 shows the enzyme activities measured from callus specimens taken at various times after fracture. The specific activities are given in U/mg total protein. The mean errors and the number of measurements are also included in Table 2.

The enzyme activities measured from extracts without ultrasonic treatment were 20 to 50% lower than those after sonication both in bone and callus samples. The protein measurements were in accord with this finding, since the protein content of extracts was increased by 10 to 30% after ultrasonic treatment.

Discussion

Despite all the investigations carried out so far, bone enzyme research is still in its early state particularly on the field of the quantitative and total enzyme content of the bone. The advantage of our method, as compared with histochemical procedures, is the quantitative evaluation of enzyme activities. The drawback of the method is that it fails to provide information on the differentiated enzyme activities of the various kinds of cells of the bone. In our opinion the two methods may favourably complement each other.

Disintegration by means of homogenization and ultrasonic treatments, is far more efficient than any of the hitherto used operation. By means of the described method not only very thin bones, such as the cranial bone of newborn animals, can be digested, but satisfactory disintegration of the diaphysis of long tubular bones of fully developed individuals can also be performed. This means that beside the enzyme content of endesmally developed bones that of enchondrally developed larger and harder bones can also be determined.

The quantity of bone needed for the measurements is very small. Even at the hardest bones it is not more than 1 g, while for callus 0.3 g is sufficient. This enables the measurement of the enzyme content of the small quantities of pathological bone removed by biopsy.

The following characteristic features were found on analysing the enzyme content of bones (Table 2):

When measuring acid phenylphosphatase outstandingly high values were obtained from guinea-pig bone, which had an enzyme activity 2.5–3 times as high as that of mouse, rabbit and rat bones.

In the case of acid β-glycerophosphatase the enzyme activity of guinea-pig and rabbit bones was 2.5–3 times as high as that of mouse and rat bones.

Rabbit bone showed an outstandingly high alkaline phosphatase activity, which was 3 to 4 times as high as that in the bones of the other animals.

In mouse and rabbit bones an about twice as high cathepsin activity was found as in the bones of guinea-pig and rat.

Guinea-pig bone has markedly high lactate dehydrogenase activity. The lowest enzyme activity of this type was observed in rat bone.

There are occasionally marked differences in the bone tissues of the various species of animals. It seems possible from these experimental data that beside

other factors – stress, biophysical influences – the differences observed in enzyme content also contribute to the development of such deviations. However, to prove this point further experiments are required.

Examination of the enzyme content of the callus as a function of the period elapsed since the fracture revealed the following changes:

In the case of acid phenylphosphatase enzyme activity increases with time. The enzyme activity measured in a 28-day-old callus was 80% higher than that of the 7-day-old one.

Acid β-glycerophosphatase activity diminishes with time, the activity of the 28-day-old callus is 40% lower than that of the 7-day-old one.

Alkaline phosphatase activity appears to decrease to a considerable degree as the callus ages, the activity of the 28-day-old callus is 50% lower than that of the 7-day-old one.

Cathepsin enzyme activity decreases with time, the activity of the 28-day-old callus is only 40% of that of the 7-day-old one.

Lactate dehydrogenase activity increases by 40% from the 7th to the 14th day, but by the 21st day it was only 20% of the activity found on the 7th day, and by the 28th day it further decreased by 5%.

The β-glucuronidase activity appeared to have increased by 20–30% on the 14th and 21st day, but on the 28th day the same value was measured as on the 7th day.

The increase or decrease of enzyme activity indicates quite clearly the enhanced or reduced activities of various kinds of cells at a given time. The cells displaying enhanced enzyme activity may be identified by histochemical method.

In certain cases there is a significant difference between the enzyme activities of the bone and callus. Since activity changes were followed up for not more than four weeks after the fracture, our investigations fail to reveal the features of the transformation of the callus into a normal bone.

The lack of significant enzymological changes in the intact bones of the body following an experimental fracture (Table 2, rat bone) seems to support the assumption that the site of the reaction elicited by the fracture and extending over the entire skeletal system is not in the fully developed bone tissue but probably in the periosteal layer.

The scattering of data obtained in the measurement of bone enzyme activities has already been noted by other authors. In this respect Fullner's explanation (1966) is probably acceptable, according to which the different activities of the various kinds of cells are responsible for the observed differences. Moreover, in a given bone volume the ratio of cells producing the various enzymes may be different from that of another bone volume. Thus the differences originate from physiological properties and reflect physiological variations.

Of the enzymes supposed to exist in the bone and callus only six were investigated. An extension of the experiments will not only provide a better understanding of the biochemical conditions of these tissues, but will also reveal the enzymological differences under physiological and pathological conditions.

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Modification of Regulatory and Catalytic Properties of Phosphorylase-*b* by Irradiation and Heat

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1. The separate and combined effects of temperature and ^{60}Co-γ-irradiation have been studied on highly purified glycogen phosphorylase-*b* (α-1,4-glucan: orto-phosphate glucosyltransferase, EC 2.4.1.1). 2. The enzyme activity is more sensitive to the allosteric effector than to the substrate between 30 and 44.5 °C. There is an opposite effect between 10 and 30 °C. 3. The irradiated phosphorylase-*b* changed its affinity to the substrate and to the allosteric activator in different manners between 15 and 44.5 °C. 4. In accordance with the Arrhenius plot, irradiation decreased the stability of the enzyme of elevated temperatures. 5. The findings suggest that irradiation enhances the temperature sensitivity of the catalytic and allosteric sites separately.

Introduction

It is known that in some enzymes the radiosensitivity of indirect, mostly allosteric, regulation is different from that of the catalytic activity. The radiosensitivity of regulation is greater in aspartate transcarbamylase, phosphorylase-*b* and DNA-dependent RNA polymerase, while the catalytic center is more radiosensitive in phosphofructokinase and fructose-1,6-diphosphatase (Kleppe et al., 1966; Damjanovich et al., 1967; Sümegi et al., 1970; Chapman et al., 1969; Little et al., 1969).

According to Sanner and Pihl (1968) the different sensitivities of catalytic and regulatory sites of enzymes to ionizing radiation in solution may be due to the high radiosensitivity of SH-groups known to play a key role in the maintenance of regulatory or catalytic function of enzymes.

In the present work a study of separate and combined effects of temperature and ^{60}Co-γ-irradiation has been made on phosphorylase-*b*. Temperature and ionizing radiation have in common their random statistical effect, although at quite different energy levels, upon the enzyme activity. As in phosphorylase-*b* regulatory sites are more sensitive to radiation than catalytic sites, we examined the effect of temperature on regulation and catalysis with enzyme samples which had been partially impaired by irradiation. We examined which of the two functions of the irradiated and native enzyme – regulation or catalysis – can be further weakened or strengthened by altering the temperature of activity assay. The question has also some importance with respect to radiosterilization.

In order to reveal the correlation between sensitivity to incubation temperature and radiation the kinetic parameters of phosphorylase-*b* have been studied under different conditions.

Materials and methods

Rabbit skeletal muscle phosphorylase-*b* was prepared and four times recrystallized as described earlier (Damjanovich et al., 1967a).

The enzyme assay was carried out in the direction of glycogen synthesis. The liberation of inorganic phosphate (P_i) from glucose-1-phosphate (G-1-P) was measured after incubation at different temperatures between 10 and 44.5 °C at pH 6.8. The phosphate release was measured after different incubation times and care was taken that not more than 15% of G-1-P be consumed by the enzyme. Phosphate release at the different temperatures was proportional to enzyme concentration and time.

The incubation mixtures (in a total volume of 0.4 ml) consisted of 2 – 10 mM G-1-P; 0.0625 – 1 mM adenosine-5'-phosphate (AMP); 1% glycogen; enzyme $1 - 5 \times 10^{-7}$ M; 0.05 M tris-HCl buffer, pH 6.8; cysteine 0.033 M. The reaction was stopped with 2.6 ml of 5% (w/v) trichloroacetic acid. P_i released in 10 minutes was determined according to Taussky and Shorr (1953).

Irradiation was carried out with a ^{60}Co-γ-source. The samples consisting of 6×10^{-6} M protein, as determined by the method of Appleman et al. (1963), were irradiated in the presence of air at 22°. Prior to enzyme assay the samples were usually diluted fiftyfold with 0.05 M tris-HCl – 0.033 M cysteine, pH 6.8.

Results

In our previous studies we found that irradiation of phosphorylase-*b* destroyed the binding capacity for the allosteric activator AMP to a greater extent than that for the substrate G-1-P (Damjanovich et al., 1967a). In the first part of the present study, experiments were carried out in which the enzyme was assayed between 10 and 44.5 °C at different substrate and activator concentrations.

Fig. 1 shows the effect of AMP on the enzyme activity at different temperatures. The separate curves indicate the activities determined at different AMP concentrations (0.0625 – 0.5 mM). All of the samples were saturated with substrate (16 mM G-1-P). It is apparent that when the incubation temperature was below 20 °C the curves representing different AMP concentrations almost coincided. All AMP concentrations resulted in a nearly exponential activity-temperature curve up to a certain maximum. With increasing AMP concentrations these maxima appeared at higher temperatures. Beyond the maxima the activities decreased depending on the actual AMP concentration and temperature.

Fig. 2 shows the relationship between enzyme activity, substrate concentration and temperature. In this case the enzyme was saturated with the activator (1 mM AMP). The enzyme activities measured at different substrate concentrations

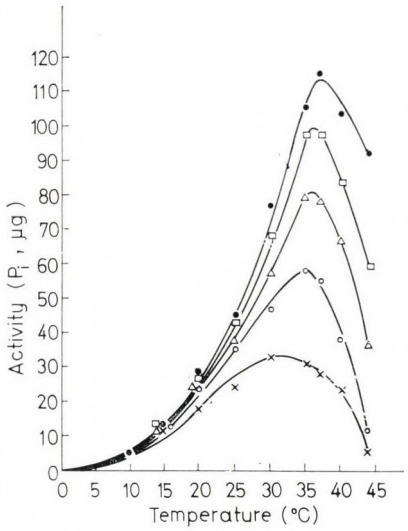

Fig. 1. Effect of AMP on the enzyme activity of phosphorylase-*b* at various temperatures. The different symbols represent various AMP concentrations in the incubation mixture: ×———× 0.0312; ○———○ 0.0625; △——△ 0.125; □———□ 0.250; ●———● 0.5 mM AMP, and 16 mM G-1-P in each. Enzyme concentration: 10^{-7} M. The ordinate shows the μg P_i released in 10 minutes in a total volume of 0.4 ml. All other constituents were as described in Materials and methods

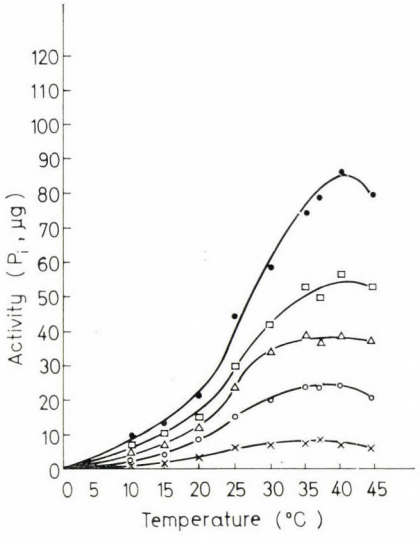

Fig. 2. Effect of G-1-P on the enzyme activity of phosphorylase-*b* at various temperatures. The different symbols represent various G-1-P concentrations in the incubation mixture: ×———× 1 mM; ○———○ 2 mM; △———△ 4 mM; □———□ 8 mM; ●———● 16 mM G-1-P and 1 mM AMP in each. Enzyme concentration 0.75×10^{-7} M. The ordinate shows the μg P_i released in 10 minutes in a total volume of 0.4 ml. All the other constituents were as described in Materials and methods

differed also at low temperature. Enzyme activity increased parallel with increasing temperature, however, the maxima of curves were more like plateaus instead of peaks, with the exception of the highest curve.

Fig. 3 demonstrates the radiosensitivity of phosphorylase-*b* measured at different concentrations of substrate and activator. The dose-response curves at

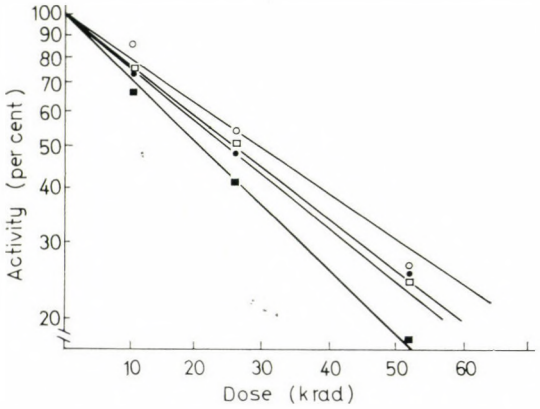

Fig. 3. Dose-response curves of phosphorylase-*b*. The activities were measured with 16 mM (○) and 2 mM (●) G-1-P, and at 0.5 mM (□) and 0.125 mM (■) AMP. When substrate concentration was varied 1 mM AMP was used, whereas 16 mM G-1-P was present when the AMP was varied. Incubation temperature: 30 °C

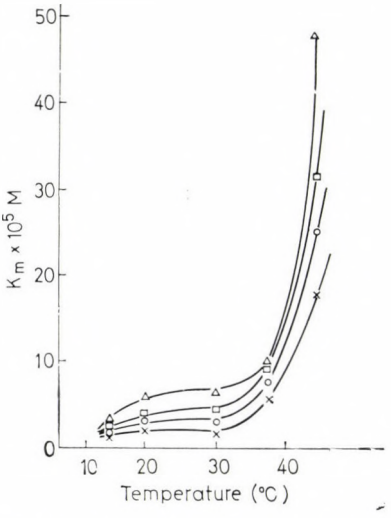

Fig. 4. K_M values for the allosteric activator AMP before and after irradiation. The K_M values for AMP were determined in the control (×) and in enzyme samples irradiated with 10.4 (○); 26 (□); and 52 KR (△). The incubation temperature of the activity assay is indicated in the figure. G-1-P concentration: 16 mM

30 °C give straight lines in the semilogarithmic plot. From this curve one cannot evaluate the different radiosensitivities of the allosteric and catalytic sites. According to our earlier investigations the K_M values of phosphorylase-*b* for AMP and G-1-P can be accepted as separate parameters for the regulatory and catalytic sites (Damjanovich et al., 1967a). To compare the separate and combined effects of temperature and irradiation, K_M values for AMP and G-1-P were determined.

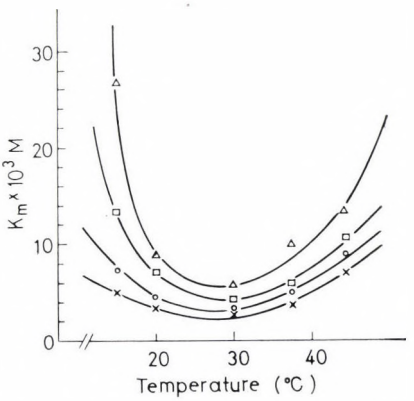

Fig. 5. K_M values for the substrate (G-1-P) before and after irradiation. The irradiation doses were as in Fig. 4 at the suitable symbols. AMP concentration: 1 mM

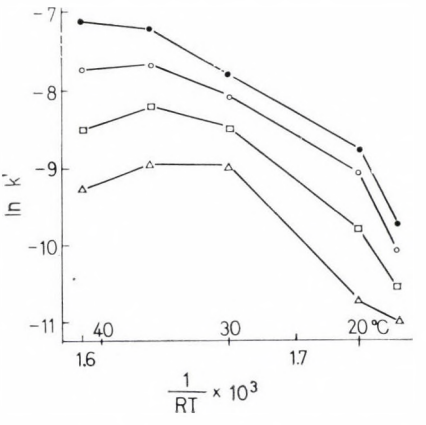

Fig. 6. Arrhenius plots of irradiated and control phosphorylase-*b*. The first order rate constants were determined at substrate and activator saturation. The irradiation doses were as described in Fig. 4 at the suitable symbols

Fig. 4 shows the dependence of K_M for AMP upon incubation temperature and irradiation dose. Between 14 and 20 °C there is a slight increase of K_M values, between 20 and 30 °C K_M values are more or less constant whereas above 30 °C K_M values rapidly increase.

Irradiation did not influence the shape of the curves to any greater extent but K_M values were higher at all temperatures as compared to the control. The higher irradiation dose was applied, the higher increase was observed. Above 37.5 °C the irradiated samples were far more sensitive to temperature than the control.

Fig. 5 shows the combined effect of temperature and irradiation on the K_M values of G-1-P. As the binding sites for AMP and G-1-P are not the same the effects may have different directions. Indeed the K_M for G-1-P was increased by low and high temperatures in the control and irradiated samples as compared to the value obtained at 30 °C. Low temperatures were even more effective in increasing the K_M of G-1-P than high ones. The shape of curves was also the same for the control and irradiated samples.

More information could be gained from the Arrhenius plots of first order rate constants. Fig. 6 shows the Arrhenius plot of control and three irradiated

samples. All ln k'-s were determined at substrate and activator saturation (16 mM G-1-P and 1 mM AMP). The control shows the usual straight line between 20 °C and 37.5 °C. Outside this temperature interval the molecular structure of native phosphorylase-*b* was changed as shown by the breaks on the straight lines. Further three lines show the effect of irradiation upon the shape of Arrhenius plot. The temperature-independent interval was shortened after irradiation as compared to the control. Deviation of the curves from straight line also increased with dose.

Discussion

The results reported here indicate that different substrate and activator concentrations affect the enzyme activity measured between 10 and 44.5 °C in different manners before and after ^{60}Co-γ-irradiation of the phosphorylase-*b*.

The native enzyme showed low sensitivity towards the changing of activator concentration at low temperature, very likely due to the greater AMP-binding capacity of phosphorylase-*b* in that temperature interval (Kastenschmidt et al., 1968). The stabilizing effect of AMP upon the structure of phosphorylase-*b* can be seen from the shift of activity maxima towards higher temperatures in the presence of higher AMP concentrations. The decreased enzyme activity between 37.5 and 44.5 °C even at high AMP concentrations indicates the sensitivity of the molecular conformation to the random collisions generated by heat.

It is interesting that enzyme activity is sensitive to the concentration of G-1-P at low temperature, whereas relatively constant activity values are found for a given G-1-P concentration between 35 and 44.5 °C in the presence of high activator concentration. The explanation of this finding probably lies in the fact that AMP can stabilize the structure of phosphorylase-*b* to a greater extent than G-1-P does, and the affinities of phosphorylase-*b* towards AMP and G-1-P at various temperatures change in a different manner.

The second question concerns the temperature sensitivity of enzyme at different AMP and G-1-P concentrations after irradiation. Our earlier experiments have shown that K_M values of phosphorylase-*b* for AMP and G-1-P can be regarded as indicators of the sensitivity of catalytic activity or allosteric regulation (Damjanovich et al., 1967a; Damjanovich, 1967).

The results presented in Figs 4 and 5 indicate, in accordance with the findings on native enzyme, that the temperature sensitivity of the irradiated enzyme is quite different with respect to catalysis and regulation. Low temperature affected the K_M values for G-1-P to a greater extent than those for AMP, while higher temperatures increased the K_M for AMP much better.

The Arrhenius plots of the control and irradiated phosphorylase-*b* proved to be very interesting. The ln k' vs. $\frac{1}{RT}$ plot gave a straight line for the control between 20 and 37.5 °C. Very likely the intramolecular bonds of the protein change with temperature and the structure of active and allosteric sites on the

enzyme is temperature-dependent outside this interval. Irradiation of the enzyme with different doses made the temperature-independent interval narrower as compared with the control curve. In accordance with other results, these findings support our assumption that irradiation with a relatively low dose does not alter the conformation of an enzyme to a great extent. On the other hand, the irradiated enzyme molecules become more sensitive to random statistical interferences and less sensitive to regulatory agents such as an allosteric effector. We suggest that temperature affects the catalytic or allosteric function of the irradiated enzyme, depending on the extent of structural changes necessary to the development of these functions. The greater the structural alteration essential for the function in question, i.e. catalysis or regulation, the greater the sensitivity towards a random statistical effect such as heat or irradiation or both.

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Papain Susceptibility of IgG Myeloma Proteins Reconstituted from Reduced, Non-Alkylated H- and L-Chains

(Short Communication)

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Several investigations have proved that the isolated H- and L-chains of reduced and alkylated IgG will associate through non-covalent interactions to form molecules which resemble the original IgG (Dorrington et al., 1967; Edelman et al., 1963; Fougereau, Edelman, 1964; Grey, Mannik, 1965; Roholt et al., 1965; Franek, Nezlin, 1963; Mannik 1967). The recombination of the polypeptide chains results in the full recovery of the antibody activity (Roholt et al. 1965; Franek, Nezlin, 1963). The restoration of native conformation, however, is proved only in case of the reassociated autologous polypeptide chains of myeloma proteins (Mannik, 1967).

The different papain susceptibilities of IgG myeloma proteins reflect on conformation differences of the molecules (Gergely et al., 1967; 1967a; Medgyesi, Gergely, 1969). As it has been shown in our previous study (Gergely et al., 1969), the papain digestibility of reassociated IgG myeloma proteins indicates the degree of recovery of molecular conformation. The results pointed to the possible importance of the reformation of interchain disulphide bonds in the recovery of conformation responsible for the papain resistance of IgG myeloma proteins. The aim of the present investigations was to study this hypothetic role of interchain disulphide bonds. To this end the papain susceptibility of the molecules recombined from autologous or heterologous polypeptide chains of reduced but non-alkylated myeloma proteins was studied.

50 to 150 mg samples of IgG myeloma proteins were reduced with 0.2 M 2-mercaptoethanol. The reduced protein was filtered through a Sephadex G-25 column equilibrated in N_2-atmosphere with a 0.075 M phosphate buffer (pH 7.0) containing 0.075 M NaCl. The reduced protein was kept in N_2-atmosphere and the H- and L-chains were separated on a Sephadex G-100 column equilibrated in N_2-atmosphere with 1 M acetic acid (Fig. 1a). The isolated non-alkylated chains were stored in N_2-atmosphere. To reassociate the autologous or heterologous H- and L-chains equimolar amounts of the polypeptide chains were mixed (protein concentration: 1 to 2 mg/ml). The reassociation was allowed to proceed over a 24-hour period by dialysis against several changes of 0.075 M phosphate buffer containing 0.075 M NaCl, pH 7.0. The reconstituted material was filtered through a Sephadex G-150 column. Three peaks were usually obtained. Aggregated chains were eluted in the first fraction. The second peak was eluted at the

same position as IgG. The immuno-electrophoretic analysis of this fraction revealed one homogeneous precipitation line (Fig. 2). Free L-chains were eluted in the third fraction. The second fraction was concentrated by pressure dialysis and the papain digestibility of this fraction was controlled by the method described earlier (Gergely et al., 1967).

To prove whether the association of reduced but non-alkylated chains results in the restoration of interchain disulphide bonds, gel-filtration of the associated polypeptide chains through a Sephadex G-100 column in 1 M acetic acid was performed. Reduced and alkylated H- and L-chains may separate in dissociating media (Edelman, 1959). As the associated material was eluted in 1 M acetic acid

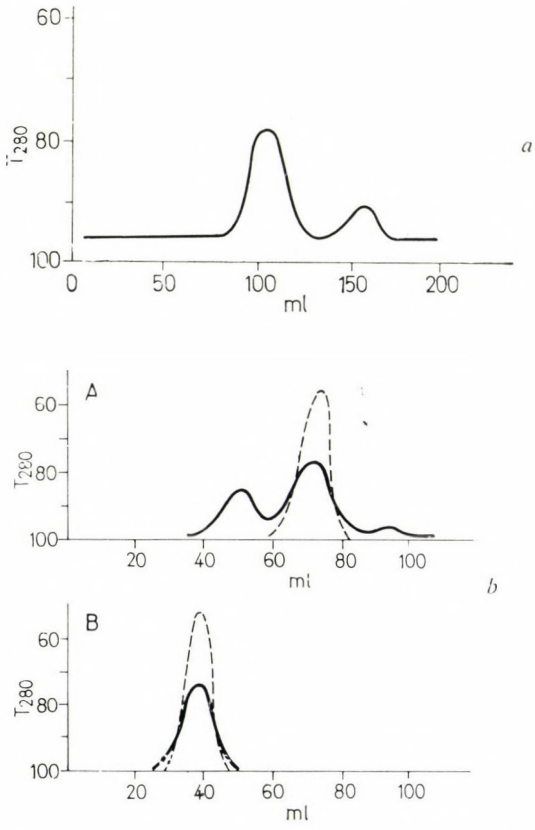

Fig. 1. Separation of H- and L-chains in N_2-atmosphere on a Sephadex G-100 column in 1 M acetic acid and reassociation of autologous reduced but non-alkylated H- and L-chains of IgG myeloma protein Sza. *a)* Separation of H- and L-chains in N_2-atmosphere on a Sephadex G-100 column in 1 M acetic acid. Column size: 2.8×52 cm. *b)* Reassociation of autologous reduced but non-alkylated H- and L-chains of IgGl myeloma protein Sza. *A* Fractionation of the reassociated material on a Sephadex G-150 column. Column size: 1.8×80 cm. *B* Gel-filtration of the reassociated protein on a Sephadex G-100 column equilibrated with 1 M acetic acid. Continuous line: protein content registered by LKB Uvicord II. Dotted line: position of native ^{131}I-labelled IgG

as one symmetrical peak at the same position as ^{131}I-labelled IgG, the disulphide bonds seem to have been restored (Fig. 1b).

The papain digestibility of associated autologous reduced and alkylated polypeptide chains was examined altogether in 15 cases (Gergely et al., 1969). Five out of six reconstituted proteins containing the reassociated reduced and

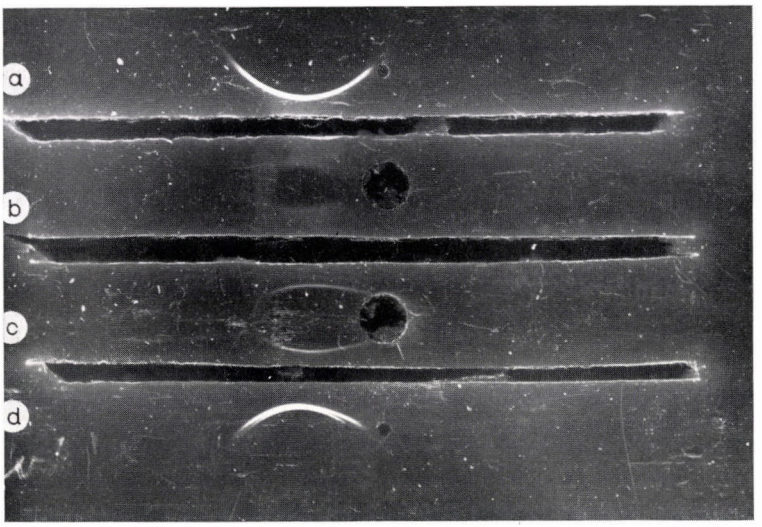

Fig. 2. Immunoelectrophoresis of native Sza (*a*) and Wih (*d*) proteins and reassociated protein of Wih H and Sza L (*b*) and Sza H and Wih L (*c*)

alkylated chains of papain-resistant IgG2 or IgG4 myeloma proteins contained papain-resistant molecules. In each case the amount of papain-resistant molecules was not more than 20% of the reassociated material.

Reassociation of reduced but non-alkylated autologous H- and L-chains of papain-resistant IgG myeloma proteins was performed in two cases. In both cases about 80% of the reassociated material was papain-resistant.

Reassociation of H- and L-chains of papain-sensitive myeloma proteins resulted almost exclusively in papain-sensitive reassociated material, both in experiments with reduced and alkylated (9 cases) and reduced but non-alkylated chains (3 cases).

The papain susceptibility of associated reduced and alkylated heterologous H- and L-chains was studied in a previous work (Gergely et al., 1969) and in the present series of experiments (22 cases altogether). In 12 cases the H-chains were prepared from papain-resistant myeloma proteins. The reconstituted material contained papain-resistant molecules only in three cases.

The susceptibility of reassociated heterologous reduced but non-alkylated H- and L-chains was examined in 12 cases. In each case the majority of the reassociated molecules containing H-chains prepared from papain-resistant IgG myeloma

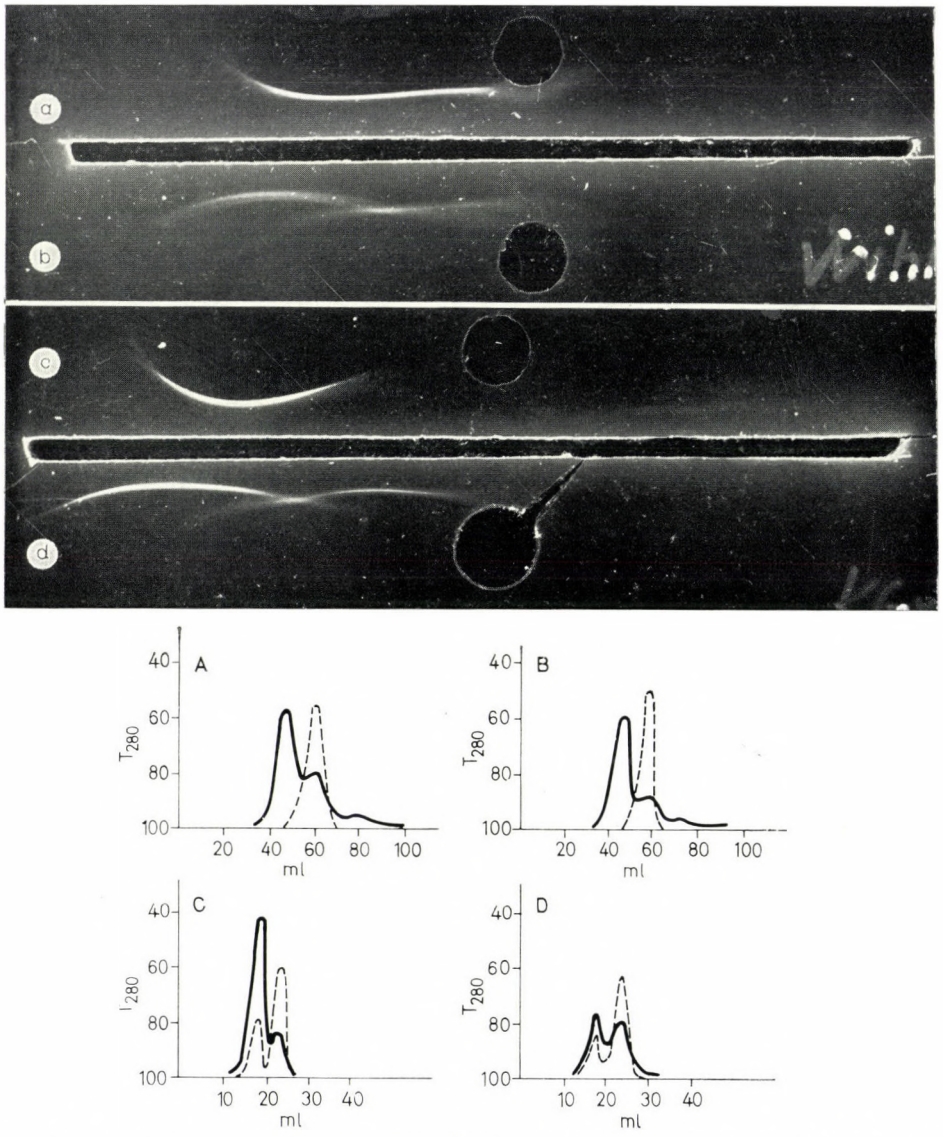

Fig. 3. Heterologous recombination of reduced but non-alkylated polypeptide chains of IgG2 Wih and IgG1 KGy myeloma proteins and immunoelectrophoretic analysis of the gel-filtration fractions of the papain-digested reassociated protein. *a*. Heterologous recombination of reduced but non-alkylated polypeptide chains of IgG2 Wih and IgG1 KGy myeloma proteins. *A* Gel-filtration of the reassociated material derived from chains Wih H and KGy L on a Sephadex G-150 column. *B* Gel-filtration of reassociated material derived from chains KGy H and Wih L on a Sephadex G-150 column. *C* Gel-filtration of papain-digested reassociated protein *A* (Wih H-KGy L) on a Sephadex G-100 column. *D* Gel-filtration of papain-digested reassociated protein *B* (KGyH-WihL) on a Sephadex G-100 column. b. Immunoelectrophoretic analysis of the gel-filtration fractions of the papain-digested reassociated proteins. *a* First fraction of gel-filtration *C*; *b* Second fraction of gel-filtration *C*; *c* First fraction of gel-filtration *D*; *d* Second fraction of gel-filtration *D*

proteins was papain-resistant (Table 1 and Fig. 3a, b). About half of the recombined molecules containing L-chains of papain-resistant proteins was papain-resistant. Less than 10% of the reassociated chains obtained from papain-sensitive myeloma proteins was papain-resistant. As the majority of the associated molecules containing autologous or heterologous H-chains of IgG2 or IgG4 myeloma

Table 1

Reassociation of H- and L-chains of reduced but non-alkylated IgG myeloma proteins

H-chain	L-chain	Papain-resistant recombination %
Pre*	Ml	80
Wih*	Ml	95
Wih*	Su	85
Wih*	KGy	80
Ml	Pre	60
KGy	Wih	50
Ml	Wih	50
Su	Wih	50
KGy	Su	10
Su	KGy	10
Kof	Su	10
Su	Kof	10

* These chains were prepared from papain-resistant IgG2 and IgG4 myeloma proteins.

proteins was papain-resistant, the results point to the decisive role of the H-chain structure and to the importance of restoration of disulphide bridges in the papain-resistant character of IgG2 and IgG4 proteins.

It is remarkable that about 50% of the reconstituted molecules containing L-chains of papain-resistant myeloma proteins was papain-resistant. This fact calls the attention to the possible influence of the L-chain structure in developing the papain-resistant conformation.

Further details of these studies will be published elsewhere.

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Thermoanalytical Characterization of the Stability of Crosslinked Proteins

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A complex thermoanalytical method, derivatography, was applied to the investigation of the thermal decomposition of proteins. A correlation could be demonstrated between the thermal decomposition of various proteins in the 450—700 °C temperature interval and the amount of covalent crosslinks present in the original protein molecule.

Introduction

Thermal decomposition of proteins takes place in two successive steps. The first process reaches its maximal rate at about 300 °C and results in the partial cleavage of peptide bonds (Lóránt, 1965; Simon et al., 1969) with the concomitant formation of peptide fragments of low molecular weight and various pirrol-type compounds (Ciamician, Silber, 1884).

Our recent investigations indicate that the thermal decomposition of these newly formed compounds depends on the amount of covalent crosslinks present in the original protein molecules.

Materials and methods

Cytochrome-c, crystalline ribonuclease (from bovine pancreas) and gelatin were purchased from Reanal (Budapest), bovine fibrinogen and fibrin from Calbiochem (California). Soluble tropocollagen was extracted from the skin of young rats with 0.15 M sodium citrate buffer pH 3.7, according to the method of Rubin et al. (1965). Insoluble mature collagen from Achilles tendon was kindly supplied by Dr I. Banga.

The effect of chemical reduction on thermal decomposition was studied with collagen samples incubated in 0.001 M ascorbic acid for 30 minutes, washed with distilled water and kept in Na_2CO_3-HCl buffer, pH 8.0, for 30 minutes, then washed and dried.

As an oxidative agent 0.001 M $KMnO_4$ was used for 30 minutes.

Thermal analysis was carried out in a Paulik–Paulik–Erdey MOM derivatograph (Paulik et al., 1958). The instrument measured and recorded simul-

taneously the weight change (TG curve), rate of weight change (DTG curve), enthalpy change (DTA curve) and the temperature (T) of the sample. The material (about 100 mg) was weighed into a platinum crucible. Heating rate was 10 °C/min up to 900 °C.

Results and discussion

Fig. 1 shows the thermal decomposition curves of cytochrome-c and ribonuclease.

The first process, indicated by the DTG curves, was the departure of water from the sample between 20 °C and 180 °C, which resulted in 14.0 and 12.0% weight losses in cytochrome-c and ribonuclease, respectively, as calculated from the TG curves.

This was followed in both proteins by the partial cleavage of peptide linkages between 180 °C and 420 °C, with maximal rates at 320 °C and concomitant weight changes of the same order (51.2% and 54.5%, as calculated for dry weight, for cytochrome-c and ribonuclease, respectively).

At 420 °C the DTG curves did not return to the base line, as the next, very exotherm thermal process started, which showed peak values at 500 °C in case

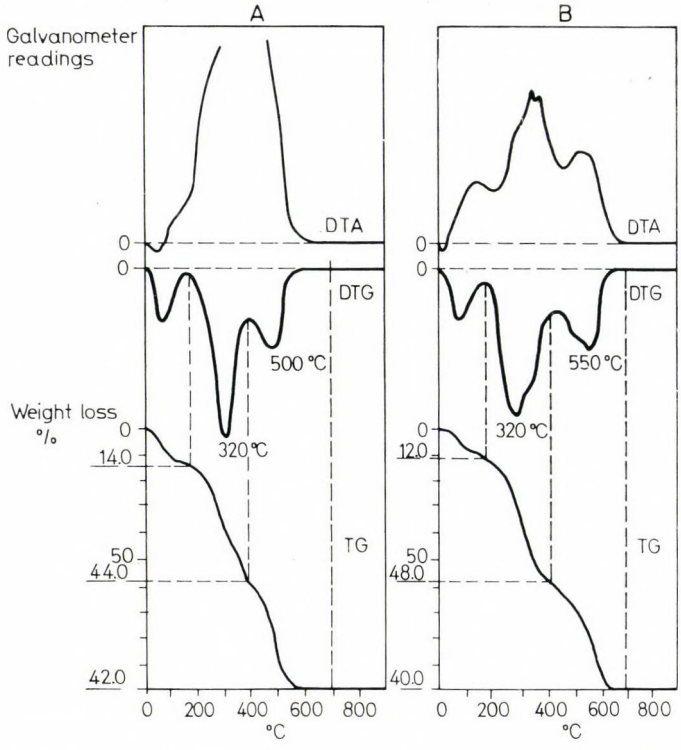

Fig. 1. Thermal decomposition curves of cytochrome-c (A) and ribonuclease (B)

of cytochrome-c and 550 °C in case of ribonuclease and resulted in 48.8 and 45.5% weight losses, respectively (calculated for dry weight). The thermal decomposition of the samples was complete at 700 °C.

Both cytochrome-c and ribonuclease are globular proteins, their molecular weight is similar. Nevertheless, there is a significant structural difference between

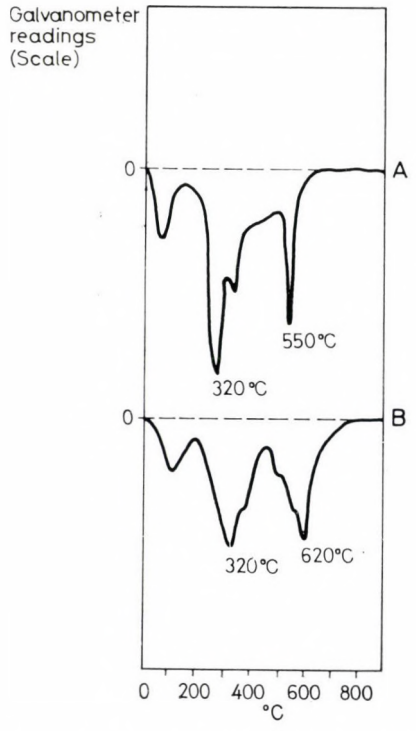

Fig. 2. Differential thermal gradient (DTG) curves of fibrinogen (A) and fibrin (B)

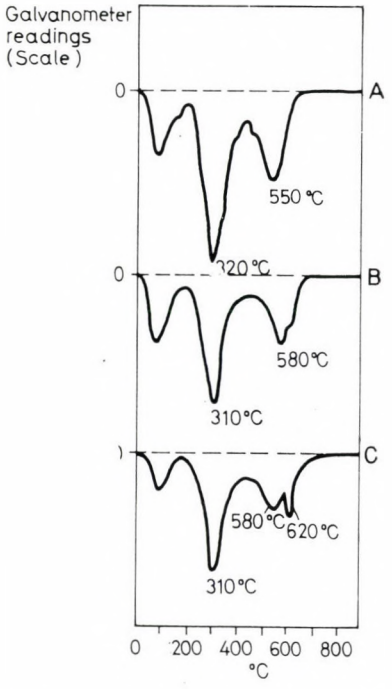

Fig. 3. Differential thermal gradient (DTG) curves of gelatin (A), soluble tropocollagen (B) and mature, insoluble collagen (C)

the two molecules: cytochrome-c does not contain covalent crosslinks, whereas ribonuclease has 4 disulfide bridges per molecule. This structural difference markedly affected the thermal decomposition of the two molecules, as indicated by the DTG peak-values in the 450–700 °C temperature interval.

Fig. 2 shows the DTG curves of two fibrillar proteins, fibrinogen and fibrin.

The first main decomposition process took place in both proteins with maximal rate at 320 °C, accompanied by weight losses of 49.4 and 50.0% (calculated for dry samples) in fibrinogen and fibrin, respectively. This process was complete at 440 °C.

On the DTG curve of fibrinogen the final thermal process resulted in a DTG peak at 550 °C (weight change: 51.7% of the dry weight). The formation

of highly crosslinked fibrin fibres increased the thermostability of the molecule and the characteristic DTG peak was shifted to 620 °C. The weight loss, calculated from the TG curve was 50.0%.

The same correlation between thermal decomposition temperatures and the amount of crosslinks could be demonstrated with gelatin, soluble tropocollagen

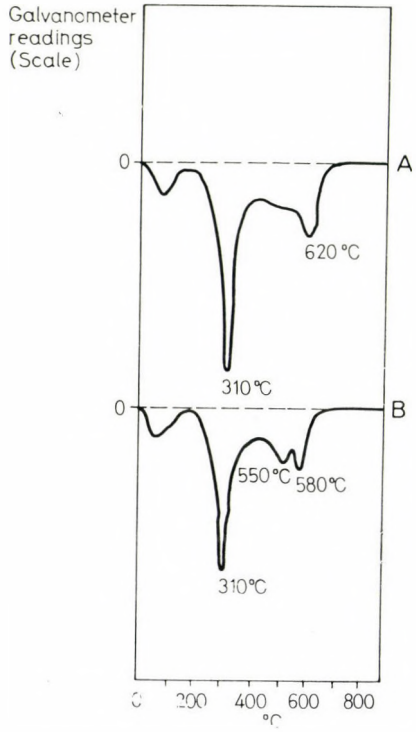

Fig. 4. Differential thermal gradient (DTG) curves of mature, insoluble collagen oxidized with $KMnO_4$ (A) and reducec with ascorbic acid (B

and insoluble mature collage. The corresponding DTG curves are presented in Fig. 3.

The percentual weight change caused by the cleavage of peptide bonds was 61.0, 53.6 and 51.5% (calculated for dry sample) in gelatin, tropocollagen and collagen, respectively.

It is generally agreed that mature collagen fibres are built up from tropocollagen molecules by the introduction of non-peptide covalent crosslinks. The number of these intermolecular crosslinks, i.e. the degree of network formation throughout the fibres determined the thermal behaviour of the samples in the 450–700 °C temperature range. Thus at DTG peak-values 550 °C and 580 °C the weight loss was 39.0 and 46.4% of dry gelatin and tropocollagen, respectively. In mature tendon collagen the final thermal process took place in two steps:

at 580 and at 620 °C causing a 24.0 and a 20.8% weight loss (expressed in per cent of the dry weight), respectively.

In order to confirm our assumption, the amount of intermolecular crosslinks in collagen samples was altered experimentally and the influence of these modifications on their thermal decomposition was studied.

As seen from the DTG curves presented in Fig. 4, treatment with $KMnO_4$ resulted in a significant elevation of decomposition temperatures of the collagen sample. The 580 °C DTG peak is not detectable on the derivatogram, the decomposition of the total amount of material takes place with maximal rate at 620 °C. Ascorbic acid had an inverse effect, the characteristic peak values were shifted towards lower temperatures, 550 and 580 °C.

These agents are known to affect the main naturally occurring aldol-type covalent crosslinks in collagen: aldehyde bridges can be formed by treatment with $KMnO_4$ and their amount reduced by ascorbic acid (Banga, 1966).

The above described correlation between thermostability of compounds decomposed in the 450 to 700 °C temperature interval and the amount of covalent crosslinks present in the molecules of fibrillar proteins could be applied to the quantitative analysis of age-related, pathological and experimentally induced changes in the stability of various mammalian proteins (Bihari-Varga, Biró, 1971, 1971a).

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Thermoanalytical Assay of Glycosaminoglycans

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1. A complex thermoanalytical method, derivatography, has been applied to the investigation of glycosaminoglycans. 2. Thermal decomposition of polycarboxylates took place with maximal rate at 220 °C, that of polycarboxysulfates at 240 °C and of keratan-sulfate at 270 °C. The three processes could be resolved and studied separately in glycosaminoglycan mixtures as well. 3. The percentual weight loss of the sample measured at the characteristic decomposition temperatures was proportional to the concentration of glycosaminoglycan. Based on this correlation, a method was developed for the quantitative assay of glycosaminoglycans. 4. The thermal decomposition process was not altered if the polysaccharide molecule was bound to structural proteins. Thus the method could be successfully used for the direct analysis of natural compounds and biological tissues.

Introduction

As reported earlier, thermal decomposition of glycosaminoglycans takes place between 200 °C and 300 °C (Simon et al., 1968, 1969). Recently, by improving the applied thermoanalytical method and by using standard compounds of high purity, we succeeded in determining the individual decomposition temperatures of a series of glycosaminoglycans and in developing a quantitative method for the measurement of their concentration.

Materials and methods

Highly purified standard hyaluronic acid, chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan-sulfate, heparan-sulfate, heparin and keratan-sulfate were isolated and kindly supplied to us by M. B. Matthews, J. A. Cifonelli and L. Rodén.

Chondroitin was prepared from chondroitin-4-sulfate, according to the method of Kantor and Schubert (1957).

Thermal analysis was carried out in a Paulik–Paulik–Erdey MOM derivatograph (Paulik et al., 1958). The instrument measured and recorded simultaneously the weight change (TG curve), rate of weight change (DTG curve), enthalphy change (DTA curve) and the temperature (T) of the sample. The samples were weighed into a platinum crucible. Heating rate was 2–10 °C/min. up to 900 °C.

Results and discussion

Fig. 1 shows the thermal decomposition curves of hyaluronic acid (*A*), chondroitin-4-sulfate (*B*), heparan-sulfate (*C*) and keratan-sulfate (*D*).

The first thermal process, indicated by the DTG curves, was the loss of water content of the samples between 20 °C and 180 °C with maximum rate at 60 °C. The resulting weight losses were 19.0%, 14.0%, 17.0% and 16.0% in hyaluronic acid, chondroitin-4-sulfate, heparan-sulfate and keratan-sulfate, respectively, as calculated from the TG curves.

Decomposition started in all the four samples at 200 °C.

In hyaluronic acid this main thermal decomposition process reached its maximum rate at 220 °C (as indicated by a very sharp DTG peak) and was accompanied by a weight loss of 48.5% (69.5%, when calculated for dry, salt-free sample). The process was very exothermal as shown by the DTA curve and resulted in the formation of intermediate oxidation products which showed the characteristic infrared absorbance of oxo groups at 1720 cm^{-1} wave number. Thermal decomposition of this intermediate took place in two additional exothermal processes with maximum urates at 340° and 550 °C, which resulted in weight losses of 11.5% (16.5% of the dry, salt-free weight) and 10.0% (14.0%), respectively. The residual 11.0% of the preparation was measured as thermostable material probably in the form of inorganic sodium salts.

On the thermoanalytical curves of chondroitin-4-sulfate (Fig. 1*B*) two processes could be distinguished, showing DTG peak values at 240 and 470 °C. The percentual weight change during the main decomposition process, when calculated for dry salt-free material, was of the same order as in hyaluronic acid (70.7%). The amount of residual inorganic compounds was higher (28.0%), due to the sulfate-content of chondroitin-sulfate.

The shape of the thermoanalytical curves of chondroitin-6-sulfate and dermatan-sulfate was very similar to that of chondroitin-4-sulfate.

The absence of sulfate groups in the chondroitin molecule resulted in a decrease in thermostability. The DTG peak, which indicates the main decomposition process, was shifted to 220 °C. The thermoanalytical curves of chondroitin thus resembled those of hyaluronic acid.

The DTG peak indicating the main decomposition in heparan-sulfate (Fig. 1*C*) was found also at 240 °C as in the chondroitin-sulfates, but the two additional steps took place with maximal rate at 420 and 470 °C.

Decomposition curves of heparin resembled those of heparan-sulfate.

The structural differences between keratan-sulfate and the 6 polyglucurans above discussed were reflected in their thermal behaviour (Fig. 1*D*). From among the glycosaminoglycans studied keratan-sulfate showed the greatest thermostability. The first DTG peak was shifted to 270 °C, followed by two maxima at 420 and 550 °C.

In mixtures containing various amounts of different glycosaminoglycans the thermal decomposition of polycarboxylates (hyaluronic acid, chondroitin), polycarboxy-sulfates (chondroitin-4- and-6-sulfate, dermatan-sulfate, heparin and

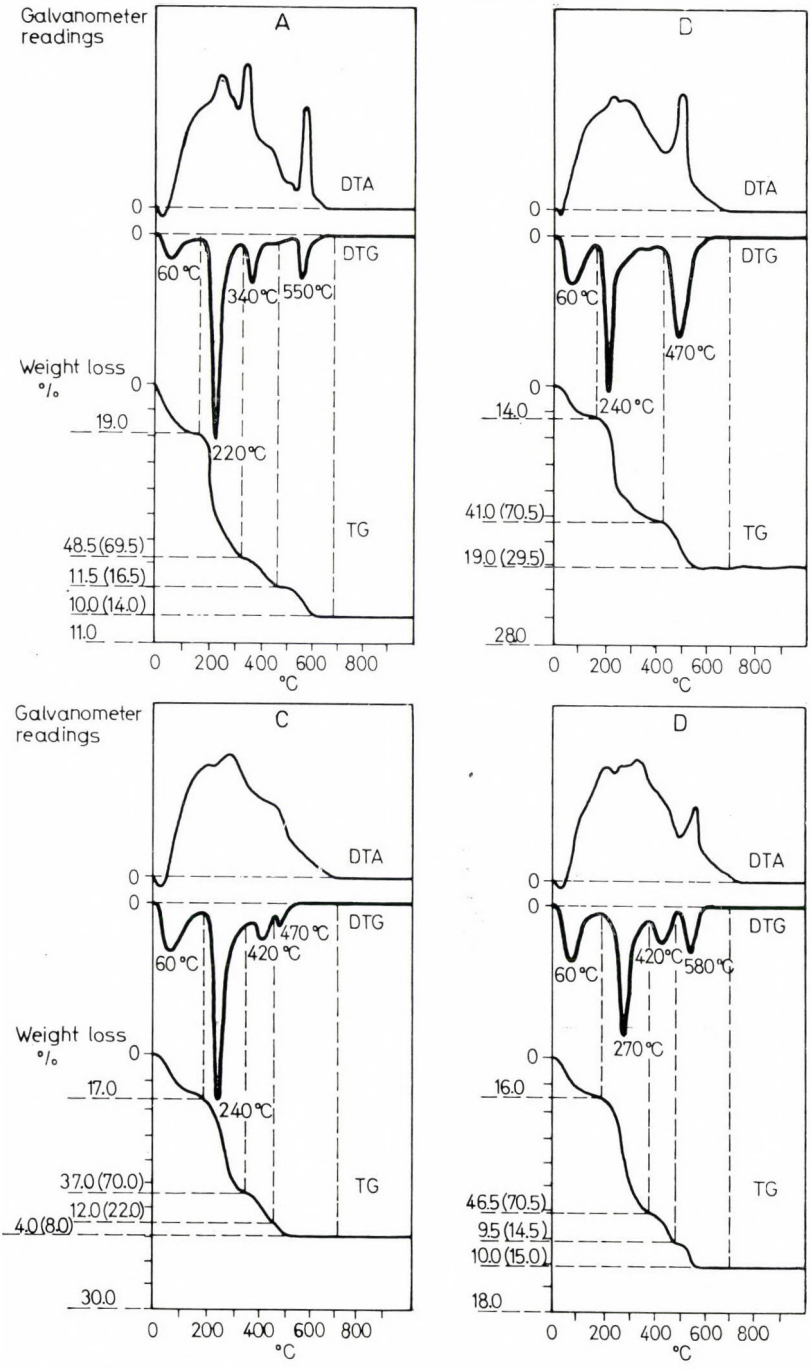

Fig. 1. Thermal decomposition curves of hyaluronic acid (*A*), chondroitin-4-sulfate (*B*) heparan-sulfate (*C*) and keratan-sulfate (*D*)

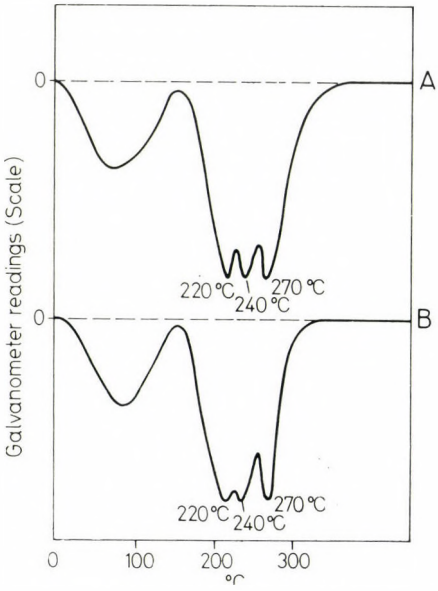

Fig. 2. Differential thermal gradient (DTG) curves of mixtures containing various amounts of glycosaminoglycans. *A*) hyaluronic acid : chondroitin-4-sulfate : keratan-sulfate = 1 : 1 : 1; *B*) hyaluronic acid : chondroitin-4-sulfate : keratan-sulfate = 2 : 1 : 2

Table 1

Thermal decomposition of glycosaminoglycans

Percentual weight change, measured in the characteristic temperature intervals. The amount of inorganic compounds could be measured in the form of thermostable residue. Data in parentheses are weight loss values, calculated for dry, salt-free samples

DTG peak Glycosaminoglycan	60 °C Departure of water	Main decomposition process			Completion of decomposition				Residue above 900 °C
		220 °C	240 °C	270 °C	340 °C	420 °C	470 °C	550 °C	
Hyaluronic acid	19.0	48.5 (69.5)			11.5 (16.5)			10.0 (14.0)	11.0
Chondroitin	17.5	58.0 (70.0)					24.5 (30.0)		—
Chondroitin-4-sulfate	14.0		48.0 (70.5)				20.0 (29.5)		18.0
Chondroitin-6-sulfate	18.0		43.0 (69.5)				19.0 (30.5)		20.0
Dermatan-sulfate	12.0		43.0 (71.0)				17.5 (29.0)		27.5
Heparan-sulfate	17.0		37.0 (70.0)			12.0 (22.0)	4.0 (8.0)		30.0
Heparin	13.5		36.5 (68.5)			11.0 (20.5)	6.0 (11.0)		33.0
Keratan-sulfate	16.0			46.5 (70.5)		9.5 (14.5)		10.0 (15.0)	18.0

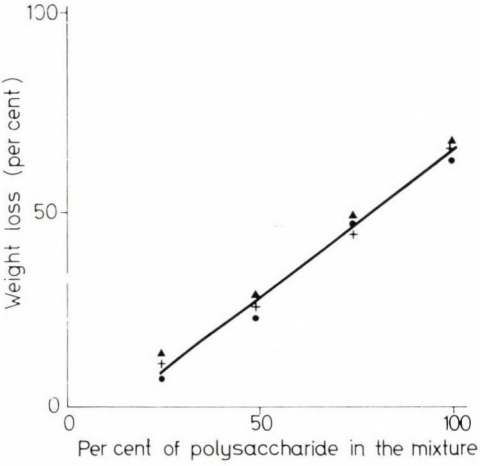

Fig. 3. Correlation between the concentration of glycosaminoglycans and the percentual weight change measured at the characteristic decomposition temperatures. + hyaluronic acid (220 °C); ▲ chondroitin-6-sulfate, heparin (240 °C); ● heparan-sulfate, keratan-sulfate (270 °C)

heparan-sulfate) and keratan-sulfate took place separately, the three characteristic DTG peaks could clearly be distinguished, as demonstrated in Fig. 2.

The derivatographic method proved suitable for the quantitative assay of glycosaminoglycans. As seen from the data in Table 1, the main decomposition process resulted in about 70% weight loss in the pure samples. The concentration of various types of polysaccharides could be determined in their mixtures, since there was a direct correlation between their concentration and the percentual weight change calculated from the TG curves in the characteristic temperature intervals. Some typical calibration curves obtained in our experiments are presented in Fig. 3.

The thermostability of glycosaminoglycans was not influenced by the presence of proteins, even if they were structurally bound (Simon et al., 1968). Thus the technique could be successfully used for the analysis of protein polysaccharide complexes as well as for the estimation of age-related, pathological and experimentally induced changes in the composition of carbohydrate-containing biological tissues (Bihari-Varga, Biró, 1971).

The author gratefully acknowledges the capable technical assistance of Mrs Amália Kardos and Mrs Mária Tóth.

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Enzymological Studies on E. coli Mutants Producing L-Serine Deaminase of High Specific Activity

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The characteristics of L-serine deaminase (E. C. 4.2.1.13) from two E. coli mutant strains are described. The substrate saturation curve of the enzyme was sigmoid. The K_m values were found to be 5.75 to 8.6×10^{-3} M for L-serine. Dilution rapidly inactivated the enzymes, nevertheless the initial velocity remained constant. The possible role of L-threonine deaminases in this anomaly was excluded.

Introduction

In earlier enzymological studies on L-serine deaminases from E. coli K-12, Salmonella typhimurium and B. cereus some results were obtained for which no satisfactory explanation was found (Alföldi et al., 1968; Raskó et al., 1969). To such phenomena belonged the sigmoid substrate saturation curve and that the initial velocity of the enzymes did not decline, despite of their rapid inactivation. Further experiments showed that about 10 to 15% of the L-serine deaminating activity of crude bacterial extracts may originate from the biosynthetic or degradative L-threonine deaminases, since these enzymes deaminate L-serine, too (Alföldi, Raskó, 1970). The observed anomalies might thus be due to the degradative L-threonine deaminase, which is always present in the cells if the bacteria are grown in a complex medium (Umbarger, Brown, 1957).

The E. coli Hfr C mutant strains investigated in this work produced L-serine deaminase of such a high specific activity that the possibly contaminating L-threonine deaminases were negligible. In addition, one of the strains studied was a biosynthetic L-threonine deaminase-less mutant.

Materials and methods

Bacterial strains: Strains E. coli Hfr C (Met$^-$), E. coli Hfr C(Met$^-$), S-2,* were isolated in our laboratory by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis from the above-mentioned strain; its second step mutant E. coli Hfr C, (Met$^-$, Ileu$^-$), S-2 was obtained by a similar treatment.

* We are greatly indebted to Dr Erzsébet Kerekes for kind supply of E. coli Hfr C, (Met$^-$), S-2 strain.

Culture media: The bacteria were grown in a synthetic salt medium containing tryptone and yeast extract (Alföldi et al., 1968) or in minimal medium without tryptone and yeast extract.

Assay of enzyme activity: The preparation of extracts and the assay of enzymic activity were described earlier (Alföldi et al., 1968).

Results

The L-serine deaminase activity of the bacteria investigated was about ten times higher than that of the parent strain E. coli Hfr C, (Met$^-$), while threonine deaminase activity was not increased. Furthermore, E. coli Hfr C, (Met$^-$, Ileu$^-$) S-2 grown in minimal medium has no biosynthetic L-threonine deaminase, but in complex medium it contained a low level of degradative L-threonine deaminase activity (Table 1). The crude extracts were diluted 100 to 200-fold before the assay, so that the threonine deaminases contributed to any detectable extent to the L-serine deaminating activity.

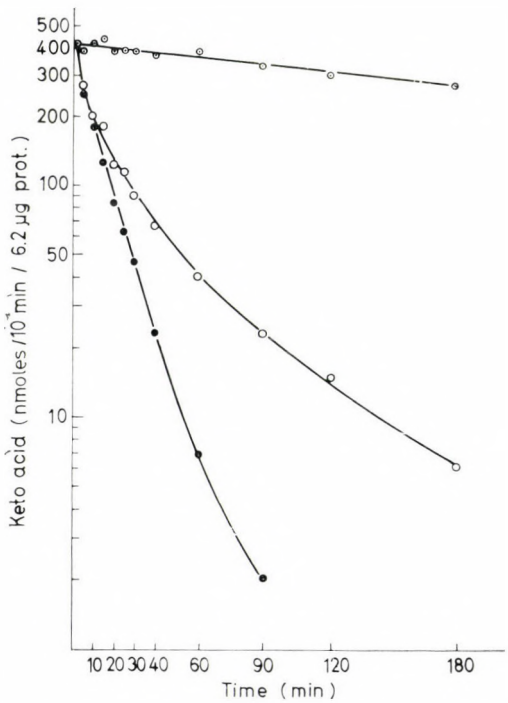

Fig. 1. Inactivation of L-serine deaminase at different enzyme concentrations. E. coli Hfr C (Met$^-$)S-2 bacteria grown in a complex medium were centrifuged sonically disrupted in 0.2 M potassium phosphate buffer (pH 7.63). The crude extract (12.4 mg/ml protein) and its 10-fold (1.2 mg/ml protein) and 50-fold (0.24 mg/ml protein) dilutions were incubated in a water bath at 30 °C. At the times shown on the graph the enzyme was diluted further (62 µg/ml protein) and 0.1 ml was pipetted into the assay mixture. Symbols: ⊙, 12.4 mg/ml protein; ○, 1.2 mg/ml protein; ●, 0.24 mg/ml protein

Table 1

The activities of L-serine and L-threonine deaminase enzymes from parent and mutant E. coli Hfr C strains

The specific activity is expressed as nanomoles of keto acid produced per 10 μg of protein per 1 hour at 30 °C (In the threonine deaminases assay the mixture contained 5 μg of pyridoxal phosphate.)

Bacterial strain	Specific activity of L-serine deaminase		Specific activity of L-threonine deaminase	
	Bacteria grown in			
	minimal medium	complex medium	minimal medium	complex medium
E. coli Hfr C (Met⁻)	32.6	232	19.1	21
E. coli Hfr C (Met⁻), S-2	353	3330	10.7	12
E. coli Hfr C (Met⁻, Ileu⁻) S-2	253	3029	2.0	3 2

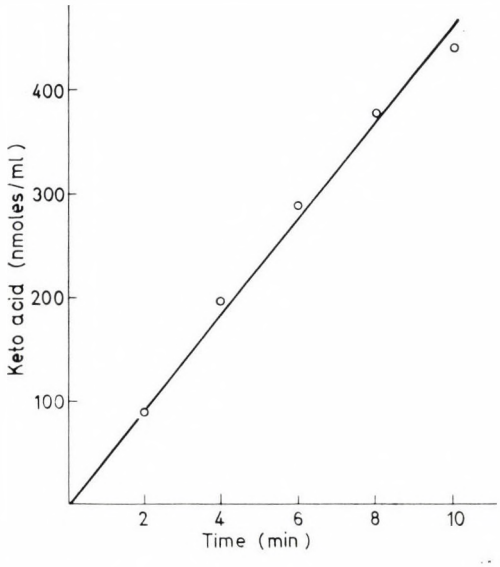

Fig. 2. Time course of keto acid production by a sonically treated bacterial suspension. E. coli Hfr C (Met⁻) S-2 cells grown in a complex medium were sonically disrupted. From an appropriate dilution (78.0 μg/ml protein) 1 ml was added to a 9.0 ml assay mixture in a 30 °C water bath and thoroughly mixed. At appropriate times 1.0 ml samples were pipetted to 1.0 ml of 2,4-dinitrophenylhydrazine solution to stop the enzyme reaction

L-serine deaminase seems to be a very labile protein, as dilution rapidly inactivates it, but is protected from inactivation by the substrate (Alföldi et al., 1968). The enzymes of the two mutant strains behave exactly in the same manner (Fig. 1). The rate of inactivation of the enzyme is a function of dilution. Neverthe-

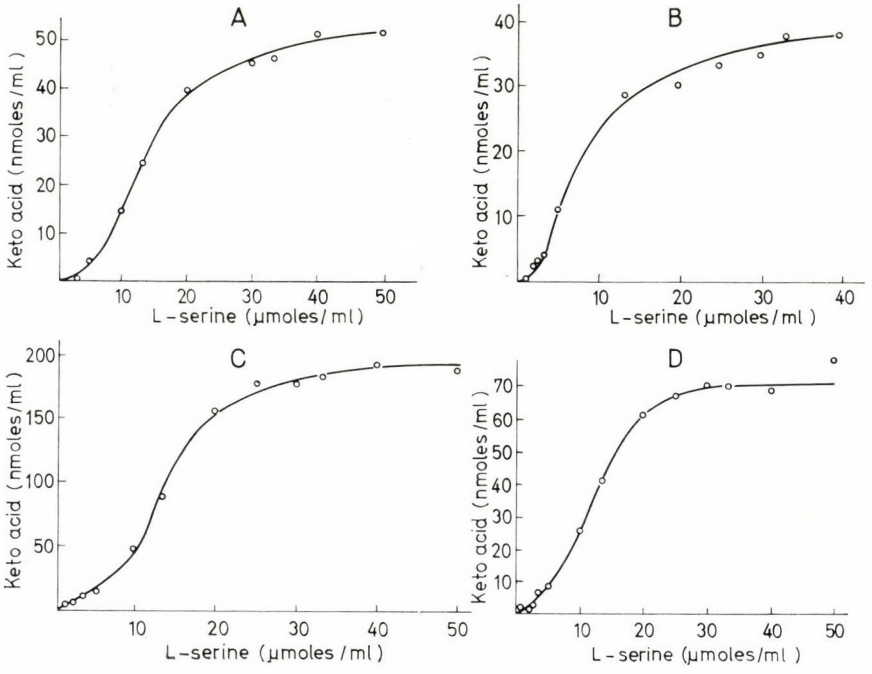

Fig. 3. Substrate saturation experiments. The mutant strains were sonically disrupted in 0.2 M potassium phosphate buffer. From appropriate dilution 0.1 ml was pipetted into the assay mixture. The reaction was stopped after 5 minutes. The keto acid production as a function of L-serine concentration is indicated in the graphs. A, E. coli Hfr C (Met⁻) S-2 grown in minimal medium (26.5 μg/ml protein); B, E. coli Hfr C (Met⁻ Ileu⁻) S-2 grown in minimal medium (22.0 μg/ml protein); C, E. coli Hfr C (Met⁻) S-2 grown in complex medium (719 μg/ml protein); D, E. coli Hfr C (Met⁻ Ileu⁻) S-2 grown in complex medium (3.7 μg/ml protein)

less, despite its high lability, the amount of substrate transformed at saturating substrate concentration was found to be proportional to the assay time up to 10 minutes (Fig. 2). The activity, therefore, can be measured under these conditions even at high enzyme dilutions.

In the substrate saturation experiments a sigmoid curve was obtained irrespective of whether the bacteria had been grown in a complex or minimal medium. The shape of the curves was the same both in the presence and in the absence of biosynthetic L-threonine deaminase (Fig. 3).

The K_m values determined from the saturation curves in the double reciprocal plot were similar to those found earlier (Alföldi et al., 1968; Raskó et al.,

1969). In these plots only the points near substrate saturation were included. The K_m values obtained are $5.75-8.6\times10^{-3}$ M for L-serine (Fig. 4).

There is no explanation for the sigmoid character of the substrate saturation curves. The possibility that the sigmoid character is caused by the rapid spontaneous inactivation of the enzymes at low substrate concentration could not be

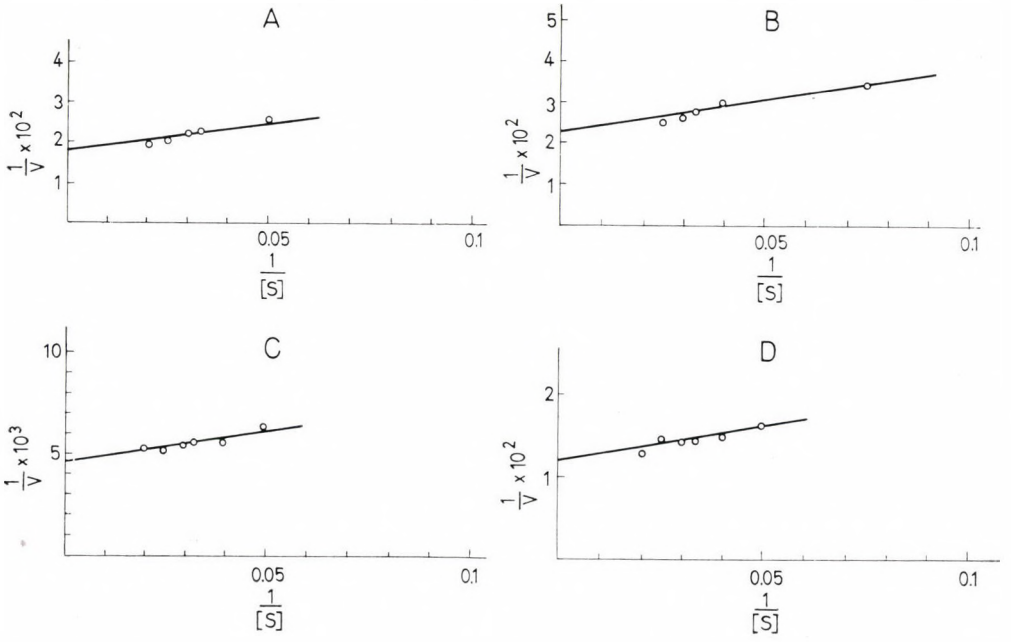

Fig. 4. Double reciprocal plots of substrate saturation data. The data are taken from Fig. 3, but in these plots only the points near substrate saturation were included. A, E. coli Hfr C (Met⁻) S-2 grown in minimal medium, $K_m = 8.6\times10^{-3}$ M; B, E coli Hfr C (Met⁻ Ileu⁻) S-2 grown in minimal medium, $K_m = 7.4\times10^{-3}$ M; C, E. coli Hfr C (Met⁻) S-2 grown in complex medium, $K_m = 5.75\times10^{-3}$ M; D, E. coli Hfr C (Met⁻ Ileu⁻) S-2 grown in complex medium, $K_m = 6.0\times10^{-3}$ M for L-serine

confirmed, as shown by the experiment illustrated in Fig. 5. The enzyme was incubated with a small amount of substrate, at different intervals samples were taken and the keto acid produced was measured. Simultaneously a sample was further incubated in an assay mixture containing high substrate concentration. If at high substrate concentration the inactivation of the diluted enzyme is stopped, then the active enzyme molecules present at the time of sampling can be determined. In the experiment the progress curve of keto acid production is almost linear in the first 10 minutes (Fig. 5A), though nearly 50% of the enzyme has been inactivated by this time as shown in Fig. 5B. Since the inactivation of the enzyme at low substrate concentrations is not reflected in the kinetics of keto acid production, the rapid inactivation of the enzyme cannot be the only cause of the sigmoid saturation curve.

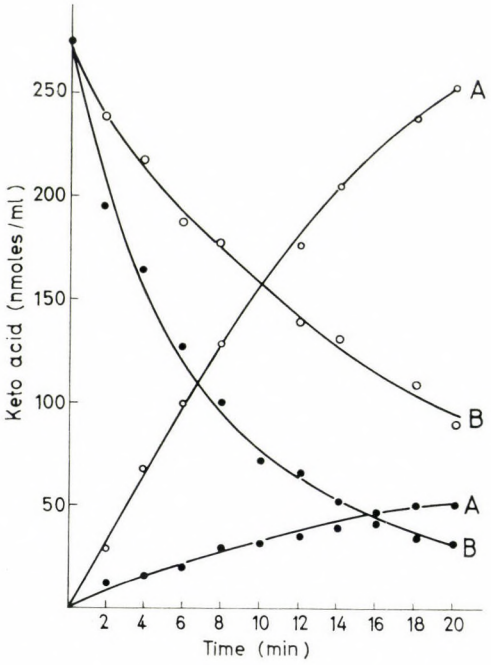

Fig. 5. L-serine deamination of the E. coli Hfr C (Met⁻) S-2 strain extract and the inactivation of L-serine deaminase at low substrate concentrations. Scheme of the experiment:

3 ml of diluted enzyme

+

27 ml of assay mixture with low substrate concentration

preincubated at 30 °C

1 ml	1 ml
+	+
1 ml 2,4-Dinitrophenil hydrazine	0.1 ml (40 μmoles) L-serine after 10 minutes further incubation
	+
	1 ml 2,4 Dinitrophenil hydrazine
("A" curves)	("B" curves)

Protein concentration of the diluted enzyme: 5.2 μg/ml. Substrate concentration of the assay mixture: 15 μmoles/ml (○), 7 μmoles/ml (●) L-serine

Discussion

The present experiments with L-serine diaminase of high specific activity have confirmed all our earlier results with this enzyme, namely the sigmoid curve of substrate saturation and the anomaly that the kinetics of keto acid production fails to reflect the inactivation of the enzyme (Alföldi et al., 1968; Raskó et al., 1969). These phenomena may be caused or influenced by the presence of L-threonine deaminases in the assay mixture. This would account for the fact that despite the marked inactivation of the enzyme at low substrate concentration the progress curve of keto acid production is linear. In fact, L-threonine deaminases may be inactivated to a lesser extent than L-serine deaminase and the initial linearity of keto acid production might be due to the L-serine-deaminating activity of the former enzyme. However, the degradative L-threonine deaminase can be responsible for only about 1 % of keto acid production on the crude extract (Table 1) and, moreover, in our experiments the crude enzyme was diluted 100 to 200-fold in the assay mixture. Therefore the amount of L-threonine deaminase present in our assays is negligible, and cannot be responsible for the observed anomalies.

We examined whether incubation of the enzyme at low substrate concentration might convert it into a conformational state which, though still enzymically active, is inhibited by high substrate concentration. This possibility could also be ruled out, since the saturation curve of the enzyme preincubated for 15 minutes at low substrate concentration (2 μmoles per ml of L-serine) had the same shape as those, shown in Fig. 3. Had our assumption been valid, then at substrate concentrations near saturation the enzymic activity should have decreased.

No explanation can yet be offered for the sigmoid substrate saturation curves of L-serine deaminase. In the case of bacteria grown in a complex culture medium any interference by the degradative L-threonine deaminase is excluded by the high dilution of the crude extract. The sigmoid saturation curve obtained with the E. coli Hfr C (Met^-, $Ileu^-$) S-2 mutant grown in minimal culture medium rules out the possible contribution of biosynthetic L-threonine deaminase.

The sigmoid substrate saturation curves may have a simple kinetic origin (Keleti, 1968) but this possibility could be tested only with purified enzyme preparations.

Similar sigmoid substrate saturation curves were obtained for the L-serine deaminase of Arthrobacter globiformis where a substrate activation mechanism was suggested (Bridgeland et al., 1965). Normal Michaelis-Menten kinetics were observed for the L-serine deaminase of C. acidi-urici (Benziman et al., 1960) and of Streptomyces rimosus (Szentirmai, Horváth, 1962). The enzyme preparation from Arthrobacter, however, was a crude extract, whereas the enzyme from Streptomyces was purified. Because of its extreme lability, we have so far failed to purify our enzyme.

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The Similarity of Trypsin Activated by Alkylammonium Ions, to α-Chymotrypsin

(Preliminary Communication)

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The most significant difference between the active center of α-chymotrypsin and trypsin is that the Ser-189 has become the Asp-189,* responsible for the different specificities. In the neighbourhood of these Ser- or Asp-189 residues the protonated α-amino group of Ile-16 occurs, which forms an internal ion-pair with the negatively charged carboxylate group of Asp-194. This Ile-16- Asp-194 ion-pair has an important effect on the spatial relation of Ser-195 to His-57 of the catalytic site. In trypsin the Ile-16 and Asp-194 ion-pair might be arranged in a slightly different way, than in α-chymotrypsin, disturbed by the $-COO^-$ group of Asp-189 (Kasserra, Laidler, 1969).** In addition to this effect, the Asp-189 might be further stabilized by other interactions.

Inagami et al. (1964a, 1969) proposed that the binding of a cationic substrate to trypsin induces a conformational change of the enzyme resulting in the activation of its catalytic site. Inagami and Murachi (1964a) then Erlanger and Castleman (1964) and recently Seydoux (1969) showed that alkylammonium ions increased the rate of the trypsin-catalyzed hydrolysis of acetylglycine esters.

These facts suggest that the positively charged substrates convert the trypsin to an α-chymotrypsin-like form, while neutral substrates have no effect. We propose that the cationic part of the substrates, owing to its ionic interaction with the $-COO^-$ group of Asp-189, abolishes the disturbing effect of this Asp-189 on Ile-16 and contributes in this way to the formation of the α-chymotrypsin-like form of the Ile-16 and Asp-194 ion-pair. This slight shift of the $-NH_3^+$ group of Ile-16 from Asp-189 towards Asp-194 initiates a similar relationship of Ser-195 and His-57 to that of α-chymotrypsin and in this way the "switching on" of the catalytic activity. We suggest that this small conformational change involves events in the substrate binding, too.

*Residues are numbered according to the sequence of bovine chymotrypsinogen A. Abbreviations used: AAEE, N-acetyl-L-alanine ethyl ester; AGEE, N-acetylglycine ethyl ester; AVEE, N-acetyl-L-valine ethyl ester; CR, α-chymotrypsin; TR, trypsin; ETA, ethylammonium; META, methylammonium, PROPA, 1-propylammonium chloride.

**Note added in manuscript: As dr. R. M. Stroud (Gates and Crellin Lab. of Chem., Calif. Inst. of Tech., Pasadena, California) kindly informed us, their crystallographic studies on trypsin showed a 6.5 Å distance between the α-amino group of Ile-16 and the carboxyl group of Asp-189.

The hydrolysis of N-acetylglycine ethyl ester, N-acetyl-L-alanine ethyl ester and N-acetyl-L-valine ethyl ester, catalyzed by trypsin in the presence and absence of alkylamines, and by α-chymotrypsin, was investigated. The initial rates of hydrolysis were determined by the pH-stat method.

Table 1

Apparent kinetic parameters

The initial rates of hydrolysis of N-acetylamino acid ethyl esters, catalyzed by trypsin, trypsin and alkylammonium ions and α-chymotrypsin were determined by pH-stat titration at pH 6.6 and 25°C in 0.1 M KCl; volume 4 ml; $[E_0] \sim 4 \times 10^{-5}$ M; titrant 0.02 N NaOH

Substrate, enzyme, effector		$k_{cat.}$ (sec^{-1})	$K_{m,app.}$ (M)	$100 \times k_{cat.}/K_{m,app.}$ (unity: CR data)
AGEE	(0.2–1.5 M)			
	TR*	0.009	1.30	44
	TR, ETA (0.50 M)**	0.10	3.50	181
	CR*	0.024	1.52	100
AAEE	(0.05–0.7 M)			
	TR	0.43	1.00	41
	TR, META (0.55 M)	1.39	0.79	167
	TR, ETA (0.50 M)	0.70	0.97	69
	TR, ETA (0.10 M)	1.00	1.48	64
	TR, ETA (0.05 M)	0.63	0.94	64
	TR-PROPA (0.05–1.0 M) ***	inhibition	—	—
	CR	1.03	0.98	100
AVEE	(0.05–0.3 M)			
	TR	0.09	2.16	14
	TR, META (0.55 M)	0.22	0.96	74
	TR, ETA (0.02–0.7 M)***	inhibition	—	—
	CR	0.21	0.69	100

* Inagami and Mitsuda (1964b) have reported values of 0.007 and 0.018 for k_3, and 0.83 and 0.40 for K_m, for TR and CR, respectively, at pH 6.6 and 25°C in 0.1 M KCl (taken from their Fig. 4, 5 and Table 1).

** Inagami and Murachi (1964a) have reported a 9.5-fold increase in the relative rate of hydrolysis in the presence of 0.34 META at pH 6.6 and 25°C in 0.1 M KCl. They state the constancy of the K_m values on activation.

*** Determined at 0.1 M AAEE and 0.2 M AVEE, respectively.

All experimental data have been analyzed and the kinetic parameters computed using a program written by P. Mezey in this Institute (to be published). The program fits experimental data directly to the Michaelis-Menten equation.

The nearly 1 : 2.5 ratio of the activity of trypsin and that of α-chymotrypsin is very conspicuous in all cases (Table 1). This was shown also by using Inagami's and Mitsuda's values, given in the footnote to Table 1. Inagami and Murachi (1964a) published a 2.4-fold rate increase for AGEE when activating the trypsin by PROPA. Our experiments revealed a similar increase of $k_{cat.}$ for AAEE and

AVEE in the presence of ETA (0.1 M) and META (0.55 M), respectively. In this way Inagami's and Murachi's results for PROPA and our $k_{cat.}$ data are near to those of CR. On the other hand, trypsin, in the presence of AGEE and ETA or AAEE and META seems to be "over-activated", as indicated by the rate constants of CR. Although the variation of the K_m, app. values seems to be disordered, the increase in $k_{cat.}/K_m$, app. data represents a good order. Namely ETA has the similar effect on AGEE, as has META on AAEE, and this is the case for the AAEE and AVEE relationship too. Inagami and Murachi (1964a) found inhibition of AGEE by 1-n-butylammonium ion, we got a similar result for AAEE by PROPA and for AVEE by ETA. All these facts prove a precise additivity of the alkyl chains of the substrate and alkylammonium ion, considering their effect on the catalytic and binding sites of trypsin. The sum of the sizes of these alkyl groups is limited by the binding site ("specificity cavity").

Although our suggested mechanism looks likely from the given data, it is impossible to determine exactly from these limited kinetic parameters, what portion of the observed effects is to be attributed to the actual differences.

The authors are indebted to Dr P. Mezey for the programming and organization of computations.

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Lattice Model for Diffusion in Liquids

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Theoretical determination of the mean life time of diffusing molecules in a certain small volume or in the neighbourhood of a given steric point may have great biological importance. The biological way of putting the question involves fluid environment for the diffusing molecules. The nowadays physical knowledge on liquid kinetics does not make possible the easy and correct theoretical answer of the question, and one is forced to live on approximations. It is the aim of the present model to make the above parameter — how long does a diffusing particle stay in a given steric point or volume — determinable theoretically with a certain approximation. The model establishes contact between the phenomenological diffusion constant of Fick's laws and the individual behaviour of diffusing particles.

The model

In an (x_1, x_2, x_3) cartesian coordinate system we assume sphere like molecules with a diameter λ, which build up a rectangular lattice network concerning the centres of the spheres. The nearby centres differ only at one coordinate value so that $\Delta x_i = \pm\lambda$, where Δx_i is the difference of two nearby centres in the i^{th} coordinate ($i = 1, 2, 3$). For sake of simplicity the origo of the coordinate system is one of the centres (lattice points). The molecules of the liquid model accomplish rotational and vibrational motion at each lattice point for τ average time (cage effect). There are six lattice points for each molecule to enter within a time negligible when compared to τ. It follows that some "empty" lattice points must exist at any time, which are not actually occupied by particles.

As such events generally do, the life time of molecules presumably follows an exponential distribution. In other words the probability that one molecule arriving to one lattice point at a time $t = 0$ will leave it in a time interval of $(t, t + dt)$ (Prékopa, 1962):

$$p(t)\,dt = \frac{1}{\tau} \cdot e^{-\frac{t}{\tau}} \cdot dt \qquad (1)$$

where $p(t)$ is the distribution density function of the exponential distribution.

The theoretical observer fixed to one lattice point can follow the motion of molecules after labelling – e.g. "painting" with isotopes – a certain ratio of them.

In studying the problems of random walk, let the c concentration of labelled molecules be defined with such properties that

$$\frac{\partial c(x_1, x_2, x_3, t)}{\partial x_1} = a \tag{2a}$$

$$\frac{\partial c(x_1, x_2, x_3, t)}{\partial x_2} = \frac{\partial c(x_1, x_2, x_3, t)}{\partial x_3} = 0 \tag{2b}$$

$$\frac{\partial c(x_1, x_2, x_3, t)}{\partial t} = 0 \tag{2c}$$

for every x_i and t. We take two lattice planes crossing the ${}_1X_1 = m\lambda$ and ${}_2X_1 = (m+1)\lambda$ lattice points at right angles to the x_1 axis (${}_1X_1$ and ${}_2X_1$ are on the x_1

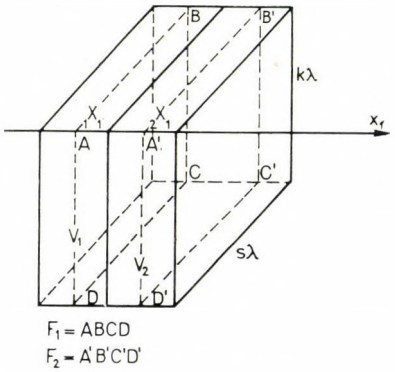

Fig. 1. A schematic figure of the model. The ${}_1X_1$ and ${}_2X_1$ coordinates are in the A and A' points. The dotted lines mark the margines of F_1 and F_2 surfaces, and the continuous lines show the margines of V_1 and V_2 volumes. For details see the text

axis), furthermore we mark out the $F_1 = F_2 = k \cdot s \cdot \lambda^2 = F$ surfaces on the planes facing each other in x_1 direction (Fig. 1). Parallel to both lattice planes we define planes in $\pm x_1$ directions at λ distances away, and cut them with planes connecting the margines of F_1 and F_2 surfaces of the lattice planes. The V_1 and V_2 volumes are constructed by this way as it is shown on the Fig. 1.

As clearly $V_1 = V_2$, the $V_1 = V_2 = V$ symbol will be used further on if only the size of the volume is taken into account.

At $t = 0$ time let N_1 and N_2 be the number of molecules in volumes V_1 and V_2, respectively. As to (2*a*, *b* and *c*) $N_2 > N_1$, there is a $j = j_1 - j_2$ diffusion stream in $-x_1$ direction through the connecting surfaces of the V_1 and V_2 volumes. j_1 and j_2 are labelled molecules diffusing from V_1 to V_2 and from V_2 to V_1 in time unit, respectively.

With these considerations one may establish the number of labelled molecules leaving their lattice points in V_1 volume within $(t, t + dt)$ time interval

$$n_1(t)\, dt = \frac{N_1}{\tau} \cdot e^{-\frac{t}{\tau}} \cdot dt \tag{3}$$

It follows from the (2*c*) assumption that

$$n_1(t)\,dt = n_1(0)\,dt = \frac{N_1}{\tau}\,dt \tag{4}$$

for every t.

Referring to the $P = \frac{1}{6}$ probability which exists for each molecule of V_1 leaving its lattice points for V_2

$$j_1 = \frac{N_1}{6\tau} \tag{5}$$

and similarly

$$j_2 = \frac{N_2}{6\tau} \tag{6}$$

From (5) and (6)

$$j = j_1 - j_2 = \frac{1}{6\tau}(N_1 - N_2) \tag{7}$$

Using Fick's I. law, the j diffusion stream crossing the plane with $x_1 = (m + \frac{1}{2})\,\lambda$ coordinate:

$$j = -DF \cdot \frac{\partial c}{\partial x_1} \tag{8}$$

Considering the (2*a*) assumption

$$\frac{\partial c}{\partial x_1} = \frac{\Delta c}{\Delta x_1} \tag{9}$$

for every x_1.

Letting $\Delta x_1 = (m + 1)\,\lambda - m\lambda = \lambda$, for $\frac{\partial c}{\partial x_1}$ must stand

$$\left.\frac{\partial c}{\partial x_1}\right|_{x_1 = (m + \frac{1}{2})\,\lambda} = \frac{1}{V\lambda} \cdot (N_2 - N_1) \tag{10}$$

But $F = k \cdot s \cdot \lambda^2$ and $V = k \cdot s \cdot \lambda^3$. Substituting these values of F and V into Eq. (10), and the obtained expression into Eq. (7):

$$j = D \cdot \frac{N_1 - N_2}{\lambda^2} \tag{11}$$

From Eq. (7) and (11):

$$\tau = \frac{\lambda^2}{6D} \tag{12}$$

Defining the steric position of sphere like large molecules by their mass centres, it is possible – knowing their diffusion constant, D – to determine the mean lifetime at one lattice point.

Similar expression was presented by Buchachenko considering the behaviour of stabile radicals in liquids (Buchachenko, 1965).

According to this model, presented above, the mean staying time of small as well as of large molecules can be determined, by good approximation in a system consisting of relatively few lattice points.

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Biology and Mathematics

III. Biology Heading for the Exact Sciences*

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According to the evidence of history the greatest achievements have been produced when pioneers succeeded in formulating their results mathematically. In contrast to that, even the best discoveries of biologists being formulated in contexts of different languages are exposed to the risk of redundancy and ambiguity inherent in every language. Notwithstanding, most biologists preferred more or less long texts to mathematical formulation as shown by the immense difference in the estimation of the works of Darwin and those of Weber—Fechner, Bernard and Mendel. Thus we are facing the task that biology should become an exact science at least for the 21st century.

1. It is our task to see that Biology steps from the twentieth into the twenty-first century as an exact science. One can be guided to the road leading to this goal by reviewing the history of science, chiefly in the last four decades.

1.1. To begin with, let us assess, from the angle of humanity's progress towards homo sapiens, the descriptions of motion and their results: the descriptions that have appeared since Zenon (fifth century before our era) up to now, on the one hand, and, in the seventeenth century, Galilei's statement concerning gravitational acceleration (g) and Huygens' finding about centrifugal acceleration $\left(\frac{v^2}{r}\right)$, on the other. On the basis of

$$g = \frac{v^2}{r},$$

the last two *mathematical formulations* led to the result

$$v = \sqrt{gr} \sim 8000 \text{ ms}^{-1};$$

if a body is to be an Earth satellite, it has to be launched at a speed of 8 km/sec. And sputniks did result.

It was in the seventeenth century, too, that Glisson stated that excitability is a fundamental property of every living system. The *words* of Glisson's formula-

* Part of a lecture given on 21st April 1971 at the request of the Free University "TIT" (Scientific Society for the Propagation of General Knowledge).

tion resulted in vitalism and they even gave rise to a fault; viz. in the next century Linné (1735, Systema Naturae) teaches: "Lapides crescunt, vegetabilia crescunt et vivunt, animalia crescunt, vivunt et sentiunt." Contrary to that, it is superfluous to emphasize that plants are also excitable, i.e. they "feel".

1.2. In general, eighteenth century Biology did not get beyond the descriptive method either and remained stuck in vitalism. A very interesting picture from the eighteenth century is given by Galvani's book published in 1791, which not only laid the foundations of the doctrine of "animal electricity" but also *described* the discovery of the electric wave. However, it was only a century later, in the nineteenth century, that the electric wave became public property in science when, on the basis of *Maxwell's mathematical formulation*, Hertz rediscovered the phenomenon, which is one of the basic elements of the life of our present-day human world.

1.3. In addition to physics, in the nineteenth century, chemistry, physical-chemistry and also colloidics moved into the domain of exact sciences finally – after proper formulation and *transmathematization*. Alas, of Biology this cannot be said, in spite of the very considerable production, of which I only mention the following works:

Lamarck (1809), Philosophie Zoologique and
Darwin (1859), The Origin of Species;
Weber (1831) – Fechner (1859), Psychophysical Law;
Bernard (1859), Physiologie et Pathologie des liquides de l'organisme;
Mendel (1865), Versuche über Pflanzen-Hybriden.

2. Thus we are entitled to raise the question why, as a result of the achievements only partly reflected in this list, Biology has not even now, after a century, become an exact science? Let a biologist from the beginning of this century answer the question (Locy: Biology and its Makers, 1909. Deutsche Übersetzung, 1915. S. 376): "Darwin's 'Origin of Species by Means of Natural Selection' von 1859 ist von unserem heutigen Standpunkt (1909!) aus das grösste klassische Werk auf dem Gebiete der Biologie,"; and further (S. 264): "die Forscher jener Zeit (around 1860) waren ganz und gar von phylogenetischen Fragen in Anspruch genommen, die durch die Publikationen Darwins hervorgerufen wurden."

And indeed, in the history of science there is hardly another book that would have achieved as great importance as Darwin's work, and which would have been surrounded by as much propaganda. Unfortunately, besides its epoch-marking scientific and social import, it must also be established that as a result of the flood of disputes in its wake the contemporary discoveries of great significance which, by virtue of their quantitative, mathematical formulation, would have been suitable for commencing the raising of Biology to the status of an exact science were eclipsed and passed unnoticed.

Although, on the whole, the possibilities indicated by the above list generally got lost for the nineteenth century, yet they were realized in splendid form in the works of Wiener in our century (Wiener, 1948, Cybernetics). Why, the initiatives

taken by the enumerated investigators are clearly demonstrable parts of cybernetics. To expound this I mention that during his stay in Budapest in 1963 Wiener expressed agreement with my suggestion according to which

$$\text{cybernetics} = \text{information theory} + \text{automation}.$$

2.1. Using mathematics information theory, as is well known, greatly reduces the large number of possibilities inherent in a certain situation to a much smaller

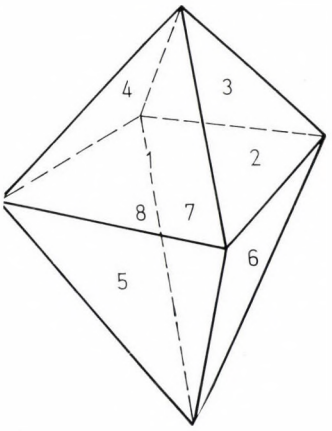

Fig. 1. Octahedron made of cardboard; the faces are marked with the numbers 1—8

number of acts of solution. As an example let us take a regular octahedron (Fig. 1); the faces are marked with the numbers 1—8. No matter how this figure happens to be lying, one of the numbers appears on the upside face; the task is to guess which of the eight it is. According to information theory it is not necessary to try to guess, say, seven times, but the person who sees that, e.g. face No 2 is on top, has to answer questions with yes or no.

1st question:	is it in the greater half?	1 2 3 4 | 5 6 7 8
Answer: no;	thus it is among	1 2 3 4
2nd question:	is it in the larger half?	1 2 | 3 4
Answer: no;	so it is among	1 2
3rd question:	is it 2?	
Answer: yes;	so *2*.	

That is: of the 8 possibilities the sure solution was obtained after 3 questions; in a formula

$$k \cdot \log 8 = 3, \quad (k = {}^{10}\log 2).$$

To prove that as the possibilities are further increased in number they can be reduced to proportionally fewer and fewer acts, I present Fig. 2, in which the horizontal lines numbered 1 – 8 form a square with the perpendicular lines 1 – 8.

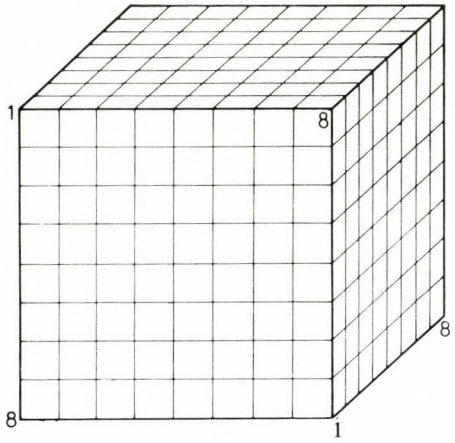

Fig. 2. The line 1—8, the square 8×8, the cube 8×8×8

The number of possibilities offered by this square amounts to $8^2 = 64$; to guess one of them, we already have the key:

$$k \cdot \log 8^2 = 2(k \cdot \log 8) = 2 \cdot 3 = 6.$$

And if the cube is divided into small cubes, then the solution to the $8^3 = 512$ possibilities is

$$k \cdot \log 8^3 = 3 \cdot 3 = 9.$$

This road is indicated by Hartley's formula (belonging to information theory)

$$I = k \log H,$$

in which I and H, respectively, are the information content and the amount of information.

2.2. Neglecting all inaccuracy deliberately, let us compare with this the "psychophysical law" of Weber – Fechner. Weber's fundamental idea, which has brought the spiritual phenomena of sensation into the province of the measuring exact sciences, is hardly less important than the Lamark – Darwinian conception. Now, according to this law

if 2 candlepowers cause a sensation of light of magnitude s,
then 2^2 candlepowers cause a sensation of light of magnitude 2 s,
2^3 candlepowers cause a sensation of light of magnitude 3 s;

and so on. I.e. if sensation (s) is to increase according to arithmetical progression then the irritation (i) or stimulus has to increase according to geometrical progression; in a formula

$$k \log i = s^*$$

Without pressing things one can recognize an analogy between the two statements: according to the Weber–Fechner formula the cardinal of the stimuli is reduced to the much smaller one of the sensations, whereas Hartley's formula reduces the large amount of information to the smaller information content. It is questionable how far the propaganda associated with the individual results, or the differences shown by this propaganda, are responsible for the fact that Weber's initiative slumbered for a century.

2.3. One of the splendid flowerings of information theory in the 1950s was the emergence of genetics as an exact science. On the basis of geometry (helix) and information theory we have now reached a point where the code of sequences of the twenty amino acids in the protein molecule is attributed to the different variations of the four bases of nucleic acid:

$$V_4^{3r} = 4^3 = 64 \ (r = \text{repetition}).$$

And it does not require force either to discover in Mendel's work the buds from which genetics can develop into an exact science. Therefore here, too, the question may be asked, how much of the century long delay along the Mendelian road is due to propaganda and how much to the biologists' degree of qualification.

3. Another sector of cybernetics, inseparable from information theory, is automation.

A decisive, weighty element of biocybernetics is automatic regulation of the processes of life, briefly called bioregulation. To give an example right here, blood pressure, blood sugar, pH, body temperature, etc., etc., do not show fixed, invariable values, but constant oscillations around certain means. If e.g. blood pressure is high, an intelligible signal, i.e. information about it, is transmitted to the centre, which, in turn, sends an intelligible signal, i.e. information for the blood vessels to dilate; about this first result, newer information goes to the centre, which sends out a newer informative instruction, etc. Such a process is termed *feed-back control.*

The foundations of this apparently new aspect can be detected in Claude Bernard's conception of the constancy of the "milieu intérieure", which was

* The formula

$$s = k i^{\frac{1}{n}} \ (n > 1)$$

of the Plateau—Stevens conception does not seem better for mathematical comprehension of the whole stimulus–sensation complex.

brought to perfection in Cannon's homeostasis. However, in the new Hungarian Encyclopaedia 0 line, 8 lines and 2 columns are devoted to Weber, Bernard and Darwin, respectively; in the new Encyclopaedia of Natural Science Weber and also Bernard received 0 lines, Darwin 3 columns. This difference in estimation can hardly be taken as an objective measure of their real scientific achievements.

4. The situation is rendered understandable – although not acceptable – by the fact that the biological entries in the above-mentioned, otherwise generally good encyclopaedias were probably written by biologists. On the basis of their mentality rooted in the past many biologists find it easier to join in a description in words rather than in mathematical formulations, which makes quantitative statements possible. If, however, (in future) Biology is to be – and it must be! – transformed into an exact science then the distinction between description in words and mathematical formulation has to be appreciated.

The Zenonian problem of motion will allow of conflicting linguistic definitions even today: 1. about the fifth century before our era: motion is contradictory and, therefore, a notion to be discarded, viz. a flying arrow *is* at this or at that point of its trajectory at different moments; if, however, it *is there*, then it is not *moving;* 2. today: Zenon only considers motion as occurring by stages, not its continuity; rest is only apparent and relative, motion is of absolute character; motion is the mode of existence of matter. When these opinions are compared with the fact of space travel – based on the formula of Galilei–Huygens – the conclusion becomes unavoidable that the redundancy and ambiguity of linguistic definitions give an indisputable advantage to mathematical formulation, which does not suffer either redundancy or ambiguity. It is through mathematization that Biology is to become an exact science.

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The Effect of Temperature on the Initial Volume Decrease and Action Potential of the Muscle

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The initial volume decrease and action potential of frog gastrocnemius was recorded simultaneously within a temperature range of 4 and 24 °C. It was concluded that the temperature dependence of the duration of maximum volume decrease and the action potential could be approximated with the function $\exp\{10^3 B/T\}$, where the value of B is (8.00 ± 0.41) °K and (7.80 ± 0.41) °K, respectively.

Introduction

Experiments investigating the initial volume decrease together with other phenomena of muscle activity, are of decisive importance in the interpretation of the initial volume decrease of the muscle. It was Ernst (1927) who recorded first simultaneously the initial volume decrease and action potential, and found that changes of the two phenomena run parallel in time during fatigue and at different temperatures (Ernst et al., 1954). The temperature dependence of volume decrease only was measured by Meyerhof and Möhle already in 1935. In spite of the difference in the experimental methods used and the quantitative data obtained by these authors, their results have unambiguously shown that the time period of the volume decrease increased with decreasing temperature. Investigating the temperature dependence of volume changes of the muscle, Abbott and Baskin (1960, 1962) as well Baskin and Paolini (1965, 1966) came to the same conclusion.

The aim of the present experiments was to determine the temperature dependence of the initial volume decrease and action potential of the muscle recorded simultaneously.

Experimental method

The investigations were performed on ischiadicus-gastrocnemius preparations of the frog (Rana esculenta), on end-free or tensionless muscles. A volumeter with the improved variety of the piezoelectric pressure transducer used originally by Ernst and his coworkers, and a dual channel oscilloscope were used for recording (Ernst et al., 1951; Aradi, Futó, 1970).

Our apparatus described previously in detail (Aradi, 1970) has appropriately been altered for the present purposes. Two pairs of platinum electrodes were

introduced into the muscle chamber through its stopper, one of these served for indirect stimulation of the muscle, and the second for recording action potentials. The nerve was hooked on the stimulating electrodes (their distance was 2 mm), and the recording electrodes were winded around the muscle at its largest diameter and 1 mm from the tendon, respectively, so, that the distance between the electrodes was about 20 mm.

On the first channel of the oscilloscope was recorded the volume decrease, and on the second the two-phase action potential of the muscle. The sweep speed was established with a time-mark generator.

The temperature of the preparation was controlled with a cooling jacket soaked with ethylene chloride around the muscle chamber. The nerve-muscle preparation was cooled down from +25 °C to +2 °C with a cooling rate of 1 °C/min, and then -- during a slow warming period – different values of temperature between 4 to 24 °C were chosen and kept for a period of 3 to 4 minutes. According to our measurements, this period of time was sufficient that the difference in temperature of the surface and the central core of the muscle should not be greater than 0.5 °C.

The temperature of the preparation was measured with a copper-to-constantan thermocouple connected to a calibrated galvanometer and fixed to the stopper of the muscle chamber. The tip of the thermocouple was placed in the close vicinity of the muscle surface. (The temperature was measured with an accuracy of 0.1 °C.)

The sensitivity to temperature of the piezoelectric quartz crystal does not interfere with the measurements, because the temperature coefficient of piezoeffect is 0.01 to 0.02 per cent/°C within the temperature range mentioned above (Spescha, Volle, 1967).

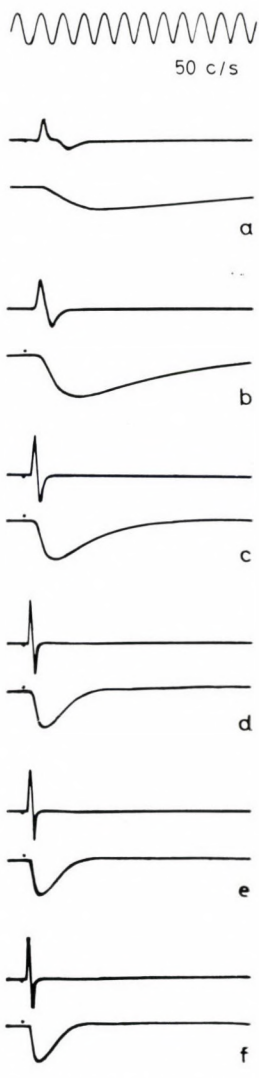
Experimental results

Fig. 1 shows the oscillograms obtained from a series of experiments performed on the same preparation. The initial volume decrease can be seen at the bottom, and the two-phase action potential at the top. The temperature of the muscle was 4, 8, 12, 16, 20 and 24 °C, respectively, in the subsequent experiments (Figs 1*a*–*f*).

The evaluation of the results is indicated in Fig. 2. t_v' and t_a' denote the latency of the volume decrease and that of the action potential, respectively; and t_v and t_a denote the duration of the maximum volume decrease and the action potential. The stimulus artefact was regarded as a reference signal.

The average values of the above data are plotted against the temperature in Figs 3 and 4. Between 8 and 24 °C the measurements were performed at every 2 °C.

The logarithm of the duration of the maximum volume decrease and that of the action potential ($\ln t_v$, $\ln t_a$) can be seen in Fig. 5 as plotted against the reciprocal of the absolute temperature (T^{-1}). The analytical forms of the



Figs 1a—f. Oscillograms of the initial volume decrease (lower curve) and action potential (upper curve) recorded from the frog's gastrocnemius at temperatures of 4, 8, 12, 16, 20 and 24 °C from *a* to *f*, respectively

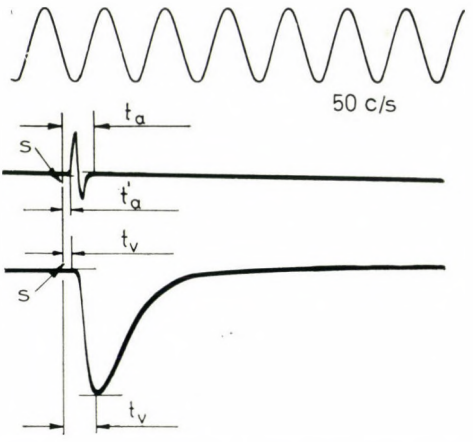


Fig. 2. Showing the time duration of the maximum initial volume decrease (t_v) and that of the action potential (t_a) of the frog's gastrocnemius. t'_v and t'_a, respectively, indicate the latency time of the two phenomena, and arrow with s points at the stimulus artefact

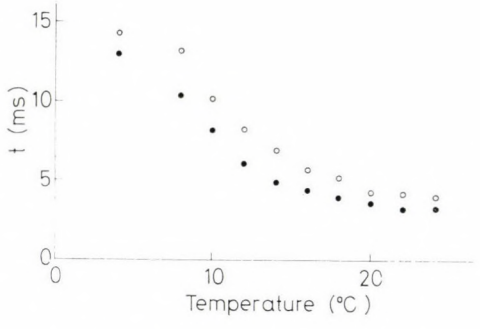


Fig. 3. Diagram showing the temperature dependence of the latency time of the initial volume decrease (○) and the action potential (●)

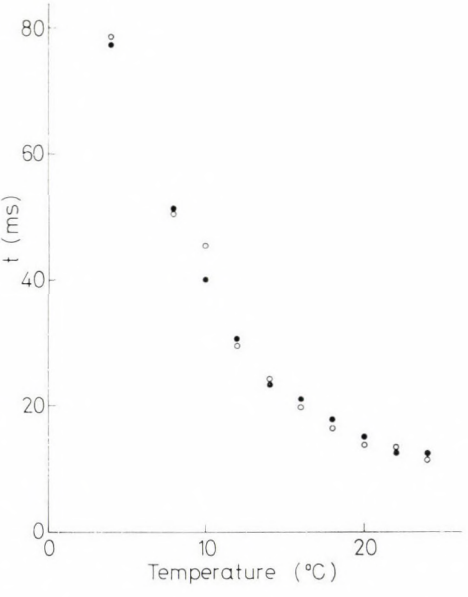


Fig. 4. Diagram showing the temperature dependence of the duration of the maximum volume decrease (○) and the action potential (●)

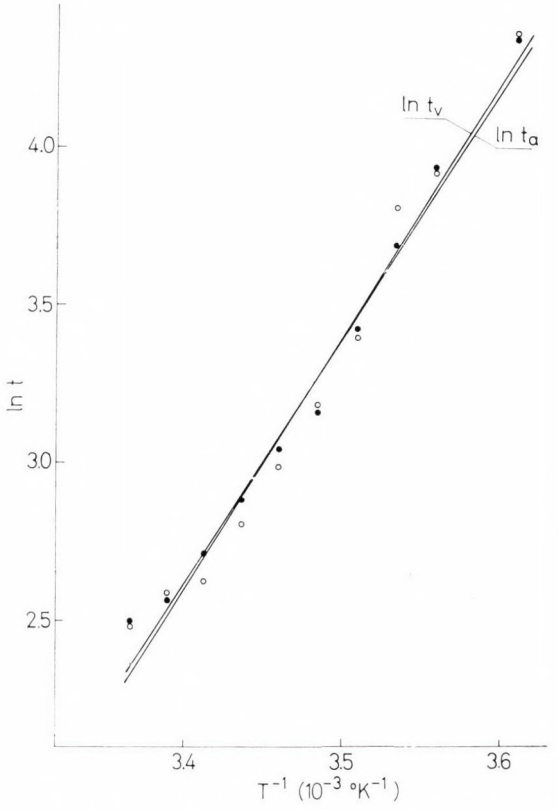


Fig. 5. Semilogarithmic plotting of the duration of the maximum volume decrease (○) and the action potential (●) against the reciprocal of the absolute temperature

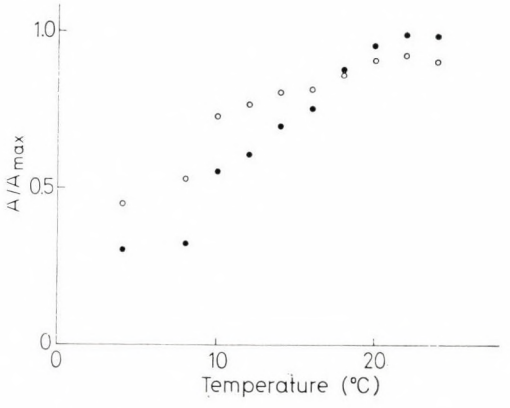


Fig. 6. Diagram showing the temperature dependence of the relative amplitude of the initial volume decrease (○) and the action potential (●)

straight lines fitted to the points of measurement by weighed least squares (where the standard deviation of the data of measurement is the weighing factor), are:

$$\ln t_v = (8.00 \pm 0.41)\, 10^3\, T^{-1} - (24.6 \pm 0.9) \quad (1)$$

and

$$\ln t_a = (7.80 \pm 0.41)\, 10^3\, T^{-1} - (23.9 \pm 1.0) \quad (2)$$

Values of the amplitudes of the volume decrease and action potential related to the maximum amplitude (A/A_{max}) are plotted against the temperature in Fig. 6.

Discussion

Data in the literature concerning the initial volume decrease of the striated muscle, and the results of our present experiments concord only with certain respects. The only unambiguous similarity is the fact that the process of the volume decrease becomes slower as the temperature decreases, significant differences, on the other hand, can be seen in the absolute time values and in the relation of the volume decrease to the action potential.

Having measured separately the volume decrease and the action potential on frog's gastrocnemius during isotonic activity, Meyerhof and Möhle (1935) came to the conclusion that at lower temperature the time course of the volume decrease was much slower than that of the electric phenomenon. In contrast with this, Ernst et al. (1954) were able to show a close relationship in the time course of the initial volume decrease and action potential recorded simultaneously from end-free muscles at different temperatures.

From Figs 3, 4, 5 and 6, and Eqs (1) and (2), observing the standard deviations of the parameters, the following conclusions may be drawn:

1) the length of the latency of the initial volume decrease and the action potential may be, to a certain extent, compared,

2) the duration of the development of the maximum volume decrease corresponds, in general, to the duration of the action potential,

3) the temperature dependence of the amplitude of the volume decrease and the action potential is of similar character.

From similar experiments of Baskin and Paolini (1966) performed on frog's sartorius, the temperature-coefficient (Q_{10}) of the volume decrease is approximately 1.5 as calculated in a temperature range between 10 and 20 °C. Determining the same Q_{10} value from our own data, it is 2.64 for the volume decrease, 2.56 for the action potential.

As we could show only a weak time correlation between the initial volume decrease and the action potential, further experiments are necessary to decide whether there is a causal relationship, or a time coincidence, between the two phenomena.

The authors are indebted to Prof. E. Ernst for his help in evaluation of the experimental data.

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X-ray Diffraction Investigation on the Growing Zone of Long Bones

(Short Communication)

G. LÉNÁRT, G. BIDLÓ, L. KÉRY

Department of Orthopaedics, Semmelweis Medical University, Budapest, and
Institute of Mineralogy and Petrology, Technical University, Budapest

Physiological ossification was investigated with X-ray diffraction in the epiphyseal disc of long bones. The results indicate that the physiological bone formation does not occur in one phase, and in this it is similar with pathological bone formation.

Longitudinal growth of long bones takes place in the proximal and distal epiphyseal discs. At these areas crystalline mineral substances are formed and deposited. At the same time the chondrocytes are arranged in columns, degenerate and are destroyed.

No experiments presenting crystallographical analysis of mineral substances formed in the process of osteogenesis are known. Investigations into this direction have double significance:

1) An insight is afforded into the process of the natural formation of the mineral substances of the bone of which very little is known.

2) They allow the comparison of physiological and pathological ossification from a crystallographic point of view.

For these purposes the proximal growing zone of the tibia was examined in 3-month-old calves (Fig. 1). The part of the growing zone shown in Fig. 1 was removed and after lyophilization ground to a grain size of 100 μ. An X-ray diffraction picture was made of the pulverized substance with a Guinier–de Wolf chamber. A quartz monochromator was applied, and so to work with a CuK_α beam defined correctly.

The analysis of the patterns (Fig. 2, Table 1) showed that in the specimen where the bone formation is just going on, two kinds of mineral can be met, i.e. apatite ($Ca_5(PO_4)_3 \cdot F \cdot OH$) and brushite ($CaHPO_4 \cdot 2H_2O$).

In another work we have pointed out the significance of brushite under pathological ossification that is in callus formation (Lénárt et al., 1968). In some of the cases monetite ($CaHPO_4$) could also be found in the mineralizing tissue of the callus, whereas it was absent in the epiphyseal disc. According to the present investigations, brushite and monetite never occurred together in the same X-ray diffraction picture, possibly because bone minerals are formed by transformation of different calcium phosphates (monetite, brushite, apatite) into each other.

The results presented suggest that the mineral substance of the new bone tissue developed under physiological conditions contains similar elements like that of a callus tissue formed under pathological conditions.

Fig. 1. Microphotograph showing the proximal growing zone of the tibia of a 3-month-old calf (110×, hematoxilin-eosin)

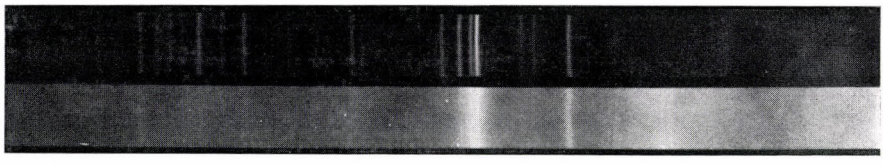

Fig. 2. X-ray diffraction picture of the mineral substance of an epiphyseal disc (above), and that of apatite (below)

Table 1

X-ray diffraction lines of the mineral substance of the epiphyseal disc as compared with the X-ray diffraction lines of apatite and brushite in the corresponding range

In the X-ray diffraction pattern of the sample the lines besides the lines of apatite corresponded to the lines of brushite, but the strongest line of brushite has not appeared

Epiphysis metaphysis d_{hkl} Å	Apatite int. d_{hkl} Å	Brushite int. d_{hkl} Å
4.64	—	4.64 s
3.88	3.88 w	—
3.42	3.44 m	3.38 w
2.814	2.814 vs	—
2.778	2.778 s	—
2.720	2.720 s	—
2.625	2.631 m	2.621 s
2.262	2.262 w	—
1.943	1.943 m	—
1.832	1.841 s	1.828 m
1.722	1.722 s	1.723 w

Symbols: Int. = intensity, vs = very strong, s = strong, m = medium, w = weak

Reference

Lénárt, G., Bidló, G., Pintér, J. (1968) Acta Biochim. Biophys. Acad. Sci. Hung. *3* 305

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An e.p.r. Study on the Nerves of the Frog

(Preliminary Communication)

A. NIEDETZKY, J. BELÁGYI

Biophysical Institute, Medical University, Pécs

(Received May 18, 1971)

The basic conditions to apply the tracing-method described by Hevesy and Paneth (1938) are as follows: 1) the system investigated be not influenced by the presence of the tracer elements, 2) the different isotopes of a given element behave in the same manner, 3) the compounds containing the isotopes suffer no change (Whitehouse, Putman, 1953). Ernst drew attention to the fact that these three conditions should not be neglected from point of view of the biological effects (Ernst, 1963, 1967). The phenomena of radioautolysis described by Hopwood and Phillips (1938), and of radio-synthesis published by Loiseleur (1955) corroborate this conception. The results of the present paper observed in surviving biological system and recorded by e.p.r. method seem to justify the effects of radioactivity. The e.p.r. investigation was performed on nerves of the frog (Rana esculenta) in order to obtain information about the yield of free radicals and to study the free radical production induced by low dose-intensity radioactive radiation.

Freshly prepared N. ischiadicus of R. esculenta were immersed in Ringer solution containing radioactive isotopes such as ^{24}Na, ^{42}K or ^{32}P; the specific activity of the Ringer solution varied between 0.26 and 3.76 mCi/ml; the absorbed dosis was chosen to be in the range of 1000 rads. The nerves kept in Ringer solution containing the radioactive isotopes and the control ones were stored in refrigerator at 4 °C for 24 hours before measurements. The e.p.r. spectra were taken up at 9370 Mc/s using a Zeiss ER 9 spectrometer. The measurements were performed at room temperature putting the nerves (2 or 3 of them; 50–150 mg wet weight) in the aqueous sample cell supplied by Zeiss or in small capillary tubes. The first derivative curve of the microwave absorption was recorded.

The preparations investigated in the above manner yielded a single absorption line. An example of our typical spectra recorded is shown by Fig. 1. On the left side of the figure the e.p.r. spectrum of the control, on the right that of a sample kept in ^{24}Na–Ringer solution can be seen. Both measurements were performed under the same experimental conditions. The g-values of the spectra are in accordance with the g-value of the organic free radicals. The line width of the spectra is in both cases about 7 G (from peak to peak). The average unpaired spin concentration was estimated to be $3 \cdot 10^{14}$ spins/g wet weight in normal nerves and $7 \cdot 10^{14}$ spins/g wet weight in nerves kept in Ringer solution contain-

ing radioactive isotopes. The use of different isotopes (sodium, potassium or phosphorus) when perfusing the nerves with radioactive isotopes revealed no change in either the e.p.r. spectra or in the unpaired spin concentrations.

The e.p.r. spectra obtained in nerves of the frog in our experiments exhibit an absorption line without any hyperfine structure. The presence of free radicals

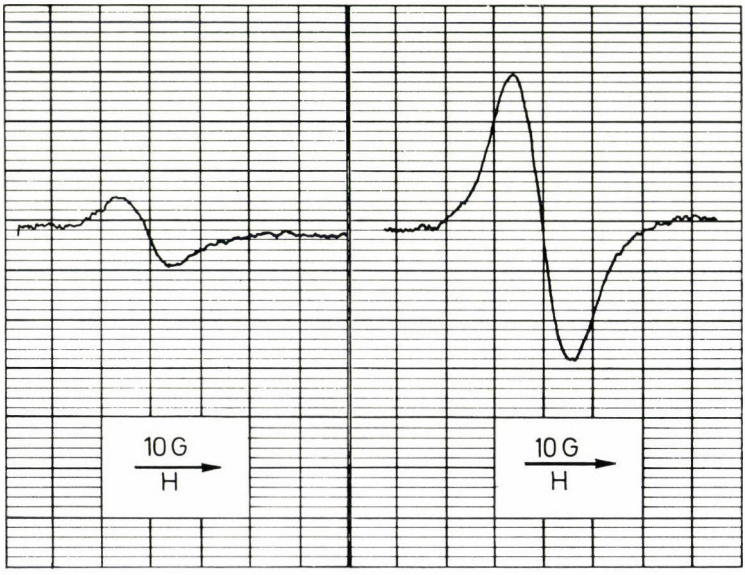

Fig. 1. The e.p.r. spectra (the derivation of energy absorption) of nerves under the same experimental conditions: a) control nerve; b) ^{24}Na incubated nerve

in animal tissues is due to the metabolic activity, especially to the reaction intermediates (Commoner et al., 1954).

Also the measurements on nerves kept in Ringer solution containing a radioactive isotope showed a single absorption line. This result differs from the experiments carried out on the nerves of rabbit by Pulatova (1962), who obtained a badly resolved dublett at room temperature. It can be imagined that the quasi-solid matrix of nerves broadens the line so that a useful resolution cannot be measured. The absence of hyperfine structure can be explained otherwise as well (Shen Pei-gen et al., 1961; Drew, Gordy, 1963).

These experimental data seem to support the view (Ernst, 1970; Niedetzky, Dalnoki, 1970) that the supposition of Hevesy and Paneth mentioned above has to be reinvestigated.

Thanks are due to Prof. Ernst for advising this work.

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Book Review

Fortschritte der experimentellen und theoretischen Biophysik. Heft 13. Hrsg. Prof. Dr. W. Beier. Leipzig. *Prinzipien der automatischen Diagnosestellung*. Dr. Dieter Barthel. VEB Georg Thieme, Leipzig, 1970, 90 pages.

In recent years it has become apparent that electronic computers may be useful devices in certain aspects of medicine, especially at diagnostic decisions. The evolving role of laboratory test results, the necessity of urgent and important decisions, the increasing number of the known symptoms require the aid of computers in the near future at the alternative diagnostic possibilities, treatment planning, statistical analysis and at the storage of patient's data. It seems the computer will be a basic tool in medicine as well.

The present number of Fortschritte der experimentellen und theoretischen Biophysik written by Dieter Barthel is a useful tool for those who are interested in the theoretical basis of automatic diagnosis production. It consists of ten chapters with the introduction and appendix together.

After a short introduction the author gives an outline about the different principles of medical diagnosis production. In Chapter 3 it is followed by the formation of the problem in the language of mathematics. In Chapter 4 and Chapter 5 the principles of exclusion diagnostics and evaluation diagnostics are discussed in detail.

Separate chapters are given to automata as learning systems for diagnosis, to exclusion diagnosis and evaluation diagnosis by automata. The last part of the periodical deals with the different strategies of diagnosis mentioned above.

The paper written by Barthel is of basically theoretical nature. Therefore the readers, for example the physicians who have not any profound mathematical qualification, cannot bridge the gap between the theory of computer science and medicine. So before applying computer technology to any operation in medical setting, a well coordinated activity is needed between physicians and mathematicians.

Neglecting this problem in relation with Barthels paper, it gives a very good survey of the principles of automatic diagnosis production.

J. BELÁGYI

The Fundación Viviana Luckhaus has instituted the "International Prize Fundación Viviana Luckhaus, 1972".

The Prize is intended to honour a report of original research related to blood platelets (morphology, physiology, biochemistry, pathology, etc.) and or their relationship to thrombosis and blood vessels, and to promote communication and interchange between research workers in different parts of the world.

For further information and rules for the 1972 contest, apply to Dr. Edgardo S. Sack, Fundación Viviana Luckhaus, Hospital Juan A. Fernández, Cerviño 3356, Buenos Aires, Argentina.

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Dissociation of Rat Liver Polyribosomes upon Treatment with Diethyl Pyrocarbonate

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Treatment of polyribosomal preparations with diethyl pyrocarbonate under certain conditions resulted in partial breakdown of polyribosomes and in the formation of particles sedimenting at a slightly lower rate than monoribosomes and ribosomal subunits. The RNA to protein ratio of the particles did not change after the above treatment.

Introduction

A new method, based on the use of diethyl pyrocarbonate (DEP) as a nuclease inhibitor for the extraction of nucleic acids from higher plant tissues has been developed by Solymosy et al. (1968). Since then DEP has been widely used for the preparation of nucleic acids from various sources (Lázár et al., 1969; Summers, 1970; Abadom, Elson, 1970; Chet, Rusch, 1970; Melera et al., 1970; Solymosy et al., 1970).

DEP has also been shown to have a protective effect on plant polyribosomes (Weeks, Marcus, 1969) during the isolation procedure. These preparations, however, were found to contain increased amounts of ribosomal subparticles as compared to the control preparations. Therefore, a detailed analysis of the effect of DEP on the sedimentation properties of polyribosomes seemed to be warranted.

In the present paper it will be shown that under certain conditions treatment of polyribosomal preparations with DEP results in the breakdown of polyribosomes and in the formation of particles sedimenting similarly to monoribosomes and ribosomal subunits. These alterations in the sedimentation properties of polyribosomes are most probably due to the reaction of DEP with ribosomal proteins.

Materials and methods

DEP ("Baycovin") was a product of Bayer Werke, Leverkusen. All other chemicals used were reagent grade and were purchased from Reanal, Budapest.

Preparation of rat liver polyribosomes. Animals of both sexes weighing 150–250 g were killed by decapitation and their livers were removed and

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Abbreviations used: DEP, diethyl pyrocarbonate; ϱ, density (g/cm^3).

immediately immersed into ice-cold physiological saline solution. Fat and connective tissue were removed and the livers were homogenized in a teflon-glass Potter-Elvehjem type tissue homogenizer with two volumes of TKM solution (0.05 M Tris-HCl, pH 7.6; 0.025 M KCl; 0.05 M magnesium acetate) containing 8.5 per cent sucrose. Nuclei, mitochondria and cell debris were removed by centrifugation at 10 000 *g* for 15 min. and 4.5 per cent sodium deoxycholate was added to the supernatant solution in a final concentration of 0.5 per cent. Ribosomes were sedimented either in the rotor No. 50.1 or No. 40 at maximum speed for 90 min. using a Spinco ultracentrifuge L2 65B or L2 65K. The pellets were suspended in TKM, clarified by centrifugation at 10 000 *g* for 10 min. and sedimented again. The washed pellets were suspended in TKM or PKM (0.025 M K-Na phosphate pH 7.6 or 6.0; 0.025 M KCl; 0.005 M magnesium acetate) buffer and clarified as described above. In the latter case in order to remove traces of Tris buffer, pellets sedimented from TKM were suspended in PKM and centrifuged at 50 000 rpm for 1 hour in the Spinco rotor No. 50, and resuspended in PKM. 60–80 per cent of the particles in these preparations sedimented more rapidly then monomers did. These preparations will be referred to as polyribosomal preparations.

Treatment of polyribosomal preparations with DEP. The preparations were treated with DEP either in TKM or PKM buffer. The amounts of DEP added per 1 ml of polyribosomal preparation are indicated in the legends to the Figures. Dilution of DEP was performed with 96 per cent ethanol. The mixtures were shaken by hand for 20 minutes in ice-cold water. (It should be noted here that when polyribosomal suspensions were kept in the presence of 50 μl/ml DEP for 24 hours at 4°C, heavier aggregates were formed.) The undissolved DEP was removed in the following way. The reaction mixtures were layered on 10 per cent sucrose solution. The undissolved DEP (spec. gravity 1.12) sedimented rapidly through the sucrose solution. The layers containing the ribosomal particles were removed and centrifuged in the Spinco rotor No. 40 at 40 000 rpm for 150 minutes at 0°C, to separate them from the dissolved DEP. The pellets were suspended in TKM or PKM buffer and clarified by centrifugation at 10 000 *g* for 15 minutes. In some experiments sedimentation analyses were performed without removing DEP.

Sucrose density gradient analysis was performed in the Spinco model L2 65K or L2 65B ultracentrifuge at 3°C as described in the legends to the Figures. The gradients were evaluated by an ISCO UA-2 type gradient analyzer.

The buoyant density of the particle was determined in the following way: Particles to be analyzed were suspended in 0.2 M phosphate buffer, pH 7.6 and fixed with 4 per cent neutralized formaldehyde for about 14 hours (Spirin et al., 1965) and then dialyzed against 0.025 M phosphate buffer, pH 7.6 containing 0.025 M KCl. The suspension was centrifuged at 10 000 *g* for 10 minutes to remove aggregates. The supernatant was mixed with CsCl in the same buffer to a concentration of 34.5 per cent w/w of CsCl and linear CsCl gradients were formed by mixing this solution with 57.5 per cent w/w CsCl solution in the same buffer. The samples were centrifuged in the rotor No. SW 41 of the Spinco ultracentri-

fuge Model L2 65B or L2 65K at 4°C for 17 hours. After centrifugation the tubes were punctured, 0.16 ml fractions were collected, diluted with water and the OD_{260} of the fractions was determined in a Beckman DU spectrophotometer. Every fifth fraction was measured refractometrically, to determine the concentration of CsCl. The density of CsCl solution was calculated as described by Meselson et al. (1964).

Results

Sedimentation properties of the reaction products formed upon treatment of polyribosomal preparations with different amounts of DEP. Polyribosomal suspensions in PKM buffer pH 7.6 were treated with varying amounts of DEP as

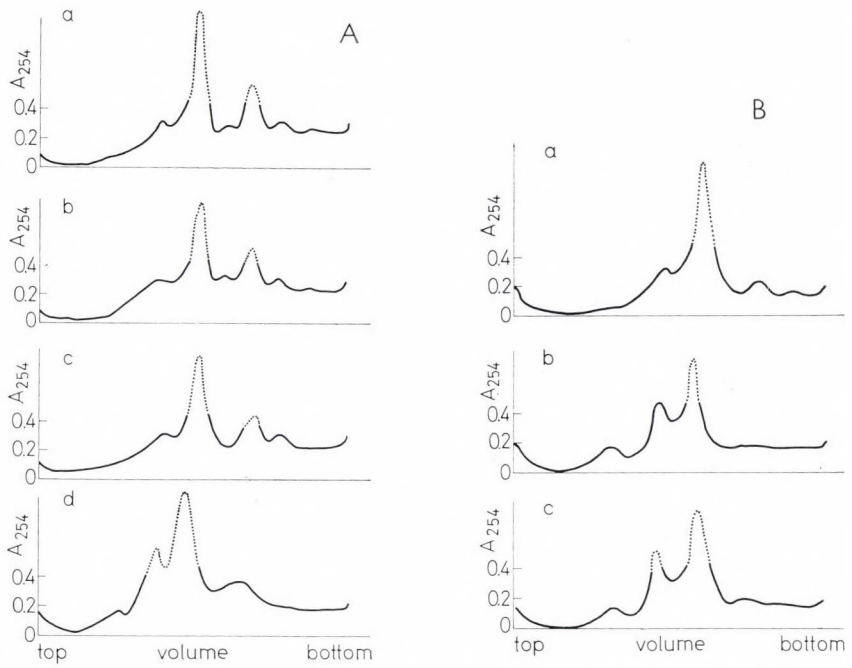

Fig. 1. Sucrose density gradient sedimentation profile of polyribosomal preparations treated with different amounts of DEP in PKM buffer, pH 7.6. *A)* DEP was added to polyribosomal suspensions (20 A_{260} units/ml) in PKM buffer pH 7.6 in a final concentration of *a)* 0, *b)* 0.05 μl/ml, *c)* 0.5 μl/ml, *d)* 5 μl/ml, and the samples were shaken for 20 minutes at 0°C. Aliquots of the samples containing 1 A_{260} unit were layered on 10 to 30 per cent w/w linear sucrose density gradients containing PKM pH 7.6 and centrifuged for 120 minutes at 38 000 rpm in the Spinco rotor No. SW 41 at 3°C. *B)* DEP was added to polyribosomal suspensions (20 A_{260} units/ml) in PKM buffer pH 7.6 in a final concentration of *a)* 0, *b)* 10 μl/ml, *c)* 100 μl/ml. The samples were shaken and analyzed as described in Legends to Figure *1A*, but centrifugation was performed for 150 minutes. The gradients were evaluated by an ISCO model UA-2 gradient analyzer using the range 0.5 (———) and the range 2.5 (·····) The results obtained by the latter range were enlarged five times

indicated in Fig. 1A. No significant changes occurred after treatment with 0.05 μl and 0.5 μl DEP per ml of reaction mixture, while 5 μl DEP per ml caused considerable changes in the polyribosome pattern: The quantity of polyribosomes decreased and that of monoribosomes and of ribosomal subunits increased. Treatment of polyribosomal preparations with 10 μl/ml and 100 μl/ml of DEP (Fig. 1B) resulted in similar changes in the polyribosome profile as treatment with 5 μl/ml DEP. It should be noted that after treating polyribosomal suspensions with 50 to 100 μl DEP per ml reaction mixture for 20 minutes at 0°C, the larger portion of DEP remained undissolved, i.e. the aqueous phase was saturated with the chemical during the reaction.

Thus treatment of polyribosomal preparations with high amounts of DEP (5 to 100 μl/ml) at 0°C for 20 minutes caused partial breakdown of polyribosomes and an increase in the quantity of monoribosomes and ribosomal subunits. In most cases treatment of polyribosomal suspensions with DEP resulted also in a shift of the peaks of both monomer and polymer fractions to zones with lower sedimentation constants (Figs 1A and B). Complete dissociation of ribosomes into subunits has never been observed under the above conditions.

The extent of decrease of the amount of polyribosomes and the increase in the amount of monomer and subunit fractions varied in different experiments. The most probable explanation of these observed variations is that the treatment of ribosomes with DEP for 20 minutes at 0°C may not be sufficient to reach the end point of the reaction, the reaction of DEP with nucleic acids in the presence of excess DEP at room temperature being completed only in several hours (Solymosy et al., 1971). Consequently, the reaction products of a 20 minute treatment are not homogeneous and their relative amount may vary in different experiments. The variations in the amount of the subunits formed may also be explained by the fact that there were some differences in the polyribosome content of the polyribosomal preparations in different experiments.

The concentration of ribosomes in the reaction mixture did not influence the changes in the sedimentation properties of ribosomes upon treatment with DEP. Similarly, the effect of DEP on the sedimentation properties of ribosomes was found to be independent whether phosphate or Tris was used as buffer, indicating that possible reactions of DEP with Tris did not influence to any appreciable extent the effect of DEP on ribosomes.

Effect of DEP on polyribosomes at different concentration of magnesium ions. As shown in Fig. 1 treatment of polyribosomal suspensions with DEP in the presence of 5×10^{-3} M magnesium acetate brought about only partial degradation of polysomes with a concomitant increase in the amount of ribosomal monomers and subunits. Lowering the concentration of magnesium ions caused in itself partial breakdown of polyribosomes. DEP treatment enhanced this effect, i.e. it caused further degradation of polyribosomes and monoribosomes, and converted them to monoribosomes and subunits, respectively (Figs 2A and B).

It can be seen in Figs 1 and 2 that the influence of DEP on the sedimentation properties of polyribosomes was similar to that of lowering magnesium ion concentration. One possible explanation of this phenomenon is that magnesium

concentration actually decreases in a reaction mixture containing DEP owing to the formation of magnesium carbonate from magnesium and CO_2 generated during the decomposition of DEP.

To test this possibility, the respective buffers were treated with DEP under the same conditions as polyribosomal preparations. These buffers were added to polyribosomal suspensions and the ribosomes were analyzed. No significant changes were found in the sedimentation properties of the particles after this treatment. Thus the decomposition products of DEP did not influence the sedi-

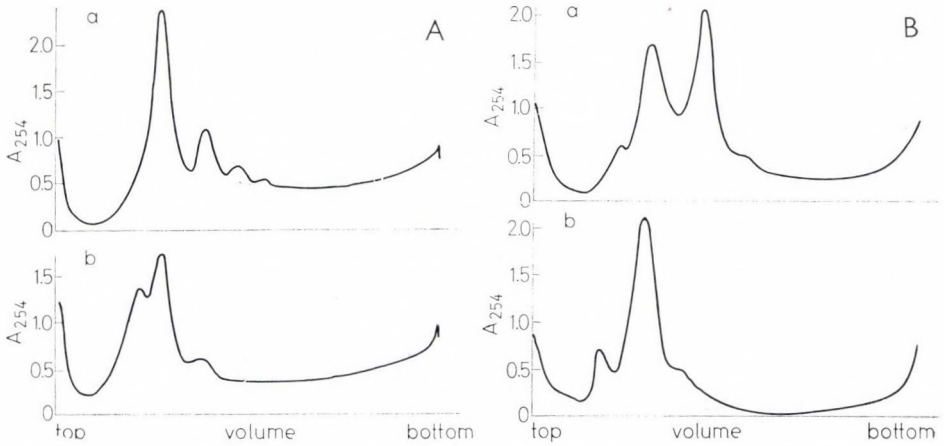

Fig. 2. Sucrose density gradient sedimentation profile of polyribosomal preparations treated with DEP at different magnesium ion concentrations. *A)* DEP was added to polyribosomal suspensions (2 A_{260} units/ml) in 0.05 M Tris-HCl, pH 7.6, 0.025 M KCl, 5×10^{-4} M magnesium acetate in a final concentration of *a)* 0, *b)* 50 μl/ml, and the samples were shaken for 20 minutes at 0°C. Aliquots containing 1 A_{260} unit were layered on 10 to 30 per cent (w/w) linear sucrose density gradients containing the same buffer as the samples and were centrifuged for 60 minutes at 41 000 rpm in the Spinco rotor No. SW 41 at 3°C. *B)* DEP was added to polyribosomal suspensions (400 A_{260} units/ml) in 0.05 M Tris-HCl, pH 7.6, 0.025 M KCl in a final concentration of *a)* 0, *b)* 50 μl/ml, and the samples were shaken for 20 minutes at 0°C, then aliquots containing 5 A_{260} units were layered on 10 to 30 per cent w/w linear sucrose density gradients containing the same buffer as the samples and were centrifuged for 90 minutes at 41 000 rpm in the Spinco rotor No. SW 41 at 3°C

mentation properties of polyribosomes. Thus a chemical reaction between DEP and ribosomes seems to be responsible for the observed breakdown of polyribosomes.

pH dependence of the reaction of DEP with polyribosomes. It has been shown earlier (Solymosy et al., 1971) that after the reaction of ^{14}C-DEP with polyribosomal preparations both ribosomal nucleic acids and proteins contained ^{14}C carbethoxy groups. The labeling of ribosomal proteins, however, was about 100 times higher than that of ribosomal nucleic acids, i.e. ribosomal proteins are responsible for the major part of the reaction between DEP and ribosomes. Since it has been shown earlier (Ovádi et al., 1967) that the reaction with DEP – of

several amino acid residues except histidine – decreased at pH 6.0, it was of interest to compare the effect of DEP on polyribosomes at pH 6.0 with that at pH 7.6.

As can be seen in Fig. 3 the changes in the sedimentation properties of polyribosomal suspensions following treatment with DEP were much less pronounced at pH 6.0 than at pH 7.6. This phenomenon most probably reflects the above-

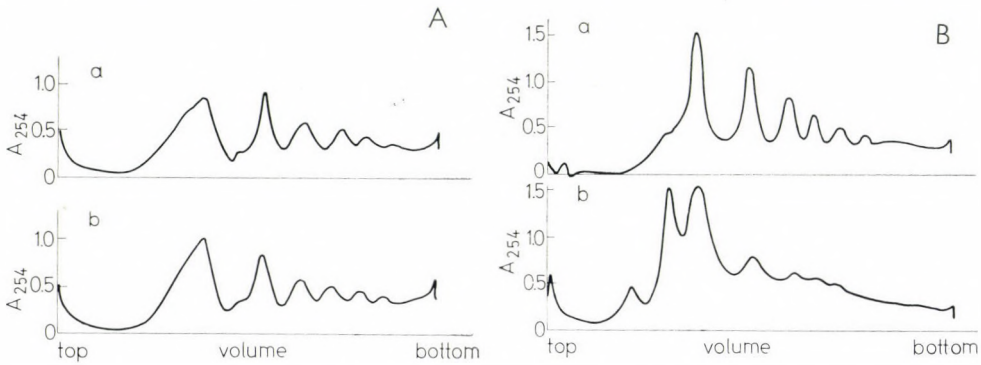

Fig. 3. Sucrose density gradient sedimentation pattern of polyribosomal preparations after treatment with DEP at pH 7.6 or 6.0. *A)* DEP was added to polyribosomal suspensions (160 A_{260} units/ml) in PKM buffer pH 6.0 in a final concentration of *a)* 0, *b)* 50 μl/ml. After shaking for 20 minutes at 0°C aliquots containing 5.6 A_{260} units were layered on 10 to 30 per cent w/w linear sucrose density gradients containing PKM buffer, pH 6.0 and centrifuged for 90 minutes at 41 000 rpm in the Spinco rotor No. SW 41 at 3°C. *B)* DEP was added to polyribosomal suspensions (260 A_{260} units/ml) in PKM buffer pH 7.6 in a final concentration of *a)* 0, *b)* 50 μl/ml. Preparation and centrifugation of the samples were performed as described in Fig. *3A*

mentioned decrease in the reactivity of several amino acid residues towards DEP at pH 6.0, since the reaction of DEP with nucleic acids was not shown to depend on changes in the pH in the above range (Kapovits, 1970).

Buoyant density of particles formed by treating polyribosomal preparations with DEP. It can be seen in Fig. 1 that the peaks of both the monomer and polymer fractions slightly shifted to zones with lower sedimentation constants upon treatment with DEP.

A decrease in the sedimentation constants may be the consequence of a decrease of molecular weight and/or changes in conformation. To test these possibilities the buoyant density of polyribosomes was determined before and after treatment with DEP. As shown in Fig. 4 the buoyant density of particles did not change upon treatment with DEP.

Discussion

It has been shown that treatment of polyribosomal preparations with DEP caused partial dissociation of polyribosomes at 5×10^{-3} M magnesium ion concentration and this dissociation could only be brought to completion by lowering

the reaction mixture to about zero. It has also been shown that the effect of DEP on polyribosomes was less pronounced at pH 6.0 than at pH 7.6.

The dissociating effect of DEP on polyribosomes is due to the reaction of DEP with ribosomal components, since (1) DEP is known to react with both ribosomal RNA and proteins (Solymosy et al., 1971), (2) the degradation products of the chemical had no effect on the sedimentation properties of ribosomes.

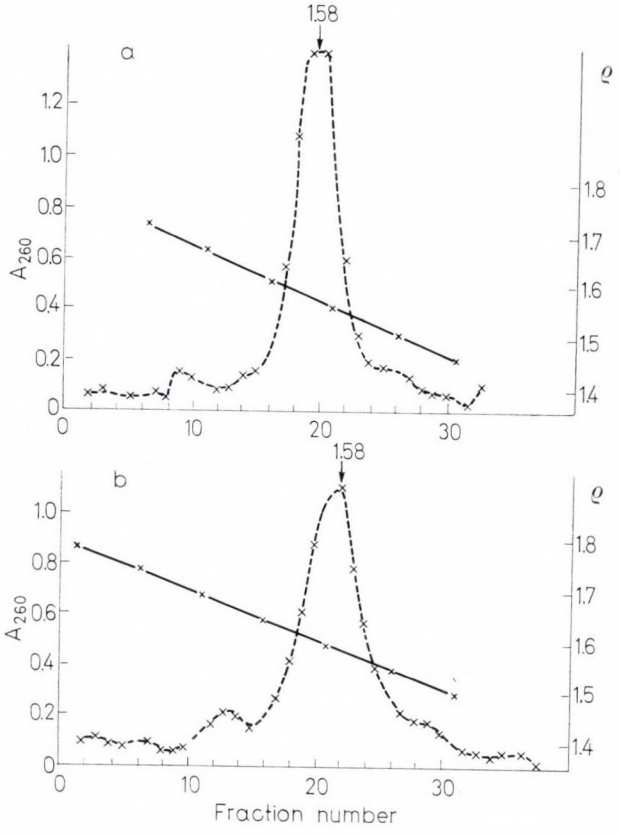

Fig. 4. Buoyant density of particles before and after treatment with DEP. DEP was added to polyribosomal suspensions (390 A_{260} units/ml) in 0.05 M Tris-HCl, pH 7.6, 0.025 M KCl in a final concentration of *a)* 0, *b)* 50 μl/ml. The samples were shaken at 0°C for 20 minutes, diluted with 0.025 M Tris-HCl pH 7.6, 0.025 M KCl and layered on 10 per cent sucrose solution to remove undissolved DEP, and ribosomes were sedimented at 60 000 rpm in the Spinco rotor No. 65 for 120 minutes at 0°C. Further treatment, preparation of the samples for the determination of buoyant density, and centrifugation were performed as described in Materials and Methods. × - - - - × A_{260}, × ——— × density of CsCl solution

It is uncertain whether the dissociating effect of DEP on polyribosomes may be attributed to a reaction with nucleotide or with amino acid residues. The latter possibility, however, seems to be more probable since the reaction of DEP with

nucleic acids was not found to be dependent on changes in pH (Kapovits, 1970) and the dissociation process was found to be dependent on it. As most of the amino acid residues react with DEP in unprotonated form (Larrouquere, 1964; Mühlrad et al., 1967) and the degradation of polyribosomes was more pronounced when treating them with DEP at pH 7.6 than at pH 6.0, it is very probable that some amino acid residues the pK values of which are about pH 7.6 play an essential role in the degradation of polyribosomes.

It should be noted that during treatment of polyribosomal preparations with 50 μl/ml DEP in PKM buffer pH 7.6 for 20 minutes the actual pH of the reaction mixture decreases by 1 pH unit, owing to the formation of CO_2. Thus, as some amino acid residues may become protonated in the course of the reaction, the reaction between DEP and amino acids proceeds at a decreasing rate.

When comparing our results to those obtained by Weeks and Marcus (1969) it must be kept in mind that during the isolation of polyribosomes cellular proteins and other cellular constituents may also react with DEP, diminishing thereby the reaction between DEP and polyribosomes. In spite of this fact, we think that 10 μl/ml DEP used in the experiments of Weeks and Marcus to isolate wheat ribosomes may have caused breakdown of polyribosomes, in addition to the prevention of degradation of polyribosome structure which is due to the well-known inhibitory effect of DEP on nucleases (Fedorcsák, Ehrenberg, 1966). Indeed, these preparations contained increased amounts of ribosomal subparticles.

Recently it has also been reported (Anderson, Key, 1970) that high concentrations of DEP resulted in the dissociation of mungbean polyribosomes into subunits. Since neither the conditions nor the concentrations of DEP were indicated, we cannot compare the results obtained by Anderson and Key (1970) with ours.

In addition to the dissociating effect of DEP on polyribosomes, we observed a shift in the peaks of ribosomal monomers and polymers to zones with lower sedimentation constants. On the other hand, the buoyant density of the particles did not change upon treatment with DEP. This means that no proteins were split off upon treatment with DEP since it was shown earlier (Hüvös et al., 1970) that the RNA profile of ribosomes treated with DEP was the same as that of control ones. Thus the decrease in the sedimentation constants may be due to conformational changes following DEP treatment.

The interest in this work, the stimulating and valuable advices of Prof. F. B. Straub, as well as the helpful discussions with Prof. F. Antoni, Dr G. L. Farkas and Dr A. Gulyás are gratefully acknowledged. The authors are also indebted to Mr K. Kovács for his skilful technical assistance.

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On the "Glucocorticoid-Like" Effects of Solanum Alkaloids

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The effect of a few Solanum alkaloids (solasodine, solasonine, α- and β-solamarine) was investigated on the inducible tryptophan pyrrolase synthesis in the liver of adrenalectomized and normal rats. The alkaloids tested failed to display any direct glucocorticoid effect on the system applied. The glucocorticoid-like action of Solanum alkaloids was found to be mainly secondary, mediated through corticoids produced in the adrenals.

Introduction

The Solanum alkaloids belong to the group of steroid alkaloids. They are endowed with multiple pharmacological activities. A number of these alkaloids exhibit antibacterial and antifungal effects (Boll et al., 1955/1956; Kuhn et al., 1950; Greathouse, Riegel, 1940; McKee, 1959; Wolters, 1966). Tomatine, α-solanine and β-solamarine exert a cytotoxic action (Tschesche, Wulff, 1965; Kupchan et al., 1965). Beside its cardiotonic, cardiac antiaccelerator and central nervous system-influencing effects solasodine also possesses antiphlogistic properties (Turova et al., 1961; Martin-Smith, Sugrue, 1940; Krayer, Briggs, 1950a, b). Owing to this latter glucocorticoid-like effect, solasodine has been tried out in the clinical practice but only small doses were used (Stopp, 1961).

The question arose whether the glucocorticoid-like effect observed was due to a direct action of steroid alkaloids or to the stimulated secretion of corticoids as in the case of many other substances. In our experiments we sought to answer this question.

Materials and methods

The alkaloids studied were the following: 1) solasodine (solanum aglucon); 2) solasonine (solanum glucoalkaloid: solasodine + L-rhamnose, D-glucose, D-galactose; $C_{45}H_{73}NO_{16} \cdot 2H_2O$); 3) β-solamarine (tomatidenol glucoalkaloid: tomatidenol + L-rhamnose, D-galactose; $C_{45}H_{73}NO_{16} \cdot 3H_2O$); 4) α-solamarine (tomatidenol glucoalkaloid: tomatidenol + 2 L-rhamnose, D-glucose; $C_{45}H_{93}NO_{15} \cdot 3H_2O$).

The alkaloids were produced by the Alkaloid Pharmaceutical Factory (Hungary).

The tryptophan pyrrolase induction test was applied by us. As a result of the enhanced synthesis of the enzyme, tryptophan pyrrolase activity in the liver was increased.

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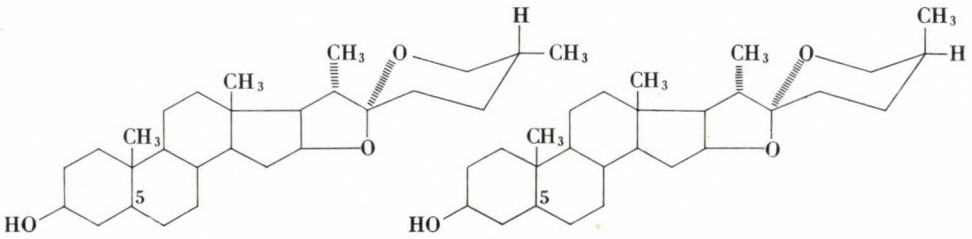

Solasodine Tomatidenol

Male Wistar rats weighing 110–140 g were used. The animals were fed standard pressed fodder containing 22% protein. In the course of a few experiments, to avoid the mobilization of corticoids from the adrenals, the animals wefd adrenalectomized under ether narcosis. The adrenalectomized rats were usere or experimental purposes either on the 4th or 5th day following adrenalectomy or immediately after the operation. The adrenalectomized rats were kept at 26–28°C, and they received 1% NaCl solution to drink.

To avoid the disturbing fluctuations during the day, the animals received the injections to induce tryptophan pyrrolase synthesis every morning between 8 and 9 a.m.

The alkaloids were dissolved in 0.5% carboxymethylcellulose suspension and then administered intraperitoneally in doses of 25 mg/kg.

The animals were decapitated, the liver was quickly removed and frozen immediately in liquid nitrogen. Liver tryptophan pyrrolase activity was determined according to the method of Knox and Mehler (1950) as modified by Feigelson and Greengard (1961) within 24 hours in a homogenate prepared with an MSE blade-homo-genizer. Tryptophan pyrrolase activity was calculated from the light absorption at 365 nm, i.e. kynurenine formed. The unit of activity is defined as 1 micromole of kynurenine formed in one hour under our experimental conditions.

Results and discussion

Fig. 1 shows that the alkaloids solasodine, solasonine, α- and β-solamarine elicit a 2.5 to 3.0-fold increase of the tryptophan pyrrolase activity in the liver as compared to the control. The animals were killed 6 hours after treatment.

To get a detailed information on the effect of the agents used, tryptophan pyrrolase activity was measured 2, 4, 8 and 24 hours after treatment.

Fig. 2 indicates the values obtained 2, 4 and 8 hours after administration. By comparing the values obtained at 4 and 8 hours, it is seen that with the two solamarines the maximal value and the decrease occurred earlier than in the case of solasodine and its glucoalkaloid. Values obtained after 24 hours are not indicated in the figure: in fact they fell back to the original level before 24 hours.

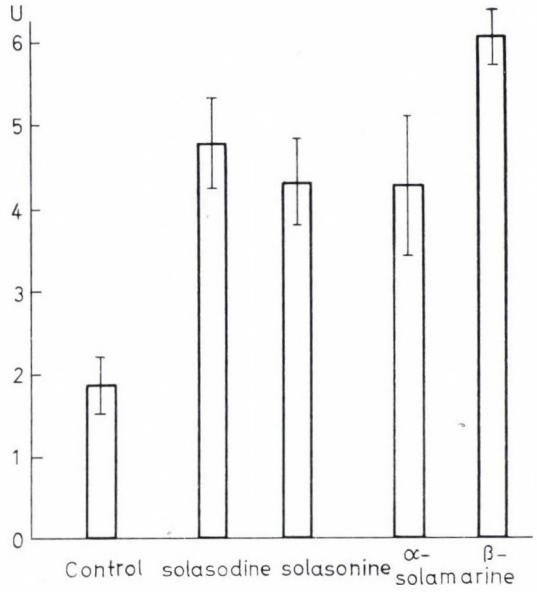

Fig. 1. Effect of solasodine, solasonine, α- and β-solamarine on tryptophan pyrrolase activity of rat liver six hours after treatment. The alkaloids were dissolved in 0.5% carboxymethylcellulose suspension and then administered in doses of 25 mg/kg intraperitoneally

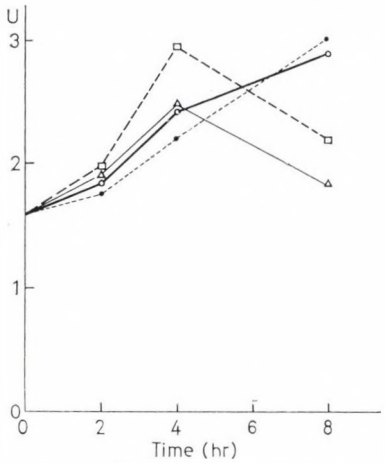

Fig. 2. Effect of solasodine, solasonine, α- and β-solamarine on tryptophan pyrrolase activity of rat liver 2, 4 and 8 hours after treatment. The alkaloids were dissolved in 0.5% carboxymethylcellulose suspension and administered in doses of 25 mg/kg intraperitoneally. ○—○, solasodine; △—△, α-solamarine; □— — —□, β-solamarine; ● · · · ●, solasonine

The glucocorticoids were found to induce tryptophan pyrrolase synthesis to an even greater extent after adrenalectomy. It is obvious that in this instance the substances which by themselves do not possess direct glucocorticoid effect but only mobilise glucocorticoids from the adrenals would prove ineffective.

Taking the foregoing into account, we have studied the changes in tryptophan pyrrolase activity induced by alkaloids in the adrenalectomized animals

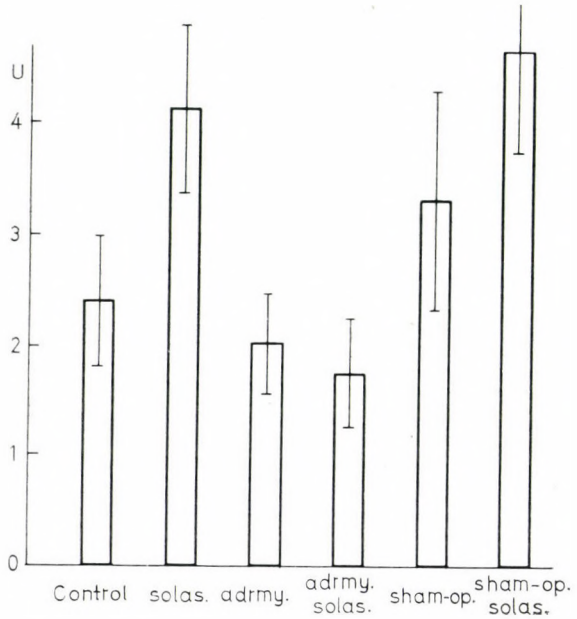

Fig. 3. Effect of solasodine in doses of 25 mg/kg on tryptophan pyrrolase activity of the liver of adrenalectomized rats, 4 hours after adrenalectomy and subsequent treatment. Solas.: solasodine; adrmy.: adrenalectomy; sham-op.: sham operation

4 to 5 days after the operation. None of these agents caused the increase of tryptophan pyrrolase activity.

In further experiments we examined the possible changes in tryptophan pyrrolase activity induced by solasodine and solamarine administered immediately after adrenalectomy. In this way we wanted to decide whether the lack of induction was due to the reduced metabolizing capacity of the liver. This would then mean that a converted form of the alkaloids was responsible for the effect in question.

Solasodine in doses of 25 mg/kg enhanced tryptophan pyrrolase activity by 75% within 4 hours in non-adrenalectomized animals. Adrenalectomy by itself induced a decrease of 15% within 4 hours. Solasodine in doses of 25 mg/kg failed to elicit an increase in adrenalectomized animals. In order to exclude the possibility that narcosis or any other effect connected with operation was the reason for the lack of induction, we performed a sham operation involving laparotomy

without removing the adrenals. The operation itself increased tryptophan pyrrolase activity, but failed to impede the effect of solasodine. It should therefore be assumed that intact adrenals are needed for the induction to occur. Solasodine and solamarine *per se* do not probably possess any glucocorticoid effect; they either mobilize glucocorticoids from the adrenals or have to be converted to active forms within the adrenals.

This hypothesis seems to be supported by our experiments on the working capacity of rat muscles. After the administration of hydrocortisone, the performance of adrenalectomized animals was enhanced, whereas it was markedly decreased by solasodine in doses as small as 1 mg/kg.

Solanum alkaloids failed to display any direct glucocorticoid effect in the tests applied. The glucocorticoid-like action of Solanum alkaloids was found to be mainly secondary, operating through the mobilization of corticoids from the adrenals.

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Effect of L-Asparaginase on the Glucocorticoid-Induced Synthesis of Tryptophan Pyrrolase in Rat Liver

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The effect of L-asparaginase on tryptophan pyrrolase synthesis induced by hydrocortisone acetate in the liver of adrenalectomized rats was investigated and compared with the effect of actinomycin D and puromycin. It was found that L-asparaginase failed to inhibit effectively the development of the induction of tryptophan pyrrolase synthesis in contrast to actinomycin D and puromycin. When, however, L-asparaginase was given 5 hours after the administration of hydrocortisone acetate, it was effective in inhibiting the enhanced tryptophan pyrrolase synthesis similarly to puromycin and in contrast to actinomycin D. It is suggested that L-asparaginase is only able to inhibit the highly increased protein synthesis of rat liver.

Introduction

In 1953 guinea-pig serum was shown to possess antitumor activity against murine lymphoma and the Murphy-Sturm lymphosarcoma in rats (Kidd, 1953). The antitumor factor was later shown to be the enzyme L-asparaginase (Broome, 1961), and an enzyme with antitumor properties was subsequently extracted from Escherichia coli (Mashburn, Wriston, 1964; Roberts et al., 1966). L-asparaginase inhibition of rodent tumors has been related to L-asparagine depletion of tumor cells that require an exogenous source of L-asparagine (Kidd, 1953; Boyse et al., 1967). It has since been demonstrated that a mechanism of L-asparaginase resistance in a nonsensitive murine tumor is the consequence of the presence of an L-asparagine synthetic pathway, mediated by the enzyme L-asparagine synthetase (Broome, Schwartz, 1967; Cooney, Handschumacher, 1968).

It has been established, however, that not only the tumor cells were liable to destruction by L-asparaginase, but other cells too, e.g. normal lymphocytes in vitro (Becker, Broome, 1967) and regenerating liver cells (Mashburn, Gordon, 1968), the latter being only temporarily affected. Hobik (1969), moreover, has observed the suppression of the graft-versus-host reaction, while Chakrabarty and Friedman (1970) have described the suppression of the antibody formation as a consequence of treatment with L-asparaginase.

In clinical practice E. coli L-asparaginase therapy was found to be associated with a decreased level of serum proteins, chiefly that of albumin, α_2- and β-globulins and a simultaneous increase of γ-globulin (Haskell et al., 1969). Oettgen et al. (1970) described increased level of serum alkaline phosphatase, serum glutamic oxaloacetic transaminase, serum bilirubin and 5′ nucleotidase. The enhanced

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bromsulphalein retention and a fatty metamorphosis of the liver pointed to an impaired function of the latter. All these changes showed up only after a prolonged administration of E. coli L-asparaginase.

The function of the liver selected by us for observation in our experiments was the one which reacted immediately and very sensitively to agents inhibiting protein synthesis. It is a well-known fact that, owing to the increased synthesis of liver tryptophan pyrrolase occurring 5–6 hours after administering glucocorticoids, a 5–10-fold enhancement of this enzyme activity is observable. According to our experiments the aforesaid increase in specific protein synthesis, i.e. the hormone induced liver tryptophan pyrrolase formation, proved to be responsive to treatment by L-asparaginase.

Materials and methods

Male Wistar rats weighing 110–140 g were used. The animals consumed standard pressed fodder containing 22% protein. To avoid mobilizing corticoids from the adrenals, the animals were adrenalectomized under ether narcosis. They were used for experimental purposes on the 4th or 5th day following adrenalectomy. The adrenalectomized rats were kept at 26–28°C, for drinking purposes they received 1% NaCl solution.

All the substances were injected intraperitoneally in 5 ml/kg water solution, or in 5 ml/kg 0.5% of carboxy-methyl-cellulose suspension. The animals were decapitated, the liver was quickly removed and frozen immediately in liquid nitrogen. Liver tryptophan pyrrolase activity was determined according to Knox and Mehler (1950) as modified by Feigelson and Greengard (1961) within 24 hours in a homogenate prepared with an MSE blade-homogenizer. The tryptophan pyrrolase activity was calculated on the basis of light absorption at 365 nm of the formed kynurenine. The activity of the enzyme was expressed in micromoles of kynurenine formed by 1 g of liver per hour.

The chemicals were purchased from the Reanal Chemical Works (Hungary). Puromycin and hemin were obtained from NBC (Cleveland, U.S.A.), while hydrocortisone acetate microcrystal suspension was prepared by the Kőbánya Pharmaceutical Factory (Hungary). L-asparaginase (Crasnitin) is a Bayer product received as a gift by Dr I. Tautz.

Results and discussion

In order to induce tryptophan pyrrolase synthesis, 15 mg/kg microcrystal suspension of hydrocortisone acetate was administered. A group of animals consisting of 5–10 rats was sacrificed at intervals of 1–2 hours for measuring liver tryptophan pyrrolase activity (Fig. 1).

The level of tryptophan pyrrolase activity was found to be 10 times that of the original 5 hours after the injection of corticoid and a further increase could be observed during the following 4 hours.

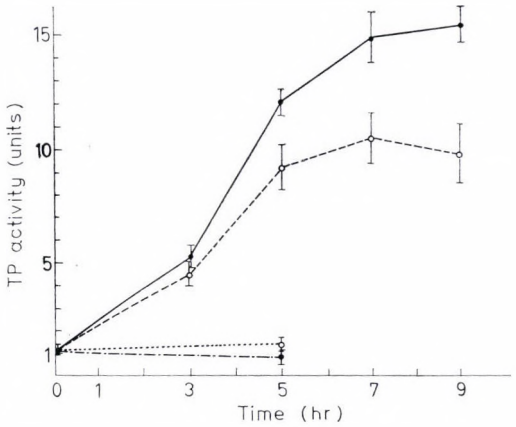

Fig. 1. The effect of L-asparaginase on tryptophan pyrrolase activity of rat liver induced by hydrocortisone acetate, administered in 1000 IU/kg simultaneously with 15 mg/kg hydrocortisone acetate. Each point represents the mean value obtained with 5—8 animals with standard deviation. 1 mg/kg of actinomycin D or 35 mg/kg of puromycin were also administered simultaneously with hydrocortisone acetate in certain groups of animals. ●——●, hydrocortisone acet. alone; ○— — —○, hydrocortisone acet. + L-asparaginase; ○ · · · ○, hydrocortisone acetate + actinomycin D; ●—·—·—●, hydrocortisone acetate + puromycin

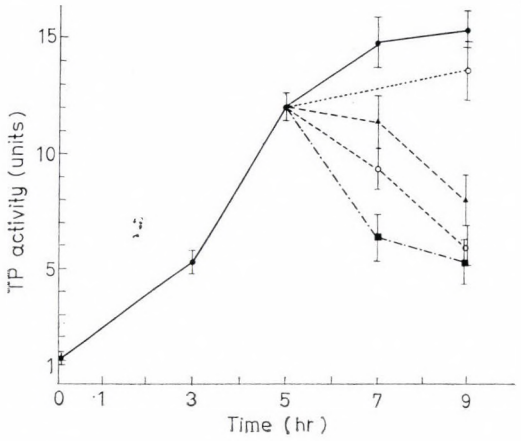

Fig. 2. The effect of L-asparaginase on the tryptophan pyrrolase activity of rat liver. L-asparaginase was given 5 hours after the administration of hydrocortisone acetate (15 mg/kg). 10 mg/kg actinomycin D or 35 mg/kg of puromycin were also given 5 hours after the administration of hydrocortisone acetate. ●——●, hydrocortisone acetate alone; ▲— — —▲, hydrocortisone acetate + 500 IU/kg L-asparaginase; ○— — —○, hydrocortisone acetate + 1000 IU/kg L-asparaginase; ○ · · · ○, hydrocortisone acetate + actinomycin D; ■—·—·■, hydrocortisone acetate + puromycin

When the administration of hydrocortisone was immediately followed by the injection of 1000 IU/kg of L-asparaginase, the activity values of liver tryptophan pyrrolase failed to reach the values obtained after giving hydrocortisone only. The difference between the two values became significant 5 hours after injecting inductor and L-asparaginase (23%, $p < 0.05$), while the greatest difference occurred 9 hours after administration (37%, $p < 0.01$).

Two further groups of animals were given 1 mg/kg of actinomycin D and 35 mg/kg puromycin, respectively, simultaneously with hydrocortisone. For measuring tryptophan pyrrolase activity in the liver, the animals of both groups were sacrificed 5 hours later. Unlike L-asparaginase, both actinomycin D and puromycin completely inhibited the inductive tryptophan pyrrolase enzyme synthesis (Fig. 1).

When actinomycin D was given only 5 hours after the injection of hydrocortisone, even a dose of 10 mg/kg failed to decrease or just barely diminished tryptophan pyrrolase activity in the course of the subsequent 4 hours. The difference was not significant. Conversely, when L-asparaginase was given 5 hours after the administration of hydrocortisone, a marked decrease of liver tryptophan pryrolase activity set in within 2 hours and failed to cease for a further 2 hours (Fig. 2). 500 IU/kg of L-asparaginase produced a 48 per cent reduction, while 1000 IU/kg resulted in a 61 per cent decrease of liver tryptophan pyrrolase activity (in both cases $p < 0.01$). 35 mg/kg puromycin given 5 hours after having administered hydrocortisone caused liver tryptophan pyrrolase activity to decrease by 65 per cent. This latter effect set in more rapidly than that of L-asparaginase. At the end of a further 4 hours, however, no marked discrepancy was noticeable between the inhibitory effect of the two agents in question.

An attempt was made to eliminate the inhibitory effect of L-asparaginase by giving 1 g/kg L-asparagine 20 minutes before the injection of the former as well as 4 hour 40 minutes after having administered the hormone. A 10 per cent protective effect can hardly be considered significant ($5\% < p < 10\%$). 1 g/kg glutamine administered as above, failed to produce any protective activity on the tryptophan pyrrolase synthesis inhibiting effect of L-asparaginase.

In our experiments L-asparaginase, unlike actinomycin D and puromycin failed to inhibit the development of the induction of tryptophan pyrrolase synthesis. When, however, L-asparaginase was given 5 hours after the administration of hydrocortisone acetate, it effectively inhibited the enhanced tryptophan pyrrolase synthesis similarly to puromycin and in contrast to actinomycin D (Goldberg et al., 1963; Smith et al., 1965).

On the basis of the data cited and the present experiments it seems probable that protein synthesis in normal liver cells can be inhibited by L-asparaginase only if protein synthesis is increased to a manifold level of its basic level. We consider it highly probable that the liver cells utilize exogenous L-asparagine for increased protein synthesis, or perhaps, having penetrated the liver cells, L-asparaginase would decompose the L-asparagine synthetized in the liver cell, reducing the L-asparagine level below that needed for increased protein synthesis.

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On the Existence of a Strongly Acid-Soluble Protein Fraction in the Eye Lens

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Experiments on the solubility of proteins of eye lenses from rats have shown that at concentrations higher than 0.5 N of hydrochloric or sulfuric acid, an appreciable part of lens proteins remains dissolved. The relative amount of acid-soluble proteins decreases with aging. The labelling of the acid-soluble protein fraction by ^{14}C-amino acids qualitatively differs from that of acid-insoluble proteins when lenses are incubated in vitro.

Introduction

The proteins of eye lens have recently been intensively investigated in many laboratories. The importance of these studies lies not only in the possibility of understanding pathological conditions and/or mechanisms but also in being an advantageous model for studies of terminal cell differentiation and concomitant changes in protein composition and synthesis.

The physiological transparency and refractional ability of the eye lens are due to the histological arrangement of lens fibres, and within them to the arrangement of macromolecules, mainly of proteins (Radnót, 1969). Mörner (1894) was the first to classify lens proteins, known as crystallines, according to their solubility. Since that time, however, mainly in the last decade, owing to the improvement of the various methods, the earlier described fractions, as the water-soluble α-, β- and γ-crystallines, as well as the water-insoluble "albuminoid" fraction themselves have proved to be heterogeneous. Thus the intrafibral molecular structure is built up not only by various protein fractions but these fractions are rather complex, i.e. they consist of different subunits. Also the grouping of proteins according to solubility furnishes a more and more complex picture in the molecular pattern of the lens.

The present paper reports on the existence of such protein fractions that remain dissolved even at high concentrations of strong acid. Some details have been reported earlier (Köteles, Antoni, 1968).

Materials and methods

Lenses from inbred white rats were used. Quantitative protein determination was done either according to Lowry et al. (1951) or to the modified biuret method. Bovine serum albumin was used as standard reference solution. To study protein synthesis in isolated lenses, these were incubated in Parker's TC 199

medium at 37°C, in the presence of various ^{14}C-labelled amino acids. ^{14}C-alga protein hydrolysate (UVVVR, Prague), ^{14}C-glutamic acid, valine or arginine (Radiochemical Centre, Amersham), in concentrations of 1 μCi/ml, were used as precursors. After appropriate incubation times, the lenses were rinsed in cold physiological saline, homogenized in a Potter-Elvehjem type teflon-coated homogenizer and the proteins from the homogenate extracted according to Siekevitz (1952). Protein content and radioactivity, using a Friesecke-Hoepfner gas glow counter, were measured in aliquots. The specific radioactivity of proteins was expressed as c.p.m. per mg protein.

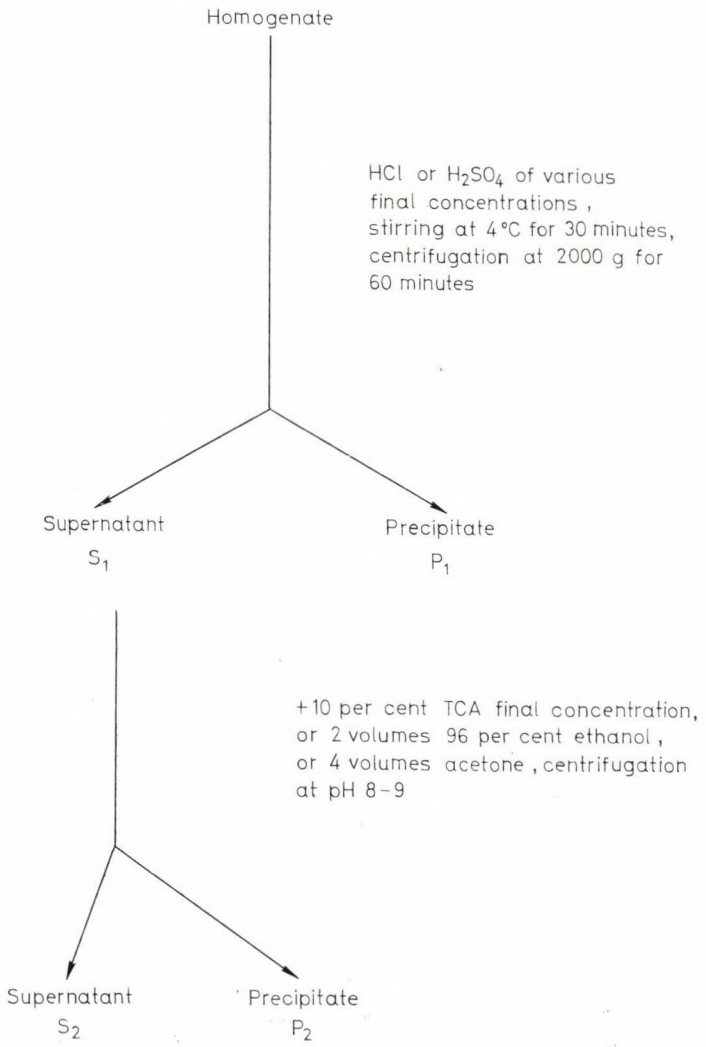

Fig. 1. Separation of various acid-soluble and acid-insoluble lens proteins

Results

The solubility of lens proteins as a function of various acid concentrations was studied according to the experimental layout shown in Fig. 1. All operations were carried out at 4°C. Both the complete and decapsulated lenses, deprived of their epithelium, were homogenized in physiological saline. Hydrochloric or sulfuric acid, in various final concentrations, was added to the homo-

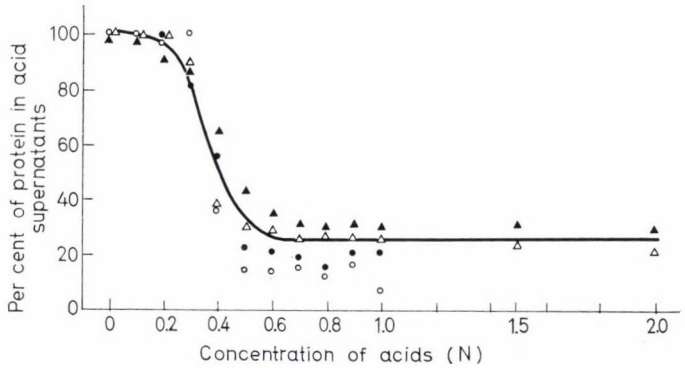

Fig. 2. Solubility of lens proteins from rats in HCl or H_2SO_4 of various concentrations: ●, complete homogenate in HCl; ○, complete homogenate in H_2SO_4; ▲, fibre proteins in HCl; △, fibre proteins in H_2SO_4

genate. The suspension was continuously stirred for 30 minutes. The supernatant (S-1) and the precipitate (P-1) were separated by centrifugation. Irrespective of the initial acid concentration, half volume of 30 per cent trichloroacetic acid (TCA) was added to supernatant S-1. This yielded a second precipitate (P-2). The protein contents in precipitates P-1 and P-2, resp., were determined after dissolution in 1 N NaOH. Fig. 2 presents, both for the complete lens and for the fibre stock, resp., the amount of protein remaining dissolved in 0.1 to 2 N acids – as a percentage of total protein content of the homogenate. In both cases the overwhelming majority of proteins precipitate at acid concentrations ranging from 0.3 to 0.5 N. The dissolved protein retained in more concentrated acids has an almost constant value.

A similar protein precipitate was obtained when supernatant S-1 was treated with 2 to 4 volumes of acetone or 2 to 4 volumes of 96 per cent ethanol, at pH 8–9. After this treatment the supernatant contained no more protein precipitable with highly concentrated acids, ethanol or acetone. The absolute deproteinization by TCA or acetone of fraction S-1, obtained by extracting lens proteins with 0.5 N HCl, is shown in Table 1.

For further studies the acid-soluble protein fraction was extracted by hydrochloric acid of 0.5 N final concentration.

The UV absorption spectrum of the acid-soluble protein fraction is shown in Fig. 3. For this measurement, the S-1 supernatant was dialyzed against acetate

Table 1

Precipitability with trichloroacetic acid or with acetone of lens proteins soluble in 0.5 N HCl

Experiment No.	Acid-soluble protein quantity mg	Protein precipitated with			
		10 per cent TCA		Acetone (4 vol., pH 9)	
		mg	%	mg	%
1	1.72	1.68	98	1.62	92
2	3.84	3.71	97	3.53	95
3	16.00	15.20	95	—	—

buffer pH 3.6 at 4°C overnight. Some characteristics of the UV spectrum of the fraction are the following: $\lambda_{max} = 278$ mμ; $\lambda_{min} = 250$ mμ; O.D. max/O.D. = 2.40; $O.D._{280}/O.D._{260} = 1.85$; $O.D._{280}/O.D._{300} = 5.55$; $O.D._{280}/O.D._{230} = 0.2$.

The described spectrum and the Lowry-positivity of the substance precipitable with TCA, ethanol or acetone are a proof for the protein content of the acidic extract.

In further experiments the respective acid-soluble protein contents in the lenses of young and adult animals were compared (Table 2). As it is evident from the data, the relative amount of acid-soluble proteins decreases with age, both in the region of the epithelial cells plus capsule and in that of the fibres. The average protein content of the lenses is also indicated in Table 2. A comparison of the average protein contents with the relative protein content clearly reveals that the absolute amount of acid-soluble proteins did not change in the course of aging.

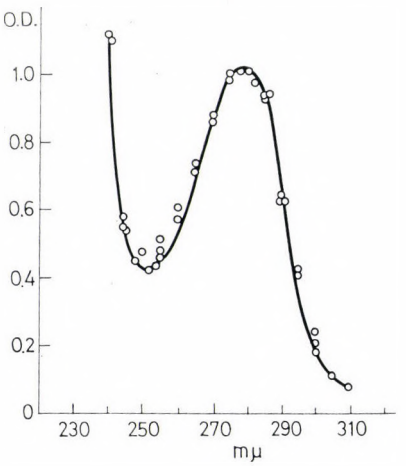

Fig. 3. UV absorption spectrum of the acid-soluble protein fraction. Solvent: acetate buffer, pH 3.6

Table 2

Acid-soluble protein content of rat lenses of different ages

Age of the animals	Protein content mg/lens	Full homogenate	Fibres	Capsule + epithelium
		in per cent of total protein content****		
Young (body weight 100 to 150 g)	15.8 13.0–18.6 (30)	20.2* 14.9–25.9** (4)***	20.8 14.9–29.0 (11)	63.8 32.0–75.2 (6)
Old (body weight 300 to 500 g)	27.1 22.8–31.5 (10)	12.1 9.16–16.5 (3)	11.2 8.0–16.5 (7)	41.9 27.1–62.0 (5)

* mean value;
** extreme values measured;
*** number of determinations, with three lenses for each determination;
**** the acid-soluble protein was extracted with 0.5 N HCl.

The next problem to be elucidated was whether the biosynthesis of this fraction resembles that of the other proteins. Lenses from young animals were incubated in the presence of ^{14}C-amino acids in vitro. After various incubation periods, the acid-soluble and acid-insoluble protein fractions were separated and the respective specific radioactivities determined. As shown in Fig. 4 the acid-soluble protein fraction could be labelled with ^{14}C-amino acid in the presence

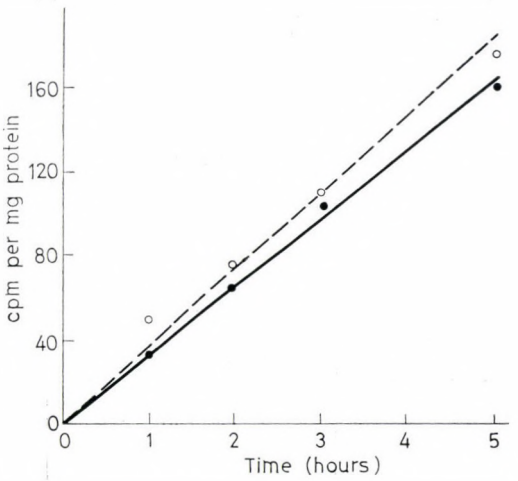

Fig. 4. Changes of specific radioactivity in acid-soluble (●———●) and acid-insoluble (○— — —○) proteins during incubation in vitro. Precursor: ^{14}C algae protein hydrolysate

Table 3

Per cent amounts and specific radioactivity values of lens proteins isolated according to their water- and acid-solubility following incubation with a mixture of ^{14}C-amino acids in vitro

Precursor: ^{14}C-algae protein hydrolysate, 1 μCi/ml. Incubation time: 3 hours. The values are the mean values of 3 experiments

Fraction	Per cent amount	Specific radioactivity cpm/mg
Complete protein mixture	100	277
Water-soluble proteins	72.9	312
acid-soluble fraction	19.2	202
acid-insoluble fraction	53.7	300
Water-insoluble proteins	27.1	109

of ^{14}C-algae protein hydrolysate, i.e. it is de novo synthesized and its metabolism can be studied in vitro. The specific radioactivity values for the acid-soluble and insoluble fractions did not differ appreciably. In the first few hours amino acid incorporation was linear in both fractions, as a function of time. However, when following in vitro labelling various protein fractions were separated as water-soluble and insoluble ones, and the water-soluble fraction was again separated into acid-soluble and insoluble fractions, different specific radioactivity values were obtained. These values are given in Table 3. As it is evident, the specific radioactivity of the acid-soluble fraction was lower than that of the acid-insoluble fraction. In this connection it should be pointed out that the acid-extractable proteins were detected in the water-soluble fraction, while the water-insoluble fraction did not contain such proteins.

Table 4

Specific radioactivities of protein fractions from rat lenses after incubation with various ^{14}C-amino acids in vitro

Precursors: ^{14}C-glutamic acid, ^{14}C-valine, ^{14}C-arginine, 1.0 μCi/ml each. Incubation time: 3 hours. A protein pool isolated from ten lenses was used for the determinations

Protein homogenate	Specific radioactivity: cpm/mg protein		
	Glutamic acid	Valine	Arginine
Complete homogenate	41	452	980
Water-soluble	56	615	1078
Water-insoluble	29	137	358
Water-soluble, acid soluble	26	267	1000
Water-soluble, acid-insoluble	55	620	1048

After these preliminary experiments with algae protein hydrolysates the incorporation of various ^{14}C-labelled amino acids into various protein fractions separated according to solubility was studied. The isolated lenses were incubated in vitro in the presence of ^{14}C-labelled glutamic acid, valine or arginine, i.e. in the presence of an acidic, neutral or basic amino acid. As shown in Table 4, the acid-soluble protein incorporated less glutamic acid or valine than the other water-soluble proteins. No difference in the incorporation level was found with the basic amino acid, arginine, i.e. identical specific radioactivity values were measured in both fractions.

Thus, it may be established that in isolated lenses, similarly to other acid-insoluble protein fractions, the acid-soluble protein fraction incorporates ^{14}C-amino acids, preferentially basic amino acids, i.e. is de novo synthesized in vitro.

Discussion

Lens proteins have for long been classified according to their solubility in water or in various salt solutions and only some protein fractions were isolated. Since then, with more refined methods, the original pattern of protein composition has become appreciably more complex, proof of which is the present paper describing a fraction with a hitherto unknown property. This property is that a considerable percentage of lens proteins remains in solution and is extractable with strong acids. The majority of this fraction occurs in the fibres and, more closely, in the water-soluble proteins. Data on the distribution of this fraction among water-soluble proteins will be given in our next paper (Köteles et al., 1971). This fact, however, raises the possibility that this fraction might be a subunit of an already known crystalline. Besides rats, this very fraction could be detected in other mammals too, as e.g. in rabbits, mice, guinea-pigs, in fact, also in humans (unpublished results). Somewhat different protein solubility conditions were found in birds, notably in the lenses from chicks of various age groups.

The relative amount of acid-soluble proteins was found to diminish with age, while the absolute protein content was identical both in young and old animals. This suggests that acid-soluble protein is formed in the course of fibre differentiation. Indeed, the in vitro experiments carried out in the presence of ^{14}C-amino acids have confirmed that in the isolated lenses of young animals this protein fraction is synthetized parallel to the other protein fractions. For the time being it is not yet clear whether this fraction incorporates labelled amino acids in the lenses of aged animals. The fact that it incorporates more basic amino acid than either of the acidic or neutral one, suggests its being a basic protein.

Experiments are in progress to further characterize this fraction and to elucidate its biological function.

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Further Studies on the Acid-Soluble Proteins of the Eye Lens

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The earlier described so-called acid-soluble protein fraction occurring in rat lenses among the water-soluble fractions is found mostly in the α-crystalline fraction. During a short time incubation in vitro of isolated lenses the acid-soluble protein fractions of any origin were labelled with ^{14}C-arginine; still, their specific radioactivity values were always lower than the values measured in the acid-insoluble parts of the respective water-soluble fractions. As against Sephadex gel filtration, polyacrylamide gel electrophoresis proved this fraction to be a heterogeneous protein mixture.

Introduction

As reported in our previous papers, among lens proteins there exists a protein fraction soluble in highly concentrated acids only (Köteles, Antoni, 1968; Köteles et al., 1971). In the present paper this fraction will be further characterized from both analytical and metabolic aspects.

Materials and methods

Lenses from young rats of 100 to 120 g weight were homogenized in 0.5 ml of distilled water at 4°C. The homogenate was centrifuged at 3500 g for 30 minutes. The supernatant contained the water-soluble, the sediment the water-insoluble proteins. The various water-soluble crystalline fractions, and the water-insoluble, but 7 M urea-soluble and insoluble fractions were isolated according to Harding (1969). Acid-soluble crystalline fractions were obtained both from the crystalline mixture and from the isolated crystalline fractions by treating them with HCl of 0.5 N final concentration (Köteles et al., 1971). Polyacrylamide gel electrophoresis was performed according to Vande and Davis (1965) and Shepherd and Gurley (1966).

For the study of ^{14}C-amino acid incorporation the isolated lenses were incubated in medium TC 199 at 37°C for 3 hours in vitro in the presence of 1 μCi/ml of ^{14}C-arginine.

Results

Water-soluble and insoluble, furthermore, α-, β- and γ-crystalline fractions and from the water-insoluble part 7 M urea-soluble and insoluble fractions were isolated from the homogenate of rat lenses. These were treated with HCl of 0.5 N

final concentration. The percentual distribution of these various fractions was determined and related to the total protein content of the lens (Table 1). The distribution values of the hitherto known components as indicated in the Table (Columns B and C) are in good agreement with the published data (e.g. Harding,

Table 1

*Percentual amount and ^{14}C-arginine content after in vitro labelling of various rat lens proteins separated by their solubilities in water, acid- and urea-solution**

Fraction	Quantity in %					Specific radioactivity cpm/mg $\times 10^{-3}$
	A	B	C	D	E	
Total protein	100					4278
Water-soluble		78.5				4990
Acid-soluble				19.6		
Acid-insoluble					58.9	
Water-insoluble		21.5				1428
Urea-soluble			9.4			
Acid-soluble				0.3		
Acid-insoluble					9.1	
Urea-insoluble			12.1			
Acid-soluble				1.0		
Acid-insoluble					11.1	
Water-soluble	100					4990
α-crystalline		29.9				5806
Acid-soluble				9.6		3046
Acid-insoluble					20.3	—
β-crystalline		35.0				4500
Acid-soluble				0.6		0
Acid-insoluble					34.4	—
γ-crystalline		35.1				3906
Acid-soluble				3.9		1680
Acid-insoluble					31.2	—

* For the details of separation and in vitro incubation see Materials and Methods.

1969). The total amount of acid-soluble proteins is obtained by adding up the values found in Column D. As it appears from the Table, these amount to about 21 per cent of the total proteins. A comparison of the details in Column D reveals that the acid-soluble protein can be extracted mainly from the water-soluble α-crystalline fraction and, to a lesser extent, from the γ-crystalline fractions. The other minor values should rather be considered as some contamination due to inadequate separation.

To study a few metabolic aspects of these fractions, the isolated lenses were incubated in the presence of 1 μCi/ml ^{14}C-arginine. The ^{14}C-arginine incorporated was determined in the various isolated fractions. As shown in Table 1, the specific radioactivity values of acid-soluble protein fractions are lower than those

of the fractions from which these had been isolated. Accordingly, the acid-soluble proteins differ from the other protein fractions not only in their solubility, but also in their labelling properties, in vitro.

The further investigations were designed to elucidate whether the acid-extracted protein fraction should be considered as homogeneous or eventually

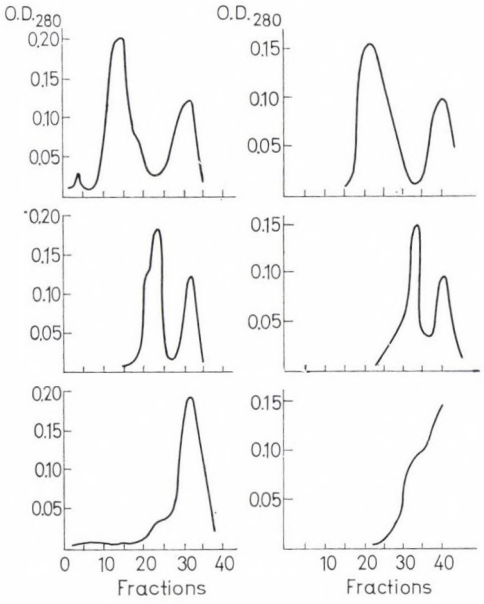

Fig. 1. Gel filtration of the acid-soluble protein fraction of rat lenses through Sephadex G-50 (upper), G-75 (middle) and G-100 (bottom) column. The profiles of the left series represent the results of experiments at pH 3.6, those in the right series the results obtained at pH 7.2. The first fraction seen in the Figure is the protein and the second is uridylic acid (UMP) added to indicate the void volume

as a mixture of several protein types. Gel-filtration and polyacrylamide gel electrophoresis were used.

The 0.5 N HCl-extracted protein was filtered through various Sephadex gels, both at acidic and neutral pH-s. For the indication of the void volume uridylic acid was used. Fig. 1 presents the profiles after filtration through G-50, G-75 and G-100 gels, resp. As it clearly appears, the G-50 gel excluded the protein both at acidic and neutral pH-s. With G-75, the protein was closer to uridylic acid, representing the inner volume, whereas the G-100 filter did not exclude it. Accordingly, in this gel filtration the protein appeared as a homogeneous fraction with a molecular weight of about 30 000. A similarly low molecular weight was obtained by using Biogel-A-0.5 m gel filter (unpublished data).

For its further characterization, the acid-soluble protein was subjected also to polyacrylamide gel electrophoresis. Both the acid- and water-soluble fractions

were electrophorized at pH 3.9 and 8.9. When performing the run at pH 3.9, the electrodes were put in an inverse position. After running and staining, the gels were fixed in acetic acid. However, it has soon been realized that the acid-soluble fraction disappears if the gel is let to stand in acetic acid. Therefore, fixing by 10 per cent trichloroacetic acid (TCA) was next attempted. As against fixing in acetic acid this time the acid-soluble fraction proved to be stable for as long as 5 days. On the other hand, TCA proved to be less advantageous for fixing the water-soluble fraction.

The results of our experiments under various conditions are presented in Fig. 2. As it can be seen from Figs 2A and 2B, both the acid- and the water-soluble fractions proved to be heterogeneous when electrophorized at pH 8.9, with about 5 and 7 easily distinguishable fractions, resp. At pH 3.9, however, a single fraction appeared but was soon dissolved when fixed in acetic acid. Consequently it cannot be seen in Figs 2A and 2B, taken on the 3rd and 5th days after fixing. When TCA was used for fixing instead of acetic acid, after electrophoresis at pH 3.9 a single component could be observed both in the acid-soluble and water-soluble fractions – as it appears from Figs 2C and 2D.

Accordingly, the so-called acid-soluble protein fraction extracted from rat lens homogenates with 0.5 N HCl and appearing to be homogeneous by gel-filtration is, in fact, heterogeneous. The fractions could be identified, i.e. the respective components could be found, when the water-soluble fractions were also electrophorized.

Discussion

In the present paper further characteristics are presented for the acid-soluble lens protein fraction described by us earlier. It has been established that the acid-extractable protein mixture contained similar fractions to those of the water-soluble fractions as revealed by gel-electrophoresis. At pH 3.9 a single protein component could be detected. The α-crystallines are known to contain basic subunits, too. Accordingly, it may also be assumed that by acidic extraction just the basic subunits of the various crystallines can be separated from fractions with different isoelectric points.

Further investigations are designed to decide whether the lens proteins soluble in strong acids play but a structural role or have some other biological function, too. As to the latter, we are reduced to conjectures only. E.g. together with the other proteins, it may participate in composing the quaternary structure of intrafibral proteins, known to be mainly responsible for the transparency and appropriate refraction of the lens. However, other macromolecules, e.g. the nucleic acids, may influence quaternary structure. The lens contains a rather high quantity of low molecular weight RNA as compared to the other organs (Köteles et al., 1968). A rather high ratio of the low molecular weight RNA has no transfer function at all (Virmaux et al., 1964). The existence of acid-soluble, presumably strongly basic proteins in the lens fits very well in with the hypothesis that the structural stability at a molecular level of the lens is

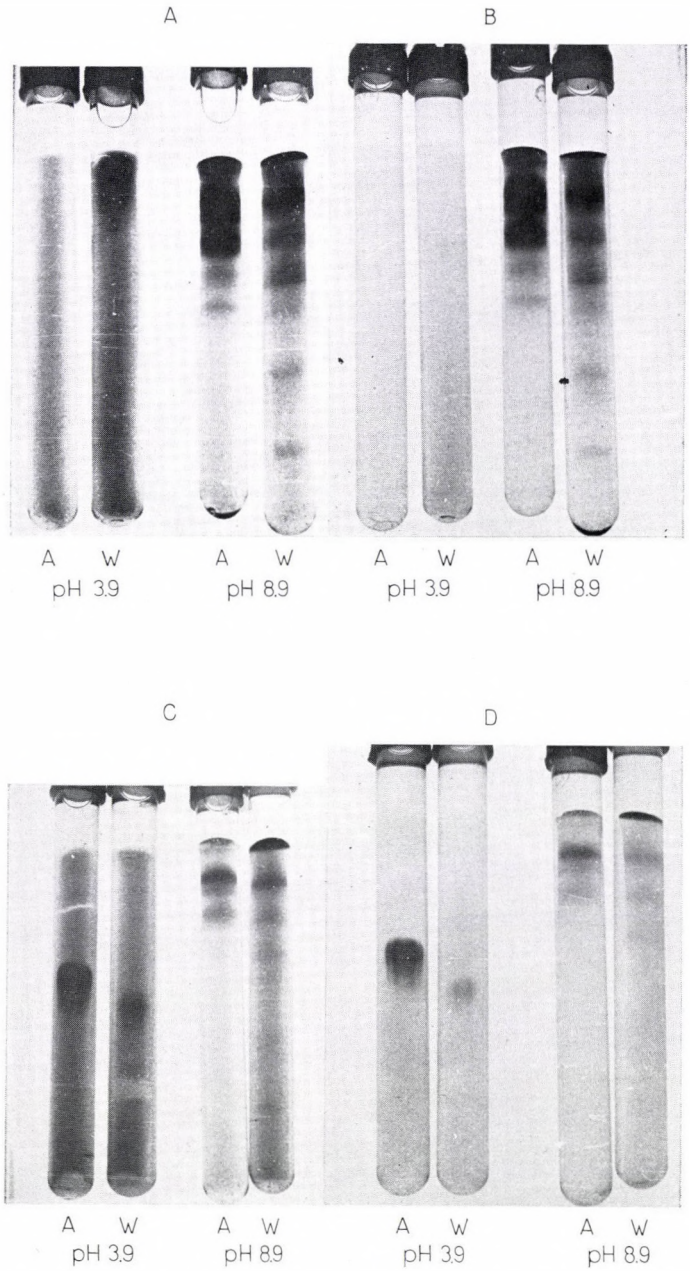

Fig. 2. Distribution of rat lens proteins following polyacrylamide gel electrophoresis. A, B: the gels 3 and 5 days, resp., after the electrophoretic run. Fixation in acetic acid. C, D: the gels 3 and 5 days, resp., after the electrophoretic run. Fixation in TCA. A: Acid-soluble fraction, W: Water-soluble fraction

ensured by both the relations among the proteins themselves and between proteins and nucleic acids. According to another hypothesis, during cell division, differentiation, regulation of transcription, intermitotic transmission of biological information, etc., basic proteins may play a central role in the lens, too.

The authors are indebted to Dr V. Várterész, director ("Frédéric Joliot-Curie", National Research Institute for Radiobiology and Radiohygiene) and to Professor Dr Magda Radnót, director (1st Clinic of Ophthalmology, Medical University, Budapest) for their support to the investigations reported. Thanks are due to Miss M. Pethő for her skilful technical assistance.

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A Molecular Enzyme Kinetic Model

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An enzyme kinetic model is presented which gives the kinetic constants a deeper physical and molecular meaning. By determining V_{max} and K_m as functions of $\frac{1}{kT}$, i.e. the temperature, the model makes the individual rate constants k_{+1} and k_{-1} available without further experimentation. The possibilities for the further extension and also the restrictions of the model are discussed.

Introduction

Since the elaborations of the Michaelis–Menten and the Briggs–Haldane theories several attempts have been made to further improve enzyme kinetics. In the last 30 years of the history of enzyme kinectics the greatest progress was obtained by the introduction of fast reaction methods and the allosteric theory (Michaelis, Menten, 1913; Briggs, Haldane, 1925; Chance et al., 1964; Monod et al., 1965).

In spite of all effort the progress is too little for a full understanding of molecular events. Since proteins cannot be handled easily even by modern quantum chemical methods, any treatment which is of some further theoretical or practical use in studying enzyme reaction seems advantageous.

The present kinetic study concerns a reaction occurring in aqueous media

$$E + n \cdot S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES_n \xrightarrow{k_2} E + n \cdot Pr \qquad (1)$$

The aim of this study was to produce a model that may give further information on the meaning of constants k_2, k_{+1} and k_{-1} and correlate them, at least in a semiquantitative manner, with the molecular events taking place in the course of the enzyme reaction.

The kinetic model

For the sake of simplicity the reservation is made that no stable enzyme-product complex is formed. Since in the following only the initial velocity of the reaction will be examined, the reaction characterized by the rate constant k_{-2} is negligible if the initial concentration of the product is taken to be zero.

It is assumed that $\frac{d[ES]}{dt} = 0$. On the measuring volume V', the free enzyme concentration is $[E]$ and the substrate concentration is $[S]$.[1]

[1] The list of principal symbols used see on page 364.

It is also assumed that the concentration of the free substrate is nearly equal to the total substrate concentration and so $[S]$ can be regarded as free substrate concentration.

Subsequently, the question how many products are formed by one enzyme molecule in a time unit will be studied.

Instead of random variables we have generally used the expectations.

It is assumed that the enzyme molecule is active and very large as compared to the substrate and during kinetic treatment it does not perform any motion. To the active centre of the enzyme molecule a volume is ordered. Within this volume the substrate can be bound by the enzyme molecule owing to specific forces acting between the two molecules. On the other hand, if the substrate is outside this volume no specific forces exert their action on it. The volume thus defined is expressed as V, or recognition volume.

The van der Waals forces are negligible as long as the substrate within the recognition volume is not optimally oriented for binding, since these forces are acting non-specifically almost in the same way on other molecules, i.e. on the solvent (in this case on water) and on the buffer molecules as well. From the foregoing it is obvious that the van der Waals forces can be excluded from the treatment and we can say that the substrate moves by free diffusion in the recognition volume until it reaches a favourable position where it is bound by specific forces forming thereby an enzyme-substrate complex or until it disappears from the recognition volume.

Assuming that the free substrate molecules in the solution follow the Poisson distribution the probability of the presence of a substrate in the recognition volume can be written as follows (Maniloff, 1969):

$$P = \lambda \cdot e^{-\lambda} \tag{2}$$

The number of substrates in the measuring volume V' is $[S]V'$, the number of volumes corresponding in size to the recognition volume is $\frac{V'}{V}$, thus

$$\lambda = \frac{[S] \cdot V'}{V'} \cdot V = [S]V \tag{3}$$

Substituting Equation (3) in (2):

$$P = [S]V \exp(-[S]V) \tag{4}$$

At the substrate concentration given in the experiments the probability of the presence of two substrates in the recognition volume is negligible. In fact, owing to the short range of action of the specific forces, V is scarcely larger than the volume of the substrate and hence $([S]V)^2$ is very small (Pollard, 1961).

If collision is meant to be the phenomenon of the appearancy of the substrate in the recognition volume, it is easy to determine the frequency of colli-

sions if, for the present, the reaction of the substrate with the enzyme is disregarded.

Let us study one enzyme molecule for time t. From time t the recognition of the enzyme is occupied during time $P \cdot t$, while during time $(1 - P) \cdot t$ it is "empty".

If the mean recurrence time is ϑ and t is sufficiently long ($t \gg \bar{t} + \vartheta$), then

$$\frac{P \cdot t}{\bar{t}} = \frac{(1-P) \cdot t}{\vartheta} \tag{5}$$

where $\bar{t}$ is the mean lifetime of the stay of the substrate in the recognition volume.

Collision frequency is:

$$\Phi = \frac{1}{\bar{t} + \vartheta} \tag{6}$$

From Equation (5) it follows that

$$\bar{t} + \vartheta = \frac{\bar{t}}{P} \tag{7}$$

Thus

$$\Phi = \frac{P}{\bar{t}} \tag{8}$$

If the substrate present in the recognition volume is recognized with probability q by the enzyme, i.e., q is the probability of complex formation occurring at the site of each collision, $(1 - q)$ is the probability of the disappearance of the substrate from the recognition volume without its binding to the enzyme, L is the probability of enzyme + product formation, and $(1-L)$ is the probability of enzyme + substrate formation, then the number of products produced by an enzyme during one time unit is:

$$\Phi' = q \cdot L \cdot \Phi = \frac{P}{\bar{t}} \cdot q \cdot L \tag{9}$$

Equation (9) is true only if all collisions can take place, i.e. if the lifetime of the complex (t') is so short as compared to ϑ that the complex breaks down before the next collision can occur.

From Equations (4) and (5) it follows that:

$$\vartheta = \bar{t}\left(\frac{1}{[S]V\exp(-[S]V)} - 1\right) \tag{10}$$[2]

[2] If the substrate does not move by free diffusion, certain parameters (t, q, F, etc.) of Equations (9) and (10) have other values than in the case of independent diffusion.

It is apparent from Equation (10) that by increasing the concentration, ϑ decreases. Since t' is the mean lifetime of the complex, Equation (9) can take place only if the condition

$$t' \ll \vartheta \tag{11}$$

is satisfied. Thus, Equation (9) is correct at low concentrations and so it must be amplified. For the amplification a new quantity will be introduced, namely the number showing how many subsequent collisions are made impossible by formation of a complex at the given $[S]$ and t'. This number will be termed F.

From the foregoing it is obvious that F is a function of the substrate concentration at a given temperature.

By means of F the initial reaction rate can be written for every concentration.

Let us examine an enzyme molecule for time t. It may be assumed that during this time the number of collisions would be $n = \frac{P}{\bar{t}} \cdot t$. If during time t the number of complexes is Q, the actual number of collisions is:

$$N = n - QF \tag{12}$$

From q it follows that

$$Nq = Q \tag{13}$$

Writing (13) in a more detailed form:

$$q\left(\frac{P}{\bar{t}} \cdot t - QF\right) = Q \tag{14}$$

The frequency of the complex formation for one enzyme:

$$\Phi^* = \frac{Q}{t} \tag{15}$$

Combining (15) with (14) and dividing the equation so obtained by t:

$$q\left(\frac{P}{\bar{t}} - \Phi^* F\right) = \Phi^* \tag{16}$$

Arranging (16) for Φ^*:

$$\Phi^* = \frac{P}{\bar{t}} \cdot \frac{1}{F + \frac{1}{q}} \tag{17}$$

From this follows

$$L \cdot \Phi^* = \Phi' \tag{18}$$

that is, the velocity of product formation for one enzyme molecule.

Writing (18) in Equation (17),

$$\Phi' = \frac{P}{\bar{t}} \cdot \frac{L}{F + \frac{1}{q}} \tag{19}$$

the equation of initial velocity, valid for all concentrations, is obtained.

If n_P and Pr denote the number of products and their concentration, respectively, in V', then from Equation (19) it follows for $\frac{\mathrm{d}n_P}{\mathrm{d}t}$ and $\frac{\mathrm{d}Pr}{\mathrm{d}t}$:

$$\frac{\mathrm{d}n_P}{\mathrm{d}t} = [E]_T V' \Phi' \tag{20}$$

$$v_2 = \frac{\mathrm{d}[Pr]}{\mathrm{d}t} = [E]_T \Phi' \tag{21}$$

Determination of recognition probability

According to our assumption the substrate moves by free diffusion until it becomes bound to the enzyme. This assumption is approximately true, considering the non-specific character of the van der Waals forces.

It is also assumed that the substrate is spherical, with a radius ϱ. If the mean collision lifetime is $\bar{t}$ and during time $\bar{t}$ the substrate traverses a mean rotary angle Θ by rotational diffusion motion, then

$$q = \frac{\Theta^2}{4\pi} \tag{22}$$

If it is a condition of recognition that the substrate should possess an energy $E \geq {}^qE$, then

$$q = \frac{\Theta}{4\pi} \cdot e^{-{}^qE/kT}$$

In case of rotational diffusion motion the mean square rotary angle is:

$$\Theta^2 = \frac{kT}{4\pi\eta\varrho^3} \cdot t \tag{23}$$

In Equation (23) k is the Boltzmann constant, T is the absolute temperature, η the viscosity, ϱ the radius of the molecule and t the time.

The formula of the translational diffusion constant is:

$$D = \frac{kT}{6\pi\eta\varrho} \tag{24}$$

Writing (24) in (23):

$$\Theta^2 = \frac{3D}{2\varrho^2} \cdot t \tag{25}$$

As the rotational diffusion is considered only for time $t = \bar{t}$, from (22) and (25):

$$q = \frac{3}{2} \cdot D \cdot \bar{t} \cdot \frac{1}{4\pi\varrho^2} \cdot e^{-{}^qE/kT} \tag{26}$$

Determination of L

It is assumed that the energy E_p required for the conversion of the substrate to a product, and the energy E_d required for the dissociation of the substrate are taken up by the ES complex from its environment by means of collisions.

Considering the possibility that the substrate possesses an energy ${}^qE$, both E_p and E_d can be zero, but they may also differ from it.

The energy uptake takes place at the surface of the ES complex and, to our present knowledge, is due to the colliding solvent molecules. A certain combination of the collisions above a certain level of energy at the right sites will most likely make the ES complex dissociate. An ES complex decomposes either to $E + Pr$ or $E + S$ with a probability

$$P^* = e^{-E_d/kT} \tag{27}$$

Assuming it has the necessary energy levels, the ES complex decomposes to the products ($E + Pr$ or $E + S$) with a probability factor 1 (Johnson et al., 1954). Therefore it may be written that

$$L = e^{-\Delta E/kT} \tag{28}$$

where L is the probability that an ES complex becomes $E + Pr$, k is the Boltzmann factor, T is the absolute temperature and

$$\Delta E = (E_p - E_d) \tag{28a}$$

Let x be the time interval between two suitable collision patterns of the solvent molecules with the ES complex. If we multiply x by the reciprocal form of (27) the lifetime of an ES complex can be obtained in the following form:

$$t' = x \cdot e^{E_d/kT} \tag{29}$$

Let τ be the mean lifetime of stay of the colliding molecule at one steric point. It is easy to accept that x is a linear function of τ, i.e. $x = k_1' \cdot \tau$, where constant k_1' is the average number of collisions at certain points of the ES complex while the right collision pattern – dissociating the complex – occurs with exp (E_d/kT) probability. From this

$$t' = k_1' \cdot \tau \cdot \exp\left(\frac{E_d}{kT}\right) \tag{30}$$

Determination of rate constants

The initial velocity of complex formation is:

$$v_{+1} = [E]\,[S]k_{+1} \tag{31}$$

where $[E]$ denotes the molar concentration of the free enzyme, and $[S]$ that of the free substrate.

On the basis of the foregoing the rate of complex formation is

$$v_{+1} = [E]\frac{P}{\bar{t}} \cdot q = \frac{[E][S]Ve^{-[S]V}}{\bar{t}}\, q \tag{32}$$

A comparison of (31) and (32) reveals that

$$k_{+1} = \frac{q}{\bar{t}} \cdot Ve^{-[S]V} \tag{33}$$

Since $[ES]$ denotes the concentration of the complex ES it follows that:

$$v_{-1} = k_{-1}[ES] \tag{34}$$

and

$$v_2 = k_2\,[ES] \tag{35}$$

i.e. the decomposition rate of the complex for E + S and E + Pr. For the determination of $[ES]$ the probability of complex formation by the enzyme P' is studied. According to Equation (17) the frequency of complex formation for one enzyme is:

$$\Phi^* = \frac{P}{\bar{t}} \cdot \frac{1}{F + \dfrac{1}{q}}$$

Considering that the mean lifetime of a complex is t', the enzyme is in complex state for time $t \cdot \Phi^* \cdot t'$ in a time interval t.

Because of the meaning of P':

$$P' \cdot t = t \cdot \Phi^* \cdot t' \tag{36}$$

Hence

$$P' = \Phi^* \cdot t' \tag{37}$$

If formation of the complex occurs with probability P', then

$$[ES] = [E]_T \cdot P' = [E]_T\, \Phi^* \cdot t' \tag{38}$$

Substituting (38) in Equations (34) and (35):

$$v_{-1} = k_{-1}[E]_T \cdot \Phi^* \cdot t' \tag{39}$$

and

$$v_2 = k_2[E]_T \cdot \Phi^* \cdot t' \tag{40}$$

On the basis of (18) and (21):

$$v_2 = [E]_T \cdot L \cdot \Phi^* \tag{41}$$

From a comparison of (40) and (41):

$$k_2 = \frac{L}{t'} \tag{42}$$

Since the complex dissociates to $E + S$ with probability $(1 - L)$, by analogy with (41):

$$v_{-1} = [E]_T(1 - L) \cdot \Phi^* \tag{43}$$

From a comparison of (39) and (43) it follows that:

$$k_{-1} = \frac{1 - L}{t'} \tag{44}$$

Using (33), (42) and (44) the following equation is obtained:

$$K_m = \frac{k_{-1} + k_2}{k_{+1}} = \frac{1}{\frac{t'}{\bar{t}} \cdot q \cdot V e^{-[S]V}} \tag{45}$$

If we accept the condition that the maximum rate of enzyme activity occurs when all the enzyme molecules are in a complex form, i.e. $[ES] = [E]_T$, then

$$v_{\max} = k_2[E]_T = [E]_T \frac{L}{t'} \tag{46}$$

The classical equation

$$v = \frac{v_{\max} \cdot [S]}{K_m + [S]}$$

can be written in a detailed form, on the basis of Eqs (26), (28), (45) and (46):

$$v = [E]_T \frac{V[S]\, e^{-[S]V}}{\bar{t}} \cdot \frac{e^{-\Delta E/kT}}{[S]V e^{-[S]V} \cdot \frac{t'}{\bar{t}} + \frac{1}{\frac{1}{4\pi\varrho^2}\left(\frac{3D\bar{t}}{2}\right) e^{-qE/kT}}} \tag{47}$$

Making use of the assumption that $\bar{t}$ – similarly to t' in Eq. (30) – is a linear function of τ

$$\frac{t'}{\bar{t}} = \frac{k_1'}{k_2'} \cdot \frac{\tau}{\tau} e^{E_d/kT} = \frac{k_1'}{k_2'} \cdot e^{E_d/kT} \tag{48a}$$

where k_2' is the average number of steps done by the substrate from one lattice point to another within the recognition volume V.[3]

[3] The τ denominators in Eqs (30) and (48a) may be taken equal to each other only if the diffusion constants of solvent and substrate are in the same order of magnitude.

According to Somogyi (1971), $\tau = \frac{\lambda^2}{6D}$, where λ is the distance between two lattice-points and D is the diffusion constant of the solvent. Hence

$$\tau = \frac{\lambda^2}{6D} = \frac{1}{kT} \cdot \lambda^2 \cdot \pi \cdot \eta \cdot \varrho \tag{48}$$

where η is the viscosity and ϱ is the radius of the solvent molecules.

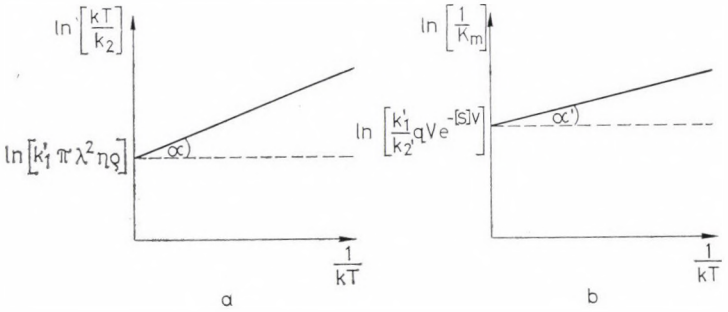

Fig. 1a. By plotting $\ln\left(\frac{kT}{k_2}\right)$ versus $\frac{1}{kT}$ a method is presented for the determination of k_1' and E_p b. A similar plot of $\ln\left(\frac{1}{K_m}\right)$ versus $\frac{1}{kT}$ makes parameters k_2' and E_d available using the k_1' of Fig. 1a

From Equation (45):

$$\frac{1}{K_m} = \frac{k_1'}{k_2'} \cdot q \cdot V e^{-[S]V} \cdot e^{E_d/kT} \tag{49}$$

and

$$\ln \frac{1}{K_m} = \ln \left(\frac{k_1'}{k_2'} \cdot q \cdot V \cdot e^{-[S]V} \right) + \frac{E_d}{kT} \tag{50}$$

$$\ln \frac{1}{k_2} = \ln (k_1' \pi \cdot \lambda^2 \cdot \eta \cdot \varrho) + \ln \frac{1}{kT} + \frac{E_p}{kT} \tag{51}$$

Plotting first $\ln \frac{kT}{k_2}$ versus $1/kT$, and then $\ln \frac{1}{K_m}$ versus $1/kT$, E_p and E_d are equal with the tg α-s. k_1' and k_2' can be calculated from the intersections of the curves with the Y axis having the values of λ, ϱ and η (Figs 1a, b). The viscosity is easily determined by standard methods while one may estimate λ and ϱ from data available or, e.g. from X-ray diffraction studies. Having got E_p, E_d, k_1' and k_2' and using Eqs (30) and (48a), t' and $\bar{t}$ are readily available. From (26) and (33) with an assumption on V, k_{+1} can be calculated. Since the values of k_{+1}, k_2 and K_m are known, k_{-1} is also known.

Discussion

The model described in this work gives a more detailed interpretation of the kinetic parameters v_{max}, K_m and the individual rate constants of the Equation (1) than other kinetics. By the proposed method the kinetic parameters generally used can be determined in a relatively simple way.

The v_{max} and K_m, using the Lineweaver–Burk plot, are available for any enzyme reaction (Lineweaver, Burk, 1934). The individual rate constants are, however, not so easily determined. The stopped flow method made k_{+1} experimentally attainable, and from K_m, k_2 and k_{+1} one can calculate k_{-1}. The only problem of the fast reactions is that they are subject to strict spectroscopic conditions. Only few enzymes are suitable for being investigated by fast reactions in native state. The application of power series in studying the differential equations of the kinetic processes gives a possibility to calculate the individual rate constants (Darvey et al., 1966a, b). However, the mathematical treatment has several difficulties in most practical situations as it has recently been shown (Walter, 1970; Barrett, Walter, 1970; Walter, Barrett, 1970).

Further objection against the above mentioned mathematical treatment is that it does not reveal deeper physical meaning of the individual constants.

Similarly, quite recently Schurr (1970a, b) presented a model, discussing the interrelationships between the rate constants of enzyme reactions likewise avoiding any molecular interpretation of the theoretical data.

The model presented here can be used for determining individual rate constants so that new parameters are introduced with molecular meaning. By determining v_{max} and K_m as functions of $1/kT$, i.e. the temperature, and using the methods demonstrated in Figs 1a and b, the individual constants k_{+1} and k_{-1} of the enzyme reactions are available theoretically without further experimentations. E_p and E_d can also be determined from the dependency of v_{max} and K_m on the temperature. The authors are aware of the difficulties, namely that V can be estimated only from the known diameter of the substrate. The mean lifetime of the stay of the substrate in the recognition volume or that of the ES complex can only be determined if one accepts equation $\tau = \frac{\lambda^2}{6D}$ (Somogyi, 1971).

From the treatment of the model it appears that the main difficulties arise from the unsolved physical problems of solution kinetics and protein chemistry. These problems made it unavoidable to use some approximations.

Nevertheless the advantages offered by our kinetics may present a new possibility of approaching the mechanism of enzyme reaction. On the other hand, it is easy to control the resolving power of the model – i.e. the errors caused by the approximations – with enzymes which are suitable for fast reaction studies.

The constants obtained by these two methods, the present proposal and the fast reactions, will help correcting our present theory.

A very promising point for further development of the model seems to be the assumption of energetically suitable and sterically synchronized collisions of

solvent molecules with the ES complex. This gives a new molecular meaning to factor $\exp(-\Delta E/kT)$ with which the enzyme reaction is proportional.

Some further energetical relationships in enzyme reactions are being considered in our laboratory and will be published elsewhere.

Appendix

Determination of F

Since at the concentrations used in our experiments $[S]V$ is very small, the condition

$$\bar{t} \ll \vartheta$$

must be satisfied.

In case the above condition is satisfied, the function $F = f([S])$ is determined.

Substrate collisions with an existing complex may occur at any time within lifetime t' of the complex. For this reason F may be

$$\left[\frac{t'}{\bar{t}+\vartheta}\right] \text{ or } \left[\frac{t'}{\bar{t}+\vartheta}\right] + 1$$

where $[x]$ is the integer value of x and further on $\{x\}$ is the fractional value of x, and x denotes any mathematical expression.

If with probability p

$$F = \left[\frac{t'}{\bar{t}+\vartheta}\right] + 1$$

then with probability $(1-p)$

$$F = \left[\frac{t'}{\bar{t}+\vartheta}\right]$$

Thus:

$$F = (1-p)\left[\frac{t'}{\bar{t}+\vartheta}\right] + p\left(\left[\frac{t'}{\bar{t}+\vartheta}\right] + 1\right) = \left[\frac{t'}{\bar{t}+\vartheta}\right] + p$$

as the above terms are exclusive events.

In case the condition of $\bar{t} \ll \vartheta$ is satisfied we may write:

$$p = \left\{\frac{t'}{\bar{t}+\vartheta}\right\}$$

Thus

$$F = \left[\frac{t'}{\bar{t}+\vartheta}\right] + \left\{\frac{t'}{\bar{t}+\vartheta}\right\} = \frac{t'}{\bar{t}+\vartheta}$$

From (6), (8) and (4) it is apparent that

$$F = \frac{t'}{\bar{t}}[S]Ve^{-[S]V}$$

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Symbols: D, diffusion constant of substrate; $[E]$, molar concentration of free enzyme. E_d, the lowest energy required to dissociate the ES complex to $E + S$; E_p, the lowest energy required to dissociate the ES to $E + Pr$; ${}^qE$, the lowest energy required for the substrate (present in the recognition volume) to form a complex with the enzyme; $[E]_T$, molar concentration of total enzyme; $[ES]$, molar concentration of enzyme–substrate complex; η, viscosity of solution; F, number of collisions that cannot take place because an ES complex has already been formed; Φ, collision frequency of substrate for one enzyme; Φ', turnover number; Φ^*, frequency of formation of ES complex; L, probability that an ES complex becomes $E + Pr$; P, probability of the presence of the substrate in the recognition volume; $[Pr]$, molar concentration of product; q, probability that the substrate present in the recognition volume is bound by the enzyme; ϱ, radius of substrate supposed to be spherical; $[S]$, molar concentration of free substrate; T, absolute temperature; $\bar{t}$, mean lifetime of stay of the substrate in the recognition volume; k, Boltzmann constant; t', mean lifetime of an ES complex; Θ, mean rotary angle traversed in time t by the substrate performing rotational diffusion motion; ϑ, mean recurrence time of the substrate in the recognition volume; τ, mean lifetime of stay of colliding molecule at one steric point; V, recognition volume ordered to the active centre of an enzyme molecule; V', measuring volume.

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Cold Sensitivity Resulting from Repression Hypersensitivity in a Mutant of Escherichia coli K12

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In a leaky histidine auxotroph mutant of Escherichia coli K12 the defect in the first enzyme of the histidine biosynthetic pathway brought about a change in the regulation of the histidine operon. When deprived of histidine, the mutant derepresses, but — in contrast to wild type bacteria — derepression ceases much before normal growth rate is attained. This repression hypersensitivity is most pronounced at low temperatures.

Since growth of the mutant in minimal medium depends on its ability to compensate for the defect in the first enzyme by derepression, growth of the mutant is cold sensitive.

Suppression of the histidine requirement of the leaky mutant by a second mutation, which allows constitutive synthesis of histidine biosynthetic enzymes, suggests that the histidine requirement is due to lack of derepression. The effect of thiazolealanine on growth of the mutant is also explained by its ability to derepress the histidine operon.

As the mutation in the gene for the first enzyme causes a regulatory alteration, it is suggested that the first enzyme may be important in the regulation of the histidine operon.

Introduction

Most of the information on the histidine operon comes from studies on Salmonella typhimurium. Recent investigations on Escherichia coli have shown that the organization of the histidine operon is similar to, if not identical with, that in Salmonella typhimurium (Goldschmidt et al., 1970; Garrick-Silversmith, Hartman, 1970).

This similarity extends to the regulation of the histidine operon since it has been revealed that histidyl $tRNA^{his}$, rather than histidine, plays a central role in repression both in S. typhimurium (Roth et al., 1966) and E. coli (Schlesinger, Magasanik, 1964). All regulatory mutations in S. typhimurium, other than those affecting the operator locus, appear to involve defective synthesis or amino-acylation of $tRNA^{his}$ (Roth et al., 1966). These studies have emphasized the role of histidyl $tRNA^{his}$ in regulation but no true regulatory gene has so far been identified.

The gene encoding the feedback sensitive first enzyme, phosphoribosyl-adenosine triphosphate: pyrophosphate–phosphoribosyl transferase (PR-ATP pyrophosphorylase), of the histidine biosynthetic pathway is adjacent to the operator gene of the histidine operon. This unique position suggested a role for the feedback sensitive enzyme in the regulation of the histidine operon (Maas, McFall, 1964; Gruber, Campagne, 1965; Cline, Bock, 1966; Koshland, Kirtley, 1966).

We were looking for changes in the repression behaviour of Escherichia coli mutants which possess an altered first enzyme. Mutants carrying feedback insensitive first enzyme could be directly isolated with the help of the histidine analogue, 2-thiazolealanine. This histidine analogue causes histidine starvation and growth inhibition in wild type bacteria since it acts as a false feedback inhibitor of the first enzyme (Moyed, Friedman, 1959). Mutants with feedback insensitive first enzymes are no longer inhibited by thiazolealanine and thus can be readily isolated.

In this paper we described further experiments on the regulatory properties of this mutant. It will be shown that lack of depression is due to a hypersensitive repression mechanism brought about by the alteration of the first enzyme. Properties of this unusual mutant are discussed with respect to repression hypersensitivity.

Materials and methods

L-Histidinol·2HCl was prepared chemically (Bauer et al., 1955) and was free of histidine as shown by thin layer chromatography. The histidine analogues DL-2-methylhistidine and DL-2-thiazolealanine were obtained from Cyclo Chemicals.

Cells were grown in minimal medium A containing 0.2 per cent glucose and the required growth factors (Davis, Mingioli, 1950).

Cultures were vigorously aerated at different temperatures and growth was followed photometrically at 490 mμ in a cuvette of a light path of 10 mm. Preparation of cell-free extracts was described in a previous article (Patthy, Dénes, 1970). Protein determinations were made by the Folin-phenol method (Lowry et al., 1951).

The activity of histidinol dehydrogenase (EC 1.1.1.23) was determined according to Shedlovsky and Magasanik (1962). One unit of enzyme activity catalyzes the formation of 1 nmole NADH per hour. Specific activities are expressed as units of enzyme activity per mg protein. Histidinol dehydrogenase specific activity is expressed relative to that in wild type Y10 and corresponds to 78 nmoles NADH formed per hour per mg protein.

Mating was carried out by the method of Taylor and Thoman (1964). In the conjugation experiments azide resistant derivatives of the recipient strains were used. Sodium azide was applied in the plates at a concentration of 1.2 mM to select against donor bacteria. The strains used were the Vhf AB311 (TL^-; Taylor, Adelberg, 1960), and the recipient strains Y10-6, Y10-6 111 (Patthy, Dénes 1970, 1971). The latter two were derived from Y10 (F^-, thr^-, leu^-, B_1^-) of E. coli K12. Recombinants were selected during the first 25 minutes of mating.

Mutation, if not indicated otherwise, was induced by UV irradiation. 5×10^8 cell/ml cultures were irradiated with a 15W Tungsram germicidal lamp. 0.1 ml of the irradiated culture was plated on selective media and mutants were isolated after 48 hours and subsequently tested for the selected marker.

Results

Nature of the altered repression behaviour

In a previous paper (Patthy, Dénes, 1970) we described the one-step mutant Y10-6 derived from E. coli K12 on the basis of its resistance to 2-thiazolealanine. In this mutant the feedback sensitive first enzyme of the histidine biosynthetic

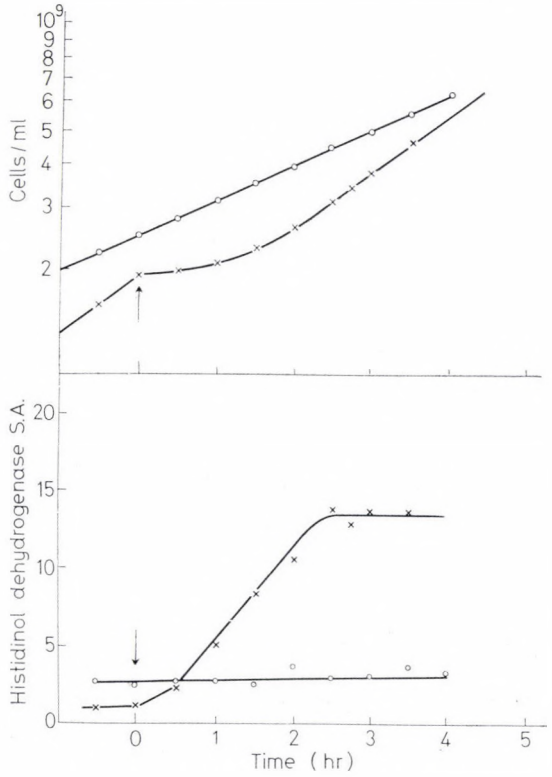

Fig. 1. Growth and derepression of Y10-6 and parent Y10 at 25°C. Y10-6 was grown in the presence of 30 μg/ml L-histidinol. Y10 was derepressed with 0.1 mM DL 2-thiazolealanine. (The arrow indicates thiazolealanine addition.) —○—○—, Y10-6; —×—×—, Y10

pathway is altered so that it has lower activity than the wild type enzyme and it is resistant to inhibition caused by the feedback inhibitor histidine or its analogue, thiazolealanine.

The leaky histidine auxotrophy caused by the low activity of PR-ATP pyrophosphorylase necessitates derepression, yet it was observed that derepression in Y10-6 mutant was retarded, especially at low temperatures, and only a severe histidine starvation elicited an increase in enzyme levels (Patthy, Dénes, 1970).

In further experiments we studied this repression anomaly under conditions where fluctuations in growth rate did not complicate the evaluation of the experi-

ments. We studied the derepression process at 25°C in media containing 30μg/ml L-histidinol where the mutant grew at a uniform rate with a doubling time of 180 minutes (compared to 102 minutes doubling time observed in the presence of histidine). Y10-6, though its growth was limited by histidine, failed to derepress, the enzyme level was kept at about three times the repressed level. By contrast, in the parent Y10 0.1 mM DL 2-thiazolealanine caused normal derepression at

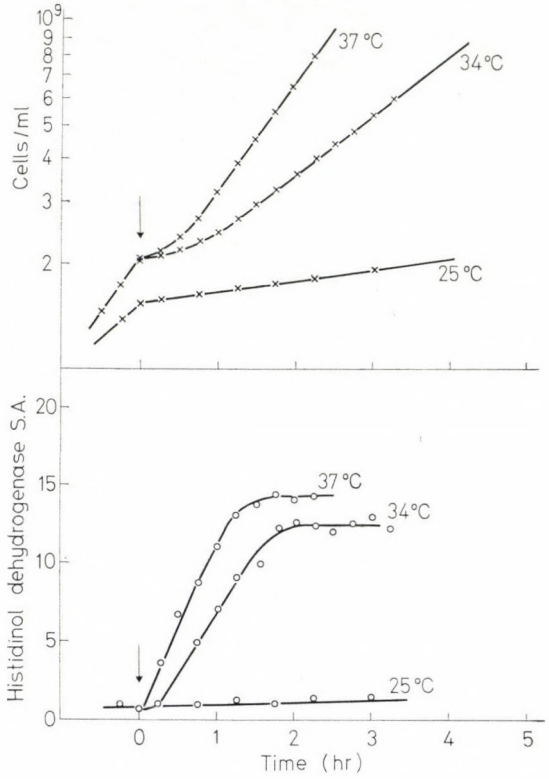

Fig. 2. Effect of 0.1 mM DL 2-thiazolealanine on growth and derepression of Y10-6 at different temperatures. Thiazolealanine was added to the cultures after exhaustion of exogenous histidine (arrow)

the same temperature as shown in Fig. 1. The fact that 0.1 mM DL 2-thiazolealanine could not derepress Y10-6 at 25°C (Fig. 2) shows that thiazolealanine was not responsible for the difference in behaviour of mutant and parent.

An important information on the nature of the altered repression behaviour was derived from studies with thiazolealanine (0.1 mM) at different temperatures.

At 37°C derepression and growth of the mutant were normal in the presence of thiazolealanine. At 34°C thiazolealanine had a similar effect with the important difference that derepression had ceased before normal growth rate was attained (100 minutes vs. 60 minutes doubling time in the presence of histidine (Fig. 2).

This regulation of enzyme synthesis at suboptimal growth rates (Fig. 1 and Fig. 2) sheds light on the nature of the altered repression behaviour. These data are compatible with the idea that a hypersensitive repression control prevents further derepression before normal growth rate is attained.

Consequences of repression hypersensitivity

The defect in PR-ATP pyrophosphorylase confers leaky histidine auxotrophy on Y10-6 and it must derepress in order to compensate this defect. Since derepression is heavily retarded at low temperatures, growth of the mutant is cold sensitive (Patthy, Dénes, 1970).

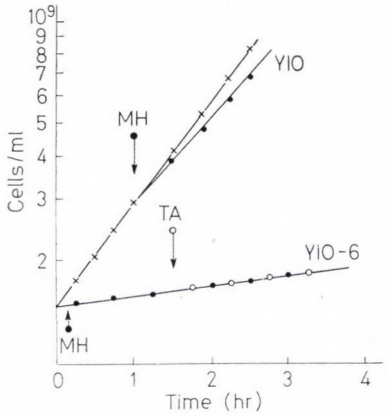

Fig. 3. Effect of 2-methylhistidine (MH) on the growth of Y10-6 and Y10 at 37°C. 2-Methylhistidine was applied at a concentration of 10 μg/ml. DL 2-thiazolealanine (TA) was used at a concentration of 0.1 mM

The effect of thiazolealanine on the growth of the mutant was attributed to its ability to help derepression. This notion is supported by the observation that thiazolealanine improved growth to an extent depending on its ability to help derepression (Fig. 2). Thiazolealanine gradually lost its effect on the growth of Y10-6 with decreasing temperature, since it could not counteract the increasing repression hypersensitivity.

The same conclusion is born out by the results of experiments with 2-methylhistidine. This histidine analogue is known to repress the histidine operon and to be incorporated into protein in place of histidine (Roth et al., 1966; Schlesinger, Schlesinger, 1969). 2-Methylhistidine repressed Y10-6 thereby preventing growth at all temperatures. 2-Methylhistidine had only a slight effect on Y10 since this parent strain produced sufficient histidine to compete with the analogue even when fully repressed.

Thiazolealanine could not counteract the effect of 2-methylhistidine on Y10-6, since 2-methylhistidine did not allow derepression of the histidine operon (Fig. 3).

Suppression of histidine auxotrophy by a second mutation

Using 2-methylhistidine we could test our supposition that the histidine requirement of Y10-6 is mainly due to lack of derepression. If this conclusion is right then a mutation which allows constitutive synthesis of histidine biosynthetic enzymes could suppress the effect of the original damage.

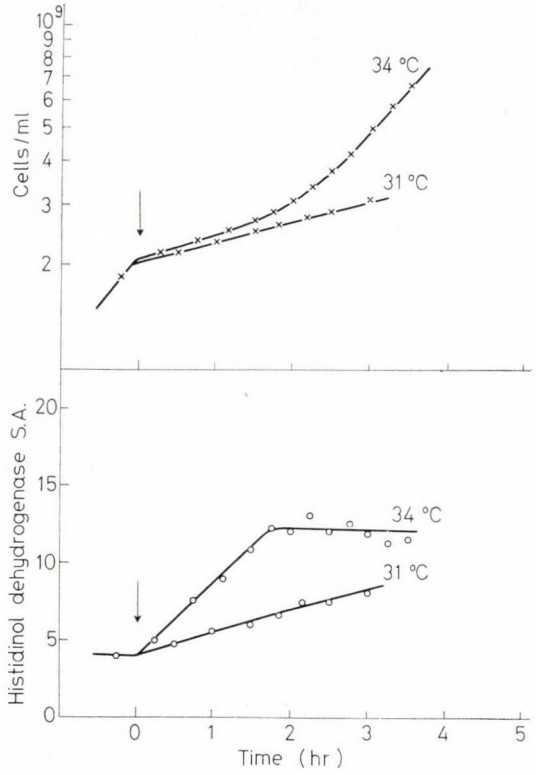

Fig. 4. Growth and derepression of Y10-6 111 at 31°C, and 34°C. After exhaustion of histidine at 31°C the mutant was grown at the temperature indicated (arrow)

A spontaneous secondary mutant – Y10-6 111 – was isolated from Y10-6 on the basis of its resistance to 2-methylhistidine (Patthy, Dénes, 1971). This mutant was shown to contain the original mutation: low activity of PR-ATP pyrophosphorylase, feedback resistance, etc. The double mutant contained high enzyme levels in the presence of exogenous histidine. Constitutive enzyme levels showed a strong temperature dependence and the level of the enzyme increased with increasing growth temperature (Patthy, Dénes, 1971).

The double mutant grew at the wild type rate even on minimal medium at temperatures above 31°C.

At 31°C (where constitutive enzyme level is low) the mutant required histidine for normal growth. This feature allowed us to study the derepression behaviour in the double mutant. The enzyme level was lowered by growing the mutant at 31°C on exogenous histidine and after the exhaustion of histidine the mutant was grown at the temperature to be studied. As can be seen in Fig. 4 derepression at 31°C and 34°C was still slow. At 37°C, however, derepression resembled that

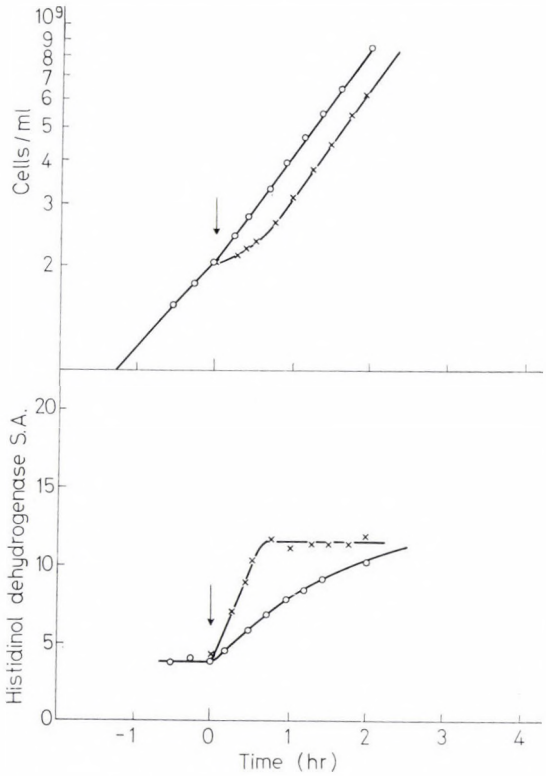

Fig. 5. Growth and derepression of Y10-6 111 at 37°C in the presence and absence of exogenous histidine. L-histidine was used at a concentration of 20 μg/ml. The arrow indicates the transfer of the cultures from 31°C to 37°C. —○—○—, in the presence of histidine; —×—×—, in the absence of histidine

of the wild type and led to rapid growth (Fig. 5). For a comparison it is shown how the same enzyme level is attained in the presence of exogenous histidine. The difference proves that the histidine operon is still under partial histidine control in the double mutant.

Temperature effects

The temperature dependence of the constitutive level of the enzyme in Y10-6 111 is similar to the temperature dependence of derepression anomaly in Y10-6. To test the possibility that this feature is conferred on the double mutant by the

defect in the first enzyme we studied recombinants which had received the wild type allele of the first enzyme. All fourteen recombinants from AB311 Vhf his^+ $\times$ Y10-6 111 (F^-, his_1^-) crosses (see Materials and Methods), selected for histidine prototrophy at 25°C, contained high repressed enzyme levels. Constitutive enzyme levels of the recombinants showed a weaker temperature dependence than those of Y10-6 111 (Fig. 6).

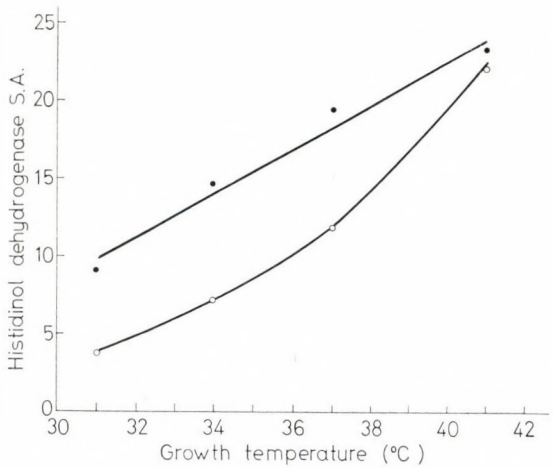

Fig. 6. Effect of temperature on constitutive histidinol dehydrogenase levels in Y10-6 111 and recombinant. Constitutive levels were determined in steady-state cultures grown for 5 generations at the given temperature in the presence of 20 μg/ml L-histidine. —○—○—, Y10-6 111; —●—●—, recombinant

Though it cannot be excluded that temperature dependence of constitutive enzyme levels is a property of the second mutation, it is probable that it is due to repression being more effective at low temperatures. This latter suggestion would explain the effect of temperature on constitutive enzyme levels in Y10-6 111 and recombinants, as well as on repression hypersensitivity in Y10-6.

Mutation in Y10-6

Of the features of Y10-6 the decreased activity and feedback resistance of PR-ATP pyrophosphorylase can be easily reconciled with a single mutation affecting this enzyme (Sheppard, 1964). This in turn explains leaky histidine auxotrophy, thiazolealanine resistance and histidine excretion.

Moreover, it has been described recently that a mutation affecting the feedback sensitivity of this enzyme can alter repressibility of the histidine operon

in S. typhimurium (Kovach et al., 1969). Thus the observed derepression anomaly could possibly be ascribed to the same mutation. This suggestion is in line with the observed effect of 2-thiazolealanine, a ligand of the first enzyme, on the derepression of Y10-6.

To test the hypothesis that all features of Y10-6 are pleiotropic to the mutation affecting PR-ATP pyrophosphorylase, we studied recombinants which had received the wild type allele of this gene.

The Y10-6 features used in the selection of recombinants were:

i) "Cold sensitivity", slow growth at 25°C on minimal medium in the absence of exogenous histidine;

ii) "2-Methylhistidine sensitivity". 2-Methylhistidine represses the histidine operon and inhibits growth of bacteria by replacing histidine in the protein. Wild type bacteria are resistant to its action since they can produce histidine at sufficient rate even when fully repressed. Mutants which, due to some defect in the histidine biosynthetic pathway, must derepress in order to produce histidine normally, are sensitive to repression by 2-methylhistidine. Thus the analogue is useful in detecting such damages.

iii) "Adenine sensitivity". Adenine was observed to cause a nearly absolute histidine requirement at all temperatures. The basis of this effect is not clear, but it seems to be specific for the defect in the first enzyme since 2-thiazolealanine can relieve this inhibition.

The AB311 Vhf strain used transfers the histidine operon about 11 minutes after mating has been started (Taylor, Adelberg, 1960). All recombinants selected for any of the wild type counterparts of the Y10-6 properties were wild type in all other respects (Table 1). These results suggest that a single mutation is responsible for the mutant features.

Table 1

Properties of recombinants from AB311 Vhf× Y10-6 F⁻ crosses

Values in parenthesis indicate number of recombinants studied. $2MH^r$: growth in the presence of 10 μg/ml 2-methylhistidine; $adenine^r$: growth in the presence of 0.5 mM adenine; $cold^r$: growth at 25°C on minimal medium

Selected marker	Nonselected markers		
	$2MH^r$	$adenine^r$	$cold^r$
$2MH^r$	(56)	56	56
$adenine^r$	47	(47)	47
$cold^r$	52	52	(52)

In reversion studies the high rate of reversion to wild type also supports the conclusion that a single mutation is responsible for the Y10-6 phenotype. Out of 56 adenine resistant revertants 37 were wild type in all respects (19 revertants were still sensitive to 2-methylhistidine).

Discussion

The histidine operon is regulated as a unit in Salmonella typhimurium (Ames, Hartman, 1963) and Escherichia coli (Shedlovsky, Magasanik, 1962). Studies on various regulatory mutants have established that histidyl $tRNA^{his}$ (or a derivative of this compound) plays an important role in the regulation of the histidine operon (Roth et al., 1966). It is generally supposed that the corepressor must interact with the product of a regulatory gene in order to exert its control over the histidine operon. However, efforts to identify a true regulatory gene for the histidine system have so far been unsuccessful.

The unique position of the gene encoding the feedback sensitive first enzyme of the histidine biosynthetic pathway (this gene lies adjacent to the operator) suggested a role for this enzyme in regulation (Cline, Bock, 1966).

Studies on various mutants have revealed that the functional state of the feedback site of the first enzyme influences the kinetics of repression (Kovach et al., 1969a). The basis and physiological significance of this alteration are unclear.

The results with the histidine analogue, triazolealanine, might have more direct bearing on the role of this enzyme in the regulation process. It has been found that the false corepressor triazolealanine cannot repress the histidine operon if the feedback site of the first enzyme is altered, though repressibility by histidine is unaffected (Kovach et al., 1969).

In a different approach we looked for regulatory alterations in mutants carrying a feedback insensitive first enzyme.

In mutant Y10-6 of E. coli K12 the alteration of the first enzyme is accompanied by changes in the repression behaviour (Patthy, Dénes, 1970). The mutant fails to derepress when its growth is limited by histidine. This regulatory anomaly is probably due to repression hypersensitivity, since under appropriate conditions it is manifested in regulation of enzyme synthesis at growth rates where growth is strongly limited by histidine (Fig. 2). The regulatory nature of this alteration is also supported by the result that the consequences of this mutation can be suppressed by a secondary regulatory mutation which allows the constitutive synthesis of histidine biosynthetic enzymes (Fig. 5). The regulatory alteration brought about by the mutation of the first enzyme suggests that this enzyme may have an important role in the regulation of the histidine operon. Recently, a specific interaction has been demonstrated in S. typhimurium between the first enzyme and histidyl $tRNA^{his}$ – the most likely candidate for corepressor (Kovach et al., 1970).

The regulatory alteration has important consequences on the growth characteristics of Y10-6. Since the mutant must derepress to compensate for the defect in the activity of the first enzyme, growth of the mutant parallels its ability to derepress. All factors which facilitate synthesis of histidine biosynthetic enzymes (higher temperatures, 2-thiazolealanine, mutation allowing constitutive enzyme synthesis) help histidine production and increase growth rate accordingly. Repression hypersensitivity is most pronounced at low temperatures, therefore

growth of the mutant is cold sensitive. Both thiazolealanine and constitutivity are ineffective at low temperatures in suppressing the increased repression anomaly. Cold sensitivity of growth resembles that arising from feedback hypersensitivity (O'Donovan, Ingraham, 1965). It seems possible that the temperature dependence of repression hypersensitivity reflects a similar temperature dependence of the regulatory interaction.

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Purification and Specificity of a Lytic Enzyme Isolated from B. cereus

(Short Communication)

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(Received May 10, 1971)

Lytic enzyme(s) formed in cultures of B. cereus irradiated by ultraviolet light have been described earlier (Csuzi, 1968). These enzymes are able to dissolve the isolated cell wall of the same and other strains of bacteria (Csuzi, 1970). After irradiation this activity considerably increases in the medium and we have attempted to isolate one or more enzymes from the irradiated culture.

The enzymes degrading bacterial cell wall have been classified by Strominger (1967) into two groups: amidases and glycosidases. We examined to which of these groups belongs the activity found in our preparations.

The lytic activity of the enzyme preparations was measured by a turbidimetric method and expressed in arbitrary units. One unit of enzyme causes a decrease of 0.001 absorbance unit in 20 minutes at 20°C if the reaction mixture consisting of the enzyme and cell wall has an initial absorbance of 0.5 units (Csuzi, 1964).

B. cereus cells were cultured on a casamine medium and lytic enzyme production was induced by ultraviolet irradiation (Csuzi, 1968). Enzyme production led to partial lysis, residual intact cells were harvested and the pellet was resuspended in one tenth of the original supernatant, then sonicated. The solution obtained was recombined with the supernatant and is referred to as "crude extract". This crude extract (2.5 l) was cooled to 0°C and stirred continuously while 2.8 l of cold acetone (−5°C) was slowly added through a capillary tube. The precipitate formed was removed by centrifugation. The enzyme was then precipitated with 2.15 l of cold acetone as before, centrifuged again and the pellet was dissolved in 250 ml of 0.01 M TRIS-HCl buffer (pH 7.8). Thus Fraction I was obtained.

Further purification was achieved by adding 150 ml of acetone to Fraction I. Inactive proteins were precipitated and removed by centrifugation. Another portion of 150 ml acetone precipitated the active fraction of proteins and a yellow substance as well. Separation was carried out by centrifugation between 5−10°C. The evaporation of acetone was prevented by sealing the tubes. A precipitate and a floating yellow substance were obtained. The last two steps have to be performed as quickly as possible, as the lytic activity is very sensitive under these circumstances. Subsequently, the sticky yellow substance was poured out carefully to avoid its being mixed with the loose precipitate. The pellet was dissolved in 25

ml of 0.01 M TRIS-HCl buffer (pH 7.6) and dialysed against the same buffer for 24 hours at 3–5°C. The solution obtained was subjected to vacuum dialysis against a 20% sucrose solution and thus concentrated to 15 ml (Fraction II).

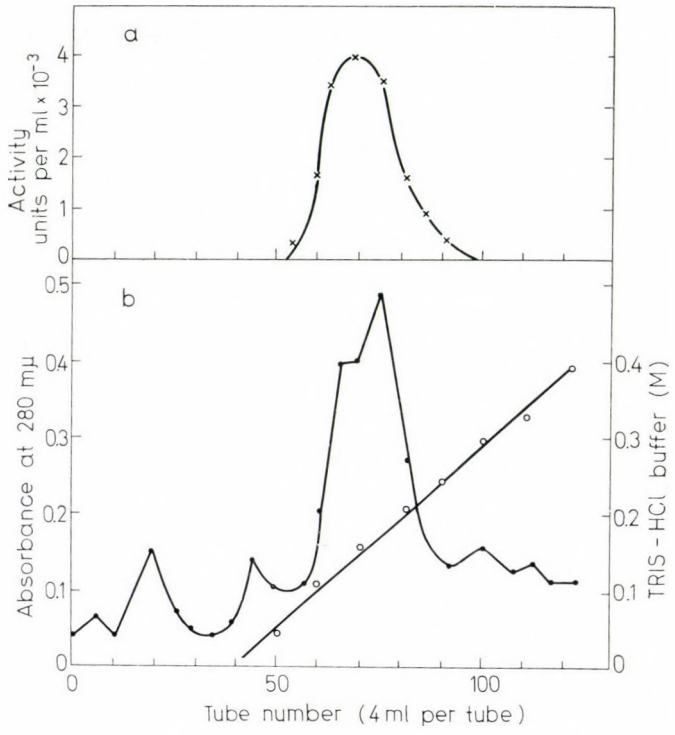

Fig. 1. DEAE-cellulose chromatography of the lytic enzyme *a:* Lytic activity of the eluted fractions. *b:* Absorbance of the proteins and the concentration of the eluting buffer. The DEAE-cellulose column (1.5×20 cm) was equilibrated with 0.01 M TRIS-HCl buffer (pH 7.6). The column was loaded with 45 mg (15 ml) of Fraction II and washed with 50 ml of equilibrating buffer. The elution of the proteins was achieved by increasing the concentration of TRIS-HCl buffer (pH 7.6) ×---× = lytic activity, ●---● = absorbance of the fractions, at 280 mμ, ○---○ = concentration of TRIS-HCl buffer

An aliquot of Fraction II was applied to a DEAE-cellulose column and the proteins were eluted with a TRIS-HCl buffer gradient, pH 7.6 (Fig. 1). Enzyme activity was found in the major protein peak eluted around 0.1 M Cl^- concentration.

The purification procedure is summarized in Table 1. As it can be seen, specific activity increased by a factor of 16.

Table 1

Scheme of purification of the lytic enzyme

Step	Total activity (units) $\times 10^{-3}$	Specific activity (units/mg protein) $\times 10^{-3}$
Crude extract	570	1.1
Fraction I	502	5.3
Fraction II	405	9.0
DEAE-cellulose chromatography	245	16.9*

* Fractions 55 to 80 were pooled and used for activity assay.

In order to obtain more detailed data about the lytic enzyme(s), their glycosidase and amidase activities have been tested. First the crude extract was examined for amidases: the quantity of amino groups was measured (Ghuysen et al., 1966). However, the effect of these enzymes was masked by the great number of intrinsic amino groups. To block these side chains, at least partially, the cell was treated by succinic anhydride (Li, Bertsch, 1960). The quantity of amino groups split off by the crude extract from the CWAM was expressed as DNP-alanine equivalent. As it is shown in Fig. 2 (curve *a*) the highest value of DNP-alanine was measured at 400 minutes and this meant an increase of about 80 μmoles as compared to the initial value. By this time lysis also ended (curve *c*). According to these data, the samples taken at the end of lysis contain the highest amount of amino groups split off by the amidases, as compared with that of the intrinsic amino groups (curve *b*). Therefore these samples were most suitable for N-terminal analysis.

The glycosidase activity of crude extract was measured by Nelson's method (Nelson, 1944) and the amount of reducing groups was expressed in glucose equivalents per g of CW. No increase could be detected in the quantity of reducing groups in the CW digested by the crude extract (curve *d*). If the extract contains small, not detectable amounts of glycosidase it still might become concentrated during purification. However, as shown in Fig. 2 (curve *e*), no increase in the quantity of reducing groups was found with the purified enzyme preparation either.

Earlier data (Csuzi, 1970) suggested that muramyl-alanine amidase activity might be displayed by the purified lytic enzyme. Assuming that only this amidase is present both in the crude extract and the purified enzyme, one would expect the amount of only DNP-alanine to increase in the digest. The N-termial amino acid was detected by paper chromatography. As shown in Table 2 the quantity of DNP-alanine increased from 6.3 to 78 μmoles per g CWAM degraded by the crude extract. Similar result was obtained when the N-terminal amino acids of the CW digested by the purified enzyme were analysed. Moreover, these values are in good agreement with the data of Fig. 2. No free DNP-amino acid could be detected in the ether extract examined by paper chromatography.

The data of Table 2 demonstrate that both in the purified enzyme and in the crude extract amidase activity alone could be found. According to our observation (Csuzi, 1970) carbohydrates and peptides were found among the fragments obtained from the cell wall digested by the purified enzyme. This finding and the fact that only the amount of DNP-alanine increases in the cell wall digested by

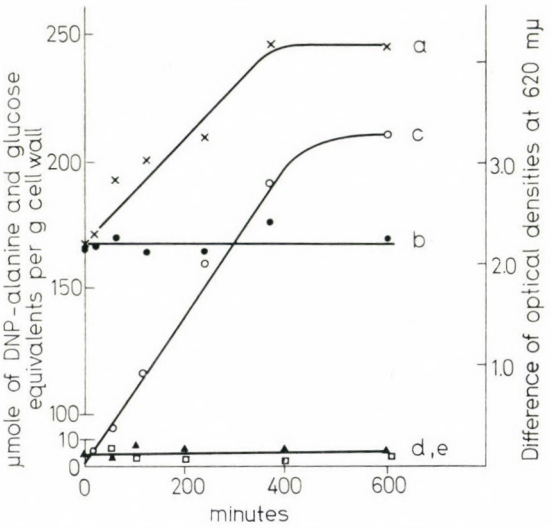

Fig. 2. The quantity of DNP-alanine and glucose equivalent in the cell wall digested by the lytic enzyme. The quantity of free amino groups was measured by the method of Ghuysen et al. (1966) and expressed as DNP-alanine equivalents. The number of intrinsic amino groups in the cell wall was reduced by succinic anhydride treatment as follows. Cell wall (160 mg) obtained by sonication (Csuzi, 1964) was reacted with succinic anhydride (Li, Bertsch, 1960) in 80 ml of 50% (v/v) aqueous methanol. The cell wall obtained was centrifuged and the pellet resuspended in 0.01 M phosphate buffer (pH 7.8). This cell wall preparation is referred to as CWAM. Reaction mixture: *a:* CWAM (4 mg/ml) and crude extract (0.4 mg/ml); reaction mixture *b:* the same as *a* but the crude extract was previously inactivated by incubation in boiling water bath for 5 minutes; reaction mixture *c:* CW (4 mg/ml) and purified lytic enzyme (0.1 mg/ml). Each tube was incubated at room temperature and 1 ml samples were taken at intervals. After addition of FDNB, the samples of *a* and *b* were hydrolyzed and their absorbancy was measured as described by Ghuysen et al. (1966). Reducing groups were measured in samples of *a* and *c* and expressed as glucose equivalents (Nelson, 1944). DNP-alanine equivalents in CWAM digested by crude extract = ×---×; DNP-alanine equivalents in CWAM incubated with heat inactivated crude extract = ●---●; glucose equivalents in CWAM digested by crude extract = △---△ (curve d); glucose equivalents in CW digested by purified enzyme = □---□: optical density differences from the initial value = ○---○

either the crude extract or the purified enzyme are in good agreement. Thus it appears that only one amidase – muramyl-alanine amidase – is responsible for the formation of peptides with alanine N-terminal residue.

Table 2

DNP-amino acids in the cell wall digested by lytic enzyme

Reaction mixture *a:* CWAM (4 mg/ml) was digested by crude extract (0.4 mg/ml) at room temperature. Reaction mixture *b:* CW (4 mg/ml) was degraded by purified lytic enzyme (0.1 mg/ml) at the same temperature as *a*. From both reaction mixtures samples of 1 ml were taken at 0 minute and at the end of lysis (60 and 600 minutes, respectively) (cf. Fig. 2). After addition of FDNB the samples were incubated (Fraenkel-Conrat, Porter, 1952). Excess of FDNB and DNP derivatives were extracted by ether, the ether insoluble substances were hydrolyzed by HCl for 16 hours, subsequently DNP-amino acids were extracted (Fraenkel-Conrat et al., 1955). The substances of the ether extract and DNP-amino acids obtained from the hydrolysate were separated by paper chromatography (Blackburn, Lowther, 1951). The spots of DNP-amino acids were cut out and eluted; the optical density of eluates was read at 360 nm. Control samples of DNP-alanine (0.2 and 0.4 μmoles) were run through the whole procedure to check recovery. Standard losses determined in this way were taken into account in calculating the amount of DNP-alanine

	Time (min)		
	0	60	600
μmoles of DNP-alanine per g CWAM	8.2		78
μmoles of DNP-alanine per g CW	6.4	82	
μmoles of DNP-amino acids in the ether extract of CW and CWAM	0	0	0

Abbreviations: DNP = dinitrophenyl; FDNB = fluorodinitrobenzene; CW = cell wall obtained from B. cereus by sonication; CWAM = cell wall treated with succinic anhydride

The author wishes to thank Prof. F. Antoni for his interest and helpful discussions.

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Thin-Layer Ion-Exchange Chromatography on Resin-Coated Chromatoplates

IV. Determination of Ornithine in Biological Fluids

(Short Communication)

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(Received July 13, 1971)

Detection and identification of aromatic and basic amino acids are very important in the early diagnosis of certain disorders in amino acid metabolism (phenylketonuria, lysinaemia, etc.). Ion-exchange thin-layer chromatography is a simple method ensuring an unambiguous detection of these amino acids (Dévényi, Zoltán, 1970; Dévényi, 1970). Lysine, however, cannot be separated by ion exchange chromatography in sodium citrate buffer (pH 5.23, 0.35 N Na^+) from the eventually present ornithine which may accumulate in significant quantities in the blood and urine. In the present work chromatographic conditions will be described which make possible the separation of all four basic amino acids (Orn, Lys, His, Arg) and aromatic amino acids.

Chromatoplates type Dowex 50X8, size 20 × 20 cm, were used in the Na^+ cycle (Dévényi, Zoltán, 1970). In some of the experiments chromatoplates type Ionex 25 SA (Macherey, Nagel & Co., Düren) were employed.

The plates, the serum and urine samples were prepared by methods described in an earlier communication (Dévényi, 1970).

The buffer used for elution (sodium citrate, pH 4.25, 0.4 N Na^+) had the following composition:

Citric acid · H_2O	14.1 g
Hydrochloric acid, 37% (sp.g. 1.19)	8.4 ml
Sodium hydroxide	8.0 g
Sodium chloride	11.7 g

The solution was made up to 1000 ml with deionized water. The ninhydrin reagent was prepared by the method described in an earlier publication (Dévényi, 1970).

Figure 1 shows the chromatograms of aromatic and basic amino acids and of serum samples from which the protein has been removed by means of trichloroacetic acid. The samples were chromatographed in two steps. The plates were first eluted with 0.01 N hydrochloric acid at 50°C for 3 hours, so that a filter paper

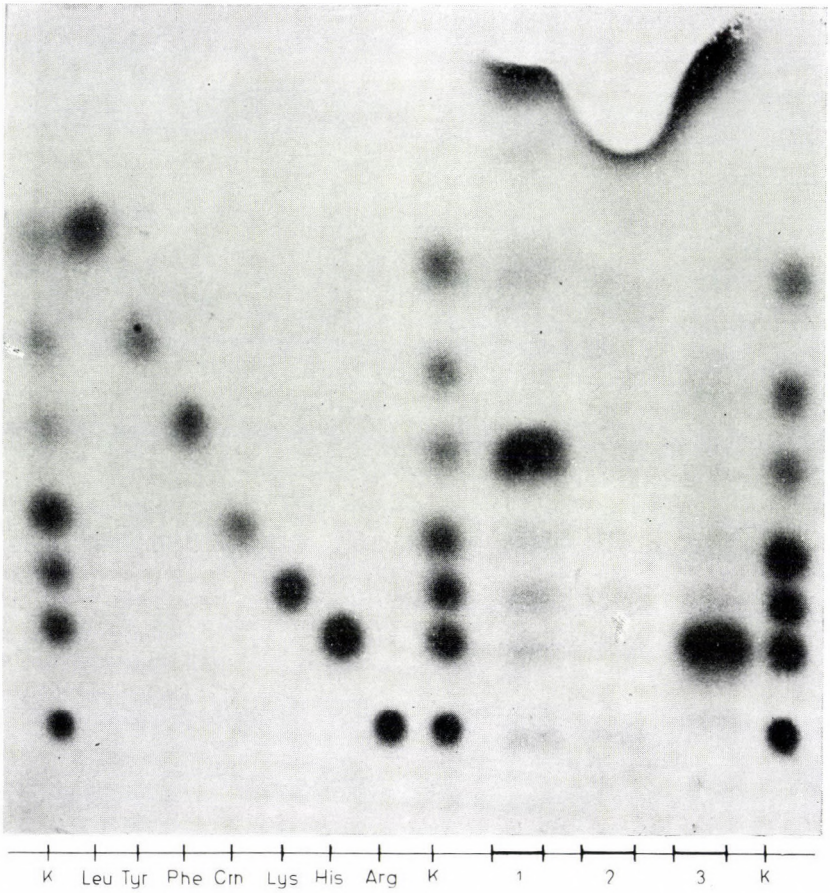

Fig. 1. Detection of ornithine on Dovex 50X8 type chromatoplate. Eluting buffer: sodium citrate, pH 4.25, 0.4 N Na^+. K: amino acid control mixture (in the order of increasing R_f values: Arg, His, Lys, Orn, Phe, Tyr, Leu). 1, 20 μl of a phenylketonuric serum from which the protein has been removed with trichloroacetic acid; 2, 20 μl of a normal serum from wich the protein has been removed with trichloroacetic acid; 3, 20 μl of an ammonia-free urine sample

strip was fixed to the upper edge of the layer (Dévényi, 1970). The plates were then dried with hot air and eluted in the sodium citrate buffer (pH 4.25, 0.4 N Na^+) at 50°C for 4 hours when again a filter paper strip was used to achieve sufficiently high R_f values. After drying with hot air the chromatograms were developed with the ninhydrin spray reagent.

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Thin-Layer Ion-Exchange Chromatography on Resin-Coated Chromatoplates

V. One-Dimensional Separation of Amino Acids

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(Received July 13, 1971)

A method is described for the one-dimensional separation of amino acids by thin-layer ion-exchange chromatography on plates coated with Dowex 50X8 type resin. In a single run all amino acids usually present in an acid hydrolysate can be separated except the Ser-Thr amino acid pair.

A method for the one-dimensional separation of amino acids could be important in structural analysis, clinical diagnosis and peptide chemistry. The one-dimensional separation of amino acids described here is rapid and simple. It might be particularly helpful for the control of reactions which result in changes in amino acid concentrations, e.g. enzymatic digestions by carboxypeptidase and aminopeptidase.

The traditional paper or thin-layer chromatographic methods do not permit the one-dimensional separation of the amino acids usually present in an acid hydrolysate of a protein. More likely, it is necessary to employ one of several two-dimensional methods. However, the thin-layer ion-exchange chromatographic method employing resin-coated chromatoplates, seems to be suitable for the one-dimensional separation of amino acids.

Materials and methods

Resin-coated chromatoplates

Chromatoplates coated with Dowex 50X8 type resin were used in the Na^+ form (Dévényi, Zoltán, 1970). In some of our experiments chromatoplates Ionex-25 SA (Macherey, Nagel & Co., Düren, Germany) were employed. Prior to use the plates were equilibrated for 24 hours with sodium citrate buffer, pH = 3.2, Na^+ = 0.004 N, as described earlier (Dévényi, 1970).

Preparation of the eluting buffer

Citric acid monohydrate	84.0 g
Sodium hydroxide	16.0 g
Hydrochloric acid, 37%, sp.gr. 1.19	5.9 ml

made up to 1000 ml with deionized water; pH = 3.3; Na^+ = 0.4 N

* Present address: Chinoin–Nagytétény, Budapest.

Ninhydrin spray reagent

1% Ninhydrin dissolved in acetone containing 10% collidine was employed.

Results and discussion

For the one-dimensional separation of the amino acids usually present in an acid hydrolysate a single run with sodium citrate pH = 3.3, Na^+ = 0.4 N and citrate = 0.4 M was found to be suitable. Fig. 1 shows a chromatogram obtained on Dowex 50X8 type resin-coated chromatoplate with this buffer at 50°C.

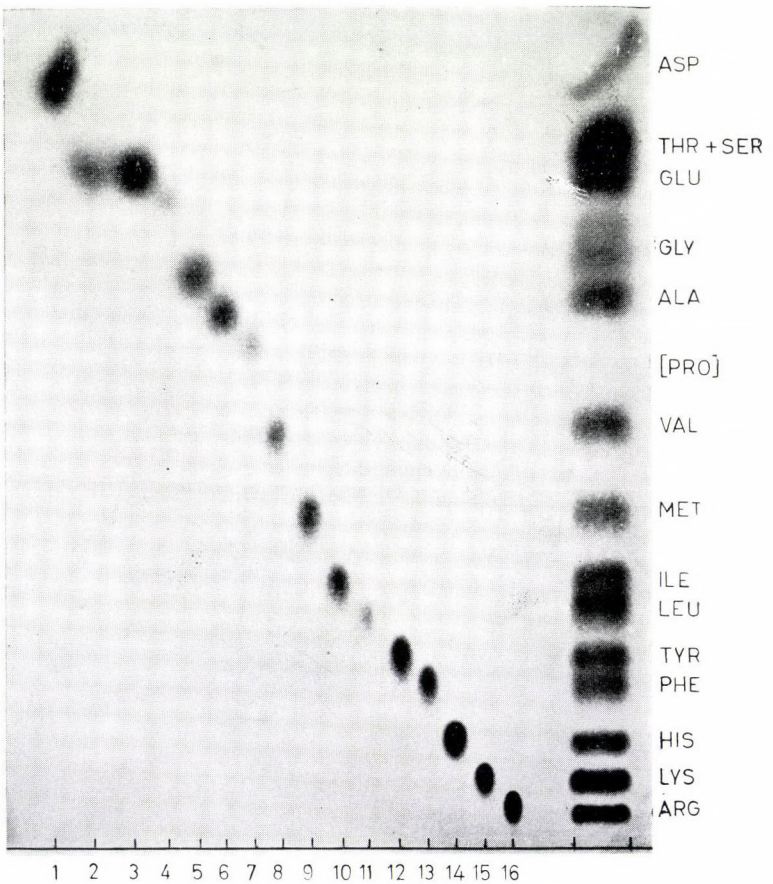

Fig. 1. One-dimensional separation of amino acids. Dowex 50X8 type resin-coated chromatoplate. Eluting buffer: sodium citrate pH = 3.3, 0.4 N Na^+, 0.4 M citrate, 50°C. 1 = Asp, 2 = Thr, 3 = Ser, 4 = Glu, 5 = Gly, 6 = Ala, 7 = Pro, 8 = Val, 9 = Met, 10 = Ile, 11 = Leu, 12 = Tyr, 13 = Phe, 14 = His, 15 = Lys, 16 = Arg

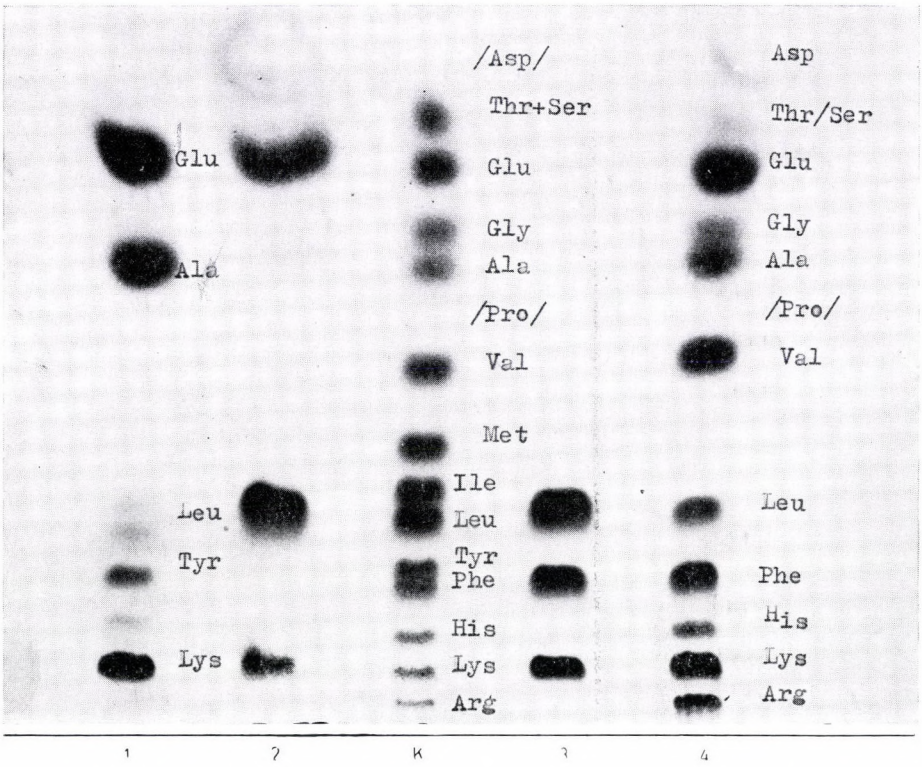

Fig. 2. One-dimensional separation of acid hydrolysates of peptides. Experimental conditions as in Fig. 1. 1 and 2, peptides isolated from the tryptic hydrolysate of rabbit muscle aldolase; 3, synthetic tripeptide Phe.Lys.Leu; 4, performic acid oxidized synthetic ACTH 1—25

Fig. 1 shows that the sequence of the separated amino acids is very similar to that which is found in amino acid analysis with the analyzer technique. Also it can be seen from Fig. 1 that only serine and threonine cannot be separated with the buffer employed, whereas the other amino acids each form a single, well-defined band or spot.

If the molar ratios of the amino acids present in the sample are close together (the maximal ratio is around 1 : 5), the experimental conditions described in Fig. 1 can be used without any difficulties.

Fig. 2 shows the one-dimensional separation of the acid hydrolysates of some peptides. The experimental conditions are the same as in Fig. 1. As it can be seen, the one-dimensional separation of amino acids gives satisfactory results even in the case of synthetic ACTH, which is composed of 25 amino acid residues.

This one-dimensional separation of amino acids on Dowex 50X8 type resin-coated chromatoplates seems to be a simple tool in protein chemistry and biochemistry for the rapid detection of amino acids. In addition to the analysis

of hydrolysates and enzymatic digests it can be used for the study of biological fluids also. In this case the deproteinized sample (sera, urine, liquor) should be extracted with ether to free it from trichloroacetic acid employed for deproteinization.

The authors wish to thank Professor F. B. Straub for his help, criticism and interest during the elaboration of the method.

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Thin-Layer Ion-Exchange Chromatography on Resin-Coated Chromatoplates

VI. Rapid Estimation of Cysteic Acid

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(Received July 13, 1971)

A simple method is described for the detection of cysteic acid by the use of Dowex 2X8 type resin-coated chromatoplates.

In fodders and plant materials among the essential amino acids an important role is played by cystine the determination of which is difficult owing to its decomposition under the conditions of acidic hydrolysis. By oxidation with performic acid cystine can be transformed into cysteic acid, which is stable during acidic hydrolysis. Cysteic acid can be readily separated from all other amino acids by thin-layer ion-exchange chromatography applying Dowex 2X8 type resin-coated chromatoplates. On the anion-exchanger plate cysteic acid is very strongly bound: its R_f value is the lowest among the amino acids. Thus relatively small amounts of cysteic acid can be detected by overloading the chromatoplate in the presence of a large excess of other amino acids.

Materials and methods

Resin-coated chromatoplates

Chromatoplates coated with Dowex 2X8 type resin were used in the CH_3COO^- form (Dévényi, Zoltán, 1970). In some of our experiments chromatoplates Ionex-25 SB (Macherey, Nagel & Co., Düren, Germany) were employed. The plates were equilibrated with 0.01 M acetic acid for 24 hours by continuous developing as described earlier (Dévényi, 1970).

Preparation of eluting buffer

Pyridine	10 ml
Glacial acetic acid	100 ml

made up to 1000 ml with deionized water; pH 3.8

Ninhydrin spray reagent was prepared as described in an earlier communication (Dévényi, 1970).

Oxidation and hydrolysis of samples

Samples were oxidized with performic acid (1 ml of 30% hydrogen peroxide + 9 ml formic acid, sp.gr. 1.22). The reagent was freshly prepared before use.

The air-dry sample (50 mg) in a pulverized state was mixed with 2 ml of performic acid. Oxidation was allowed to proceed for 2 hours at room temperature, then the samples were freeze-dried.

The samples were hydrolyzed in 6 N hydrochloric acid in sealed tubes at 105°C for 48 hours. After hydrolysis the acid was removed in vacuo and the dry material was redissolved in deionized water to give a final protein concentration of 5 mg/ml and filtered.

Results and discussion

Fig. 1 shows the chromatogram of the acid hydrolysate of fodder-pea samples oxidized with performic acid. The anion exchanger plate (in acetate form) was run in pyridine–acetic acid buffer, pH 3.8, up to 15 cm front height. The duration of the run was about 60 min.

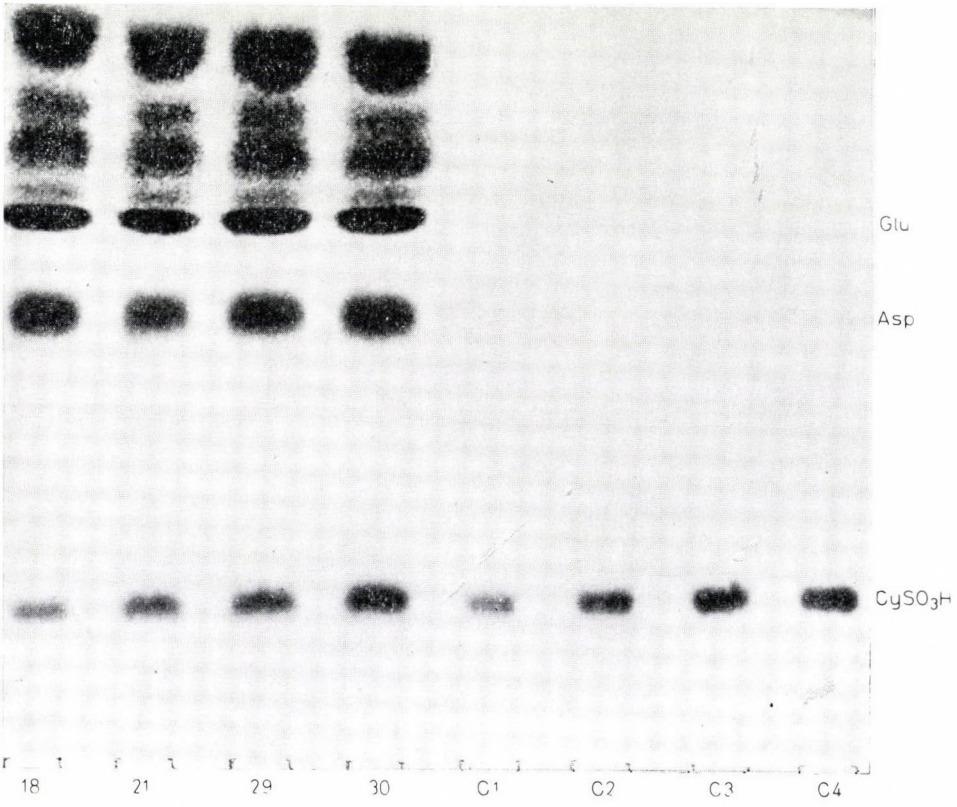

Fig. 1. Rapid estimation of cysteic acid content of various fodder-pea samples on Dowex 2X8 type resin-coated chromatoplates. 18, 21, 29, 30: fodder-pea hydrolysates (cf. the text). C1, C2, C3, C4: cysteic acid controls containing 0.5, 1.0, 1.5 and 2.0 μg of cysteic acid, respectively. Eluting buffer: pyridine–acetic acid, pH 3.8

Samples of the same protein content were applied to the plate (20 μl, 0.1 mg of protein per 1 cm). The amount of cysteic acid, run as a control, has to be chosen to fall between the maximal and minimal amounts to be expected on the basis of the protein quantity applied. Considering the average cystine content of fodder-pea, in the present experiment the amounts of cysteic acid controls (C1, C2, C3, C4) were 0.5, 1.0, 1.5 and 2.0 μg, respectively (i.e. 0.5, 1.0, 1.5, 2.0% calculated on a crude protein basis). Samples of unknown cysteic acid content can thus be selected by comparing them to the controls visually or densitometrically in a semi-quantitative manner. By this preliminary screening samples can be selected the quantitative analysis of which seems worthwhile.

It is conspicuous on simple inspection that the cysteic acid content of sample No. 18 (Vigna sinensis L.) is about one half that of sample No. 30 (Pisum sativum L.). By quantitative analysis the cysteic acid content of samples 18 and 30 proved to be 1.0 and 2.4%, respectively. The cysteic acid content of samples No. 21 (Cicer ariethinum G-172) and No. 29 (Pisum sativum L.) in accordance with quantitative analyses (1.4 and 1.8%, respectively), falls between these two values.

Due to its simplicity and rapidity the method is suitable to the simultaneous testing of a great number of samples, which could considerably reduce the number of quantitative analyses.

The method is also suitable for the rapid detection of acidic components in peptide chemistry and sequence analysis.

The authors wish to thank Mrs Judit Báti for the performance of the amino acid analyses. We should also like to thank Professor E. Kurnik and Mrs Szánthó (Research Institute of Forage Corps.) for the seed samples.

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Enzymatic Degradation of Peptides Containing α-Aminooxycarboxylic Acids

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Tripeptide heterologs containing α-aminooxycarboxylic acids were synthetized and their enzymatic degradation by carboxypeptidase, aminopeptidase, trypsin and chymotrypsin was investigated. The resistance of aminooxy-heterologs to enzymatic hydrolysis is similar to that of D-amino acids.

In earlier studies α-aminooxycarboxylic acid derivatives showed a pronounced tuberculostatic activity (Kisfaludy et al., 1969, 1971). In connection with this work it was of interest to investigate the susceptibility of the $-CO-NH-O-$ bond against different proteolytic enzymes. The tripeptide Phe-Lys-Leu was chosen as a model peptide in which the Phe and Leu residues were replaced by OPhe and OLeu. Therefore the peptides Phe-Lys-Leu (LLL), Phe-Lys-Leu (DLL) and their heterologs OPhe-Lys-Leu (LLL), OPhe-Lys-Leu (DLL), Phe-Lys-OLeu (LLL) and Phe-Lys-OLeu (DLL) were synthetized and subjected to enzymatic hydrolysis.

Materials and methods

Thin-layer chromatography was carried out on Merck's chromatoplates (DC Plastikfolie, Kieselgel F 254) with solvent systems (1) chloroform : methanol = 9 : 1, (2) EtOAc : (pyridine : acetic acid : water, 20 : 6 : 11) = 95 : 5, (3) EtOAc : (pyridine : acetic acid : water, 20 : 6 : 11) = 9 : 1, (4) EtOAc : (pyridine : acetic acid : water, 20 : 6 : 11) = 6 : 4. All melting points are uncorrected.

1. *L-Phenylalanyl-L-lysyl-L-leucine*

1a. *Methyl N^{α}-benzyloxycarbonyl-N^{ε}-t-butyloxycarbonyl-L-lysyl-L-leucinate.* Z-Lys(BOC)-OSu (9.6 g; 20 mmoles) was added to a suspension of Leu-OCH_3 · HCl (3.8 g; 21 mmoles) in DMF (15 ml) containing triethylamine (2.95 ml; 21 mmoles). After standing for 4 hours the reaction mixture was diluted with 200 ml of 8% $NaHCO_3$ solution. The precipitated oil crystallized in a few minutes. The crude material was recrystallized from EtOAc-petroleum ether. Yield: 8.0 g (79%); m.p.: 112–114°C; R_f^2 0.79.

1b. *N-benzyloxycarbonyl-N-t-butyloxycarbonyl-L-lysyl-leucine.* Z-Lys(BOC)-Leu-OCH_3 (8.0 g; 15.8 mmoles) was dissolved in methanol (70 ml) and then 1 N NaOH (23 ml) was added. After standing for 1 hour at room temperature 1 N HCl (23 ml) was added to the solution and the methanol was removed by distil-

lation. EtOAc (140 ml) was added to the residue and the solution obtained was extracted with 3×70 ml of 8% $NaHCO_3$ solution and 3×70 ml of water. The combined washings were acidified (pH 3) with citric acid. The precipitated oil was extracted with EtOAc (150 ml), and after washing with water the solution was evaporated to dryness. The oily residue solidified by triturating with petroleum ether. Yield: 6.1 g (78.5%); R_f^1 0.38; R_f^2 0.40; R_f^3 0.57.

1c. *N^{ε}-t-butyloxycarbonyl-L-lysyl-L-leucine.* Z-Lys(BOC)-Leu (6.1 g; 12.4 mmoles) was hydrogenated over 10% Pd charcoal in 80% acetic acid (140 ml). After completion of the reaction the catalyst was removed by filtration and the solution was evaporated to dryness. The oil obtained was first solidified with ether, then crystallized with boiling methanol (40 ml). Yield: 2.73 g (61%); R_f^4 0.46.

1d. *N-t-butyloxycarbonyl-L-phenylalanyl-(N^{ε}-t-butyloxycarbonyl)-L-lysyl-L-leucine.* To a suspension of Lys(BOC)-Leu (1.8 g; 5 mmoles) in DMF (20 ml) triethylamine (0.7 ml) and BOC-Phe-OSu (1.55 g; 4.3 mmoles) were added. After strirring for two hours the homogeneous mixture was kept overnight at room temperature, then the solvent was evaporated. The remaining substance was dissolved in a mixture of EtOAc (50 ml) and 1 N HCl (20 ml) and the organic phase was washed with 1 N HCl and water, then dried. Evaporation of the solvent gave an oil which solidified by triturating with petroleum ether. Yield: 1.95 g (75%); R_f^1 0.38; R_f^2 0.50; R_f^3 0.57.

1e. *L-phenylalanyl-L-lysyl-L-leucine acetate.* BOC-Phe-Lys(BOC)-Leu (0.30 g; 0.5 mmoles) was dissolved with shaking in trifluoroacetic acid (1.5 ml). After standing for 20 min, the solution was diluted with dry ether (20 ml) and the precipitate was filtered and washed with dry ether. The tripeptide trifluoroacetate (0.25 g) was dissolved in water (2 ml) and the solution was passed through a column of Dowex 1X8 (5 ml, acetate cycle), then the column was washed with 20 ml of water. Lyophilization of the eluate gave amorphous Phe-Lys-Leu. Yield: 0.16 g; R_f^4 0.44. Amino acid ratios in acid hydrolysate: Phe 0.92; Lys 1.04; Leu 1.00.

2. *D-phenylalanyl-L-lysyl-L-leucine*

2a. *Benzyloxycarbonyl-D-phenylalanyl-N^{ε}-t-butyloxycarbonyl-L-lysyl-L-leucine.* Z-D-Phe-OSu (0.55 g; 1.4 mmoles) was added to a suspension of Lys(BOC)-Leu (0.5 g; 1.4 mmoles) in DMF containing triethylamine (0.195 ml; 1.4 mmoles). After stirring for 2 hours the solvent was evaporated and the residue was dissolved in a mixture of EtOAc (30 ml) and water (20 ml). The organic phase was washed with 1 N HCl and water. Evaporation of the solvent gave a resinous residue. Yield: 0.65 g (73%); R_f^3 0.60.

2b. *D-phenylalanyl-N^{ε}-t-butyloxycarbonyl-L-lysyl-L-leucine.* Z-Phe-Lys(BOC)-Leu (DLL) (0.65 ; 1.02 mmoles) was hydrogenated in 80% acetic acid (20 ml) in the presence of 10% Pd charcoal (0.3 g). After completion of the reaction the catalyst was removed by filtration and the solvent was evaporated. The solid residue was triturated with ether, then crystallized by treatment with boiling 96% ethanol (10 ml). Yield: 0.4 g (78%); m.p.: 215–217°C; R_f^4 0.46.

2c. *D-phenylalanyl-L-lysyl-L-leucine di-trifluoroacetate.* Phe-Lys(BOC)-Leu (DLL) (0.3 g; 0.59 mmoles) was dissolved in 1.2 ml of trifluoroacetic acid. After

30 min dry ether (2 ml) was added to the reaction mixture, then the precipitate was filtered and washed with dry ether. Chromatographically homogeneous Phe-Lys-Leu was obtained in the form of di-trifluoroacetate. Yield: 0.37 g (97%); R_f^4 0.05. Amino acid ratios in acid hydrolysate: Phe 1.00; Lys 1.07; Leu 1.00.

3. *L-α-aminooxy-β-phenylpropionyl-L-lysyl-L-leucine*

3a. *N-t-butyloxycarbonyl-L-α-aminooxy-β-phenylpropionyl-N^{ε}-t-butyloxycarbonyl-L-lysyl-L-leucine.* BOC-OPhe-OSu (0.6 g; 4.3 mmoles) was reacted with Lys(BOC)-Leu (1.9 g; 5 mmoles) in DMF (20 ml) containing triethylamine (0.7 ml; 5 mmoles) as described in 1d. The oil obtained (2.4 g) was dissolved in ether, cyclohexylamine (0.5 ml) was added to the ethereal solution and the crude salt was recrystallized twice from EtOAc. Distribution of the purified salt (1.7 g; m.p.: 161–163°C) between ether (50 ml) and 1 N H_2SO_4 (20 ml) gave the free acid, which was obtained as a crystalline substance after evaporation of the solvent. The product was then recrystallized from EtOAc-petroleum ether. Yield: 1.2 g; R_f^3 0.54. Anal. calculated from $C_{31}H_{50}N_4O_9$ (622.74) C 59.8; H 8.1; N 9.0. Found C 59.9; H 9.1; N 8.7.

3b. *L-α-aminooxy-β-phenylpropionyl-L-lysyl-L-leucine dihydrochloride.* BOC-OPhe-Lys(BOC)-Leu (LLL) (0.75 g; 1.2 mmoles) was dissolved in EtOAc (3 ml) by mild heating and 5.5 N HCl in EtOAc (2 ml) was added to the solution. After a few minutes white powder precipitated from the solution which, after diluting the reaction mixture with dry ether, was filtered and dried in vacuo. OPhe-Lys-Leu (0.35 g; 59%) was obtained in the form of dihydrochloride. M.p.: decomp. at 115°C, melts at 204°C. R_f^4 0.27. Amino acid ratios in acid hydrolysate: Lys 1.22; Leu 1.00.

4. *D-α-aminooxy-β-phenylpropionyl-L-lysyl-L-leucine*

4a. *N-t-Butyloxycarbonyl-D-α-aminooxy-β-phenylpropionyl-N^{ε}-t-butyloxycarbonyl-L-lysyl-L-leucine.* BOC-D-OPhe-OSu (1.62 g; 4.3 mmoles) was reacted with Lys(BOC)-Leu (1.8 g; 5 mmoles) in DMF (20 ml) containing triethylamine (0.7 ml; 5 mmoles) as described in 1d. The oily product (2.1 g) was dissolved in dry ether and dicyclohexylamine (0.8 ml) was added to the solution. The crude product was recrystallized twice from EtOAc. Yield 1.6 g (60%); m.p.: 169–170°C. Anal. calculated for $C_{43}H_{73}N_5O_9$ (804.05) C 64.3; H 9.1; N 8.7. Found C 64.3; H 9.1; N 9.0. The dicyclohexylamine salt (1.1 g) was dissolved by vigorous shaking in a mixture of ether (50 ml) and 1 N H_2SO_4 (20 ml). The solution was washed with water, dried and evaporated to dryness. After recrystallization of the residue from EtOAc-petroleum ether 0.70 g (38%) product was obtained. M.p.: 68–92°C. R_f^1 0.36; R_f^2 0.40; R_f^3 0.55.

4b. *D-α-Aminooxy-β-phenylpropionyl-L-lysyl-L-leucine dihydrochloride.* BOC-OPhe-Lys(BOC)-Leu (DLL) (0.55 g; 0.88 mmoles) was dissolved in EtOAc (5 ml) and to the solution 5.5 N HCl in EtOAc was added. The procedure is the same as described in 3b. Yield: 0.3 g (69%); m.p.: 188°C (decomp.); R_f^4 0.24. Amino acid ratios in acid hydrolysate: Lys 1.16; Leu 1.00.

5. *L-Phenylalanyl-L-lysyl-L-α-aminooxy-γ-methylvaleric acid*

5a. *N^{α}-benzyloxycarbonyl-N^{ε}-t-butyloxycarbonyl-L-lysyl-L-α-aminooxy-γ-methylvaleric acid.* Z-Lys(BOC)-OSu (2.38 g; 5 mmoles) was added to a suspension of OLeu. HBr (1.11 g; 5 mmoles) in methylene chloride (10 ml) containing triethylamine (0.7 ml; 5 mmoles). After stirring for 5 min the solution became homogeneous. The reaction mixture was allowed to stand at room temperature for 20 hours. After evaporating the solvent the residue was dissolved in a mixture of EtOAc (30 ml) and water (20 ml) and the organic layer was washed twice with 1 N HCl and water. Evaporation of the solvent gave chromatographically homogeneous oil. Yield: 2.5 g (98%); R_f^1 0.29; R_f^2 0.38; R_f^3 0.50.

5b. *N^{ε}-t-butyloxycarbonyl-L-lysyl-L-α-aminooxy-γ-methylvaleric acid.* Z-Lys(BOC)-OLeu (2.5 g; 4.9 mmoles) was dissolved in 80% acetic acid (50 ml) and hydrogenated in the presence of 10% Pd charcoal (1.0 g). After the completion of the reaction (controlled by thin-layer chromatography) the catalyst was removed by filtration and the filtrate was evaporated to dryness. The crystalline residue was triturated with ether, filtered and dried. Yield: 1.65 g (90%); m.p.: 163–165°C. R_f^4 0.35.

5c. *t-Butyloxycarbonyl-L-phenylalanyl-N^{ε}-t-butyloxycarbonyl-L-lysyl-L-α-aminooxy-γ-methylvaleric acid.* BOC-Phe-OSu (0.77 g; 2.12 mmoles) was added to a solution of Lys(BOC)-OLeu (0.8 g ; 2.13 mmoles) and triethylamine (0.295 ml; 2.12 mmoles) in DMF (5 ml). The reaction mixture was kept at room temperature for 20 hours. The solvent was then evaporated and the residue was dissolved in a mixture of EtOAc (30 ml) and 1 N HCl (10 ml). The organic phase was washed with 1 N HCl and water, then evaporated to dryness. The resinous material obtained was purified in the form of dicyclohexylamine salt (1.15 g; m.p.: 136–139°C). The salt was distributed between ether (30 ml) and 1 N H_2SO_4 (20 ml) and the ethereal phase was washed with water, dried and evaporated. The amorphous residue was crystallized from EtOAc-petroleum ether. Yield: 0.83 (63%); m.p.: 105–111°C; R_f^1 0.27; R_f^2 0.35; R_f^3 0.50. Anal. calculated from $C_{31}H_{50}N_4O_9$ (622.74) C 59.8; H 8.1; N 9.0. Found C 59.8; H 8.1; N 9.4.

5d. *L-phenylalanyl-L-lysyl-L-α-aminooxy-γ-methylvaleric acid dihydrochloride.* BOC-Phe-Lys(BOC)-OLeu (LLL) (0.6 g; 0.96 mmoles) was dissolved in 2.95 ml EtOAc and to the solution 3.4 N HCl in EtOAc (3.05 ml) was added. After 5 min white powder precipitated from the solution. To complete the separation of the product, after 1 hour dry ether was added to the reaction mixture and the product was filtered off. Yield: 1.15 g (97%) m.p.: 210°C (decomp.); R_f^4 0.05; Amino acid ratios in acid hydrolysate: Phe 1.00; Lys 1.07.

6. *L-Phenylalanyl-L-lysyl-D-α-aminooxy-γ-methylvaleric acid*

6a. *N^{α}-benzyloxycarbonyl-N^{ε}-t-butyloxycarbonyl-L-lysyl-D-α-aminooxy-γ-methylvaleric acid.* The procedure described in 5a was used with the following materials: D-OLeu (1.14 g; 5 mmoles); Z-Lys(BOC)-OSu (2.38 g; 5 mmoles); triethylamine (0.7 ml; 5 mmoles) and methylene chloride (10 ml). The oily crude product (2.4 g) was converted into dicyclohexylamine salt and recrystallized twice from

EtOAc. Yield: 2.15 g (62.5%); m.p.: 142–143°C; R_f^3 0.53. Anal. calculated for $C_{37}H_{62}N_4O_8$ (690.90) C 64.3; H 9.1; N 8.1. Found C 64.7; H 9.3; N 8.2.

6b. *N^{ε}-t-butyloxycarbonyl-L-lysyl-D-α-aminooxy-γ-methylvaleric acid.* The dicyclohexylamine salt of Z-Lys(BOC)-OLeu (LD), described in 6a (2.15 g; 3.12 mmoles) was converted into free acid and was hydrogenated in 80% acetic acid (20 ml) in the presence of 10% Pd charcoal. After completion of the reaction (controlled by thin-layer chromatography) the catalyst was removed by filtration and the solution evaporated to dryness. The residue was solidified by triturating with dry ether. Yield: 0.97 g (82%); R_f^1 0.34.

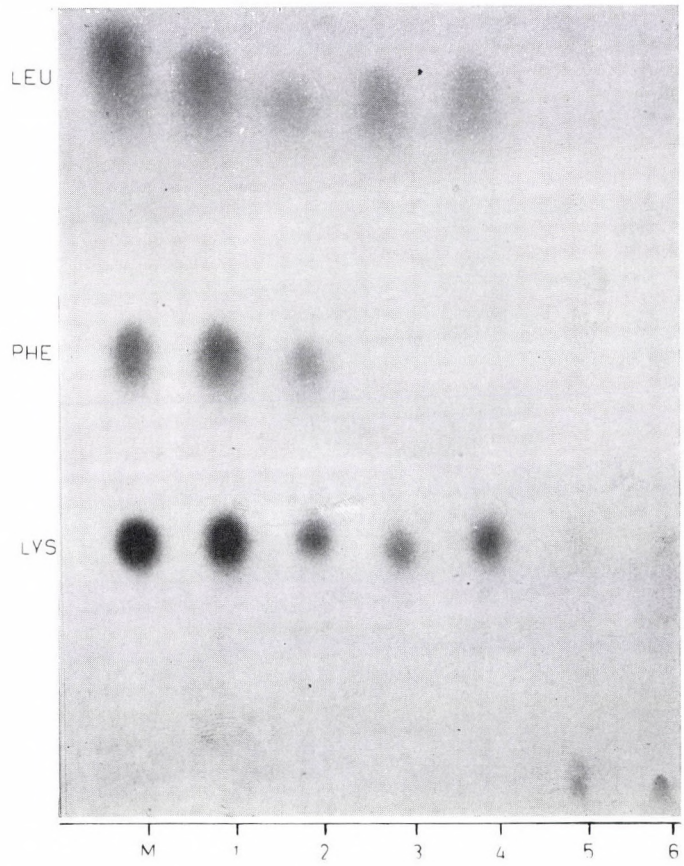
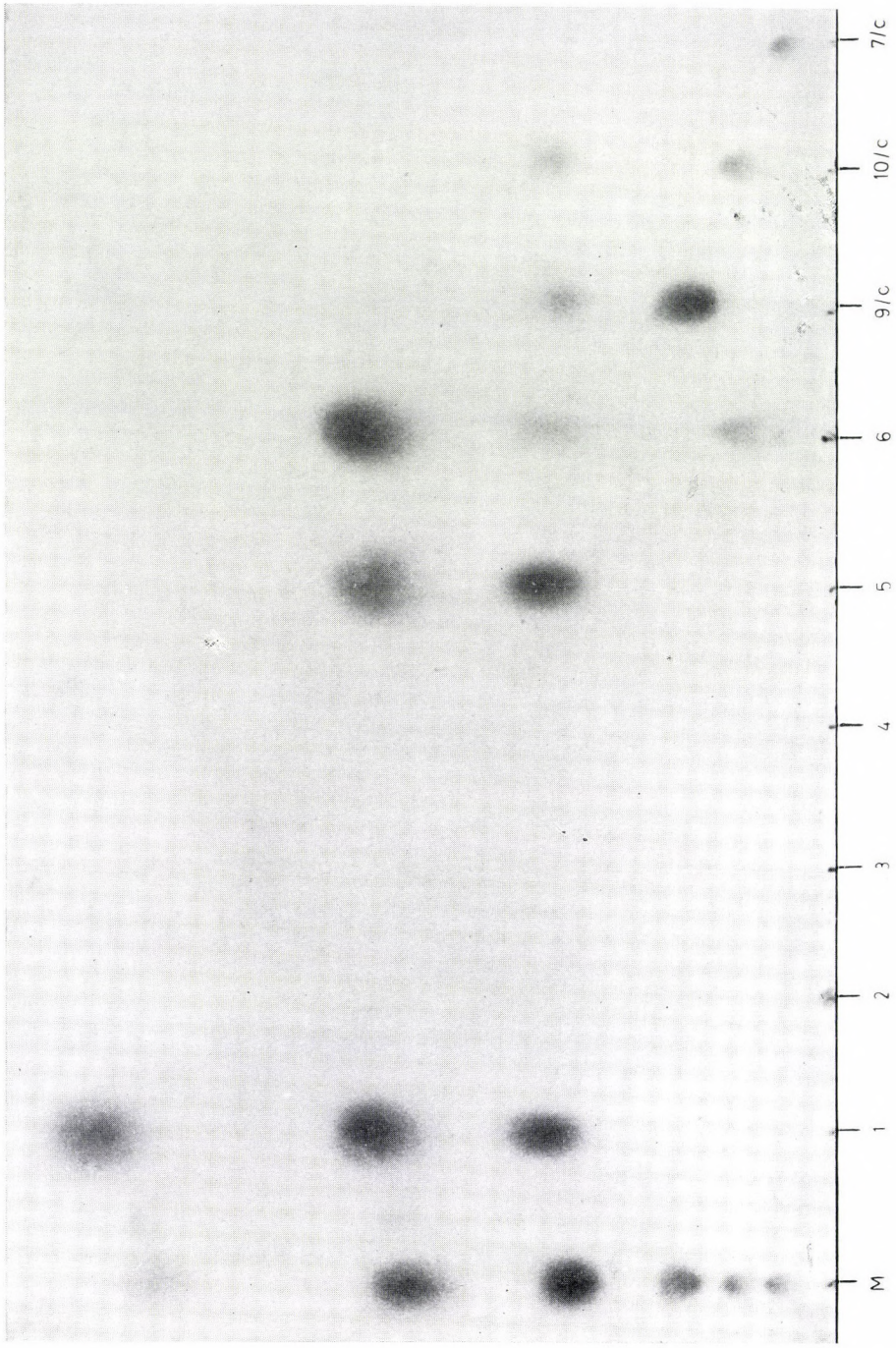


Fig. 1. Digestion of tripeptides with a mixture of carboxypeptidase A and B.
M = control amino acids

1 = Phe-Lys-Leu (LLL)
2 = Phe-Lys-Leu (DLL)
3 = OPhe-Lys-Leu (LLL)
4 = OPhe-Lys-Leu (DLL)
5 = Phe-Lys-OLeu (LLL)
6 = Phe-Lys-OLeu (LLD)

Eluting buffer: sodium citrate pH = 5.23; 0.35 N Na^+


7/c
10/c
9/c
6
5
4
3
2
1
M

6c. *N-t-butyloxycarbonyl-L-phenylalanyl-N^{ε}-t-butyloxycarbonyl-L-lysyl-D-α-aminooxy-γ-methylvaleric acid.* Lys(BOC)-OLeu (LD) (1.9 g; 5.07 mmoles) was pissolved in DMF (10 ml) containing triethylamine (0.7 ml; 5 mmol) and to the solution BOC-Phe-OSu (1.8 g; 4.96 mmoles) was added. The reaction mixture was allowed to stand at room temperature for 20 hours, then the solvent was evaporated. The residue obtained was distributed between EtOAc (50 ml) and 1 N HCl (20 ml). The organic phase was washed with 1 N HCl and water and after drying it was evaporated to dryness. The solid residue was crystallized from EtOAc-petroleum ether. Yield: 2.1 g (68%); m.p.: 103–106°C; R_f^1 0.29; R_f^2 0.35; R_f^3 0.49; Anal. calculated for $C_{31}H_{50}N_4O_9$ (622.74) C 59.8; H 8.1; N 9.0. Found C 59.5; H 8.0; N 9.0.

6d. *L-Phenylalanyl-L-lysyl-D-α-aminooxy-γ-methylvaleric acid dihydrochloride.* BOC-Phe-Lys(BOC)-OLeu (LLD) (1.5 g; 2.4 mmoles) was dissolved in EtOAc (7.3 ml) and to the solution 3.4 N HCl in EtOAc (7.7 ml) was added. Within a few minutes white powder precipitated from the solution. After standing for 1 hour the reaction mixture was diluted with dry ether and the product was filtered off. Yield: 1.15 g (97%); m.p.: 210°C (decomp.); R_f^4 0.04. Amino acid ratios in acid hydrolysate: Phe 1.00; Lys 1.04.

Dowex 50X8 type resin-coated chromatoplates

Strong cation exchanger chromatoplates (Dowex 50X8 type) 20×20 cm in size were used in Na^+ cycle, as described earlier (Dévényi, Zoltán, 1970). In some of our experiments chromatoplates Ionex-25 SA (Macherey, Nagel & Co., Düren) were used.

Enzymatic hydrolysis and chromatography

Carboxypeptidase A DFP (Sigma), carboxypeptidase B (Sigma), aminopeptidase M (Röhm and Haas), trypsin (Calbiochem, B grade, essentially free of chymotrypsin with PTCK), chymotrypsin (Calbiochem, A grade) were applied.

5 mg peptide sample was dissolved in 4 ml 0.1 M NH_4HCO_3 and 0.1 mg enzyme was added to the solution. In the case of carboxypeptidase digestion a mixture of A and B enzymes was applied. The hydrolysis was carried out at 37°C for 24 or 72 hours.

Fig. 2. Digestion of tripeptides with aminopeptidase (chromatogram of the 24-hour hydrolysate)

1 = Phe-Lys-Leu (LLL)
2 = Phe-Lys-Leu (DLL)
3 = OPhe-Lys-Leu (LLL)
4 = OPhe-Lys-Leu (DLL)
5 = Phe-Lys-OLeu (LLL)
6 = Phe-Lys-OLeu (LLD)
9/c = Lys-OLeu (LL)
10/c = Lys-OLeu (LD)
7/c = Lys-Leu
M = control amino acids and peptides

Eluting buffer: sodium citrate pH = 4.25; 0.4 N Na^+

Five microliter aliquots were used for the thin-layer ion-exchange chromatography. The hydrolysate could be directly applied to the strong cation exchanger plates under hot air, as the predominant part of ammonium hydrocarbonate sublimated and did not disturb the chromatography. Sodium citrate pH = 5.23, 0.35 N Na^+ (Dévényi, 1970) and sodium citrate pH = 4.25, 0.4 N Na^+ (Hrabák, Ferenczi, 1971) buffers were used for the chromatography.

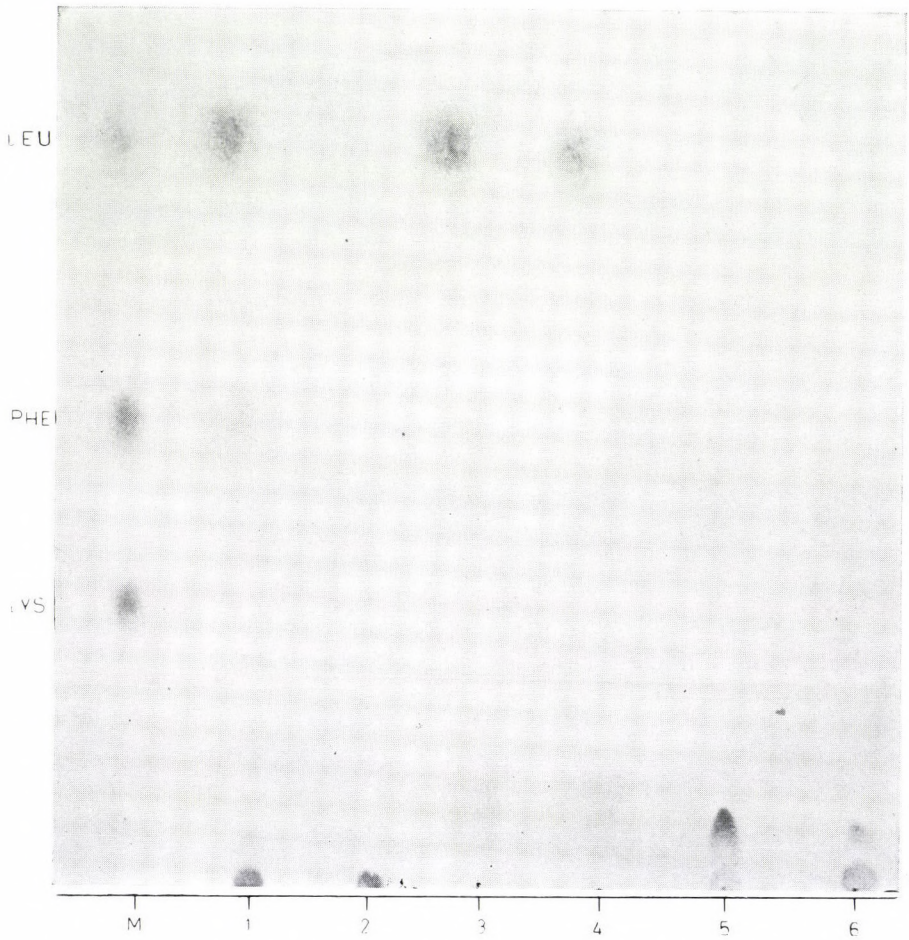


Fig. 3. Chromatogram of tryptic hydrolysates of tripeptides

1 = Phe-Lys-Leu (LLL)
2 = Phe-Lys-Leu (DLL)
3 = OPhe-Lys-Leu (LLL)
4 = OPhe-Lys-Leu (DLL)
5 = Phe-Lys-OLeu (LLL)
6 = Phe-Lys-OLeu (LLD)

Eluting buffer: sodium citrate pH = 5.23; 0.35 N Na^+

Results and discussion

The enzymatic digestibility of the peptides obtained in the above described manner was investigated with carboxypeptidase (mixture of A and B), aminopeptidase, trypsin and chymotrypsin. The digestibility was measured after the completion of the reaction (after 24 hour-, or in the case of aminopeptidase 72 hour-digestion). The composition of the mixture was analyzed by thin-layer ion-exchange chromatography.

Fig. 1 shows the chromatogram of the hydrolysates of peptides obtained by digestion with carboxypeptidase (mixture of A and B). The chromatogram was obtained with sodium citrate pH = 5.23, 0.35 N Na^+ buffer. As can be seen the Phe-Lys-Leu (LLL), Phe-Lys-Leu (DLL) and both of their OPhe analogues proved to be readily digestible with carboxypeptidase A + B. Their digestibility is demonstrated by the equal ratios of lysine and leucine (samples 3 and 4 in Fig. 1),

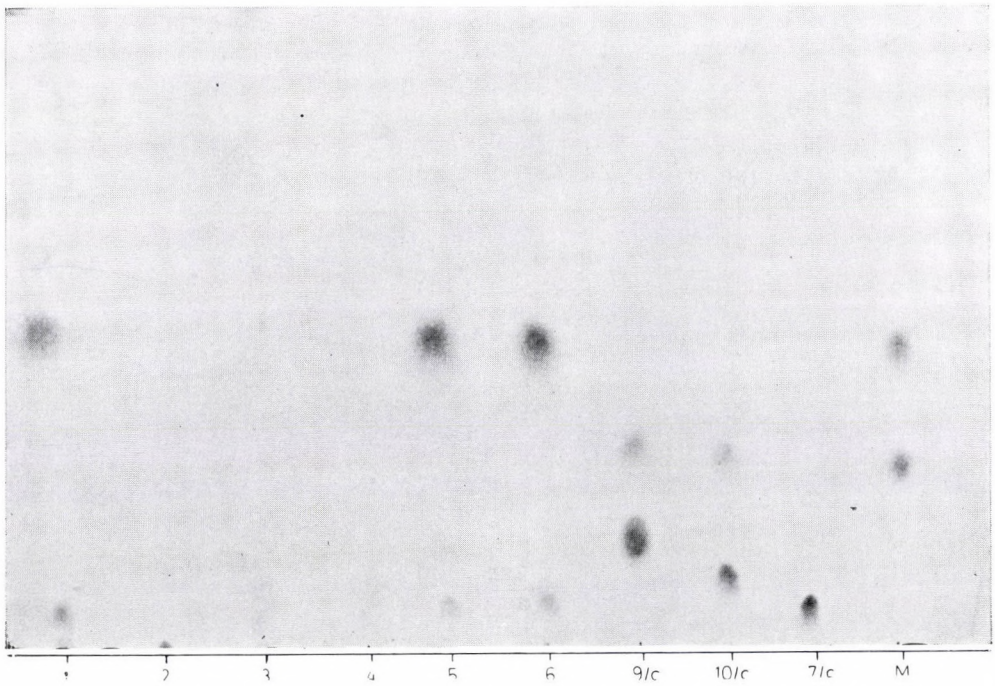


Fig. 4. Chromatogram of the chymotryptic hydrolysates of tripeptides

1 =	Phe-Lys-Leu (LLL)	5 =	Phe-Lys-OLeu (LLL)
2 =	Phe-Lys-Leu (DLL)	6 =	Phe-Lys-OLeu (LLD)
3 =	OPhe-Lys-Leu (LLL)	9/c =	Lys-OLeu (LL)
4 =	OPhe-Lys-Leu (DLL)	10/c =	Lys-OLeu (LD)
		7/c =	Lys-Leu

Eluting buffer: sodium citrate pH = 4.25; 0.4 N Na^+

whereas the aminooxy analogue of phenylalanine cannot be detected with ninhydrin.

Fig. 2 shows the chromatogram of the hydrolysate of the peptides obtained by aminopeptidase. It can be seen from the chromatogram that the Phe-Lys-Leu tripeptide (sample 1) is readily digestible, while the DLL form and both of the OPhe analogues are completely resistant to the action of aminopeptidase (samples 2, 3 and 4). The Phe-Lys-OLeu analogue (sample 5) is readily digestible since the amount of phenylalanine and lysine is equal in the hydrolysate. The Phe-Lys-OLeu (LLD) analogue (sample 6) could only be partially hydrolysed since beside the N-terminal phenylalanine only the heterodipeptide Lys-OLeu (LD) was detectable (sample 6).

Fig. 3 shows the chromatogram of the tryptic hydrolysate of the peptides and analogues. Tryptic digestion was detected in the case of Phe-Lys-Leu (LLL) and of both OPhe analogues (samples 1, 3 and 4). Phe-Lys-Leu (LLD) and both OLeu analogues are resistant to tryptic digestion (samples 2, 3 and 6).

Fig. 4 shows the chromatogram of the chymotryptic hydrolysis. Phe-Lys-Leu (LLL) and both OLeu analogues (samples 1, 5 and 6) are readily digested by chymotrypsin, which is demonstrated by the presence of phenylalanine in the hydrolysates. The Phe-Lys-Leu (DLL) and both OPhe analogues are resistant to chymotrypsin (samples 2, 3 and 4).

The peptide bonds which proved to be resistant to enzymatic hydrolysis are summarized in Table 1.

Table 1

Peptide bonds resistant to enzymatic hydrolysis

Carboxypeptidase A + B	Phe-Lys-OLeu	(LLL)
	Phe-Lys-OLeu	(LLD)
Aminopeptidase	Phe-Lys-Leu	(DLL)
	OPhe-Lys-Leu	(LLL)
	OPhe-Lys-Leu	(DLL)
	Phe-Lys-OLeu	(LLD)
Trypsin	Phe-Lys-Leu	(DLL)
	Phe-Lys-OLeu	(LLL)
	Phe-Lys-OLeu	(LLD)
Chymotrypsin	Phe-Lys-Leu	(DLL)
	OPhe-Lys-Leu	(LLL)
	OPhe-Lys-Leu	(DLL)

From the data of Table 1 it appears that with respect to their resistance to hydrolysis the aminooxy derivatives are similar to the peptides containing D-amino acids.

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Abbreviations: Designations of amino acids, peptides and their derivatives are abbreviated as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature in July 1965 and July 1966; Biochemistry (1967) *5* 2485. The symbols OPhe, OLeu mean the α-aminooxy analogues of Phe and Leu, i.e. OPhe = α-aminooxy-β-phenylpropionic acid, $H_2N—O—CH(CH_2C_6H_5)$ COOH; OLeu = α-aminooxy-γ-methylvaleric acid, $H_2N—O—CH(CH_2CH)CH_2CH(CH_3)COOH$. All amino acid and aminooxy acid residues are of L-configuration if not stated otherwise. Further abbreviations: Z = benzyloxycarbonyl, BOC = t-butyloxycarbonyl, OSu = N-succinimidoxy, DMF = dimethylformamide, EtOAc = ethylacetate.

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Susceptibility of Human γG Immunoglobulins to Tryptic Fragmentation

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Tryptic digests of normal human γG globulins were resolved into three fractions by gel filtration. The first fraction contained a fragment with a molecular weight intermediate between that of fragments Fab and Fc and the native γG globulins. The second fraction contained fragments similar to, but not identical with fragments Fab and Fc (called tFab and tFc). The third fraction was composed of two fragments, one antigenically related to Fc (tFc'), the other related to the Fab part of the protein (tFab'). Antigenic deficiency of tFc' compared with Fc was demonstrated. Experiments carried out on γG myeloma proteins revealed marked differences between the four heavy chain subclasses.

Introduction

Human γG globulins were found to be heterogeneous with respect to sensitivity of papain proteolysis (Gergely et al., 1967). The portion of γG globulins split into fragments Fab and Fc without additional cysteine in the digestion mixture is called papain-sensitive, the other, cleaved only in the presence of a certain concentration of -SH compound is called papain-resistant. The papain-sensitive or papain-resistant character is related to subclass-heterogeneity as revealed by studies on γG myeloma proteins (Rivat et al., 1967; Jefferis et al., 1968; Gergely et al., 1970).

Our present investigations are concerned with the tryptic fragmentation of human γG globulins. The fragments obtained from normal and myeloma γG globulins have been characterized by comparison with the well known fragments produced by papain-hydrolysis. Human γG globulins were found heterogeneous with respect to their trypsin sensitivity and the relationship between tryptic susceptibility and subclass specificity was investigated.

Materials and methods

Normal γG globulins were isolated by the DEAE Sephadex batch technique (Baumstark et al., 1964). Myeloma γG globulins were separated by DEAE Sephadex column chromatography (Sela et al., 1963). To eliminate transferrin, DEAE Sephadex chromatography was preceded by filtration through a Sephadex G-200 column when the protein to be isolated had a high anodic mobility.

Typing of the myeloma proteins for subclass specificity and Gm factors was kindly performed by Dr H. H. Fudenberg (Section of Hematology and Immunology, Department of Medicine, University of California School of Medicine, San Francisco) and Dr Erna von Loghem (Central Laboratory of the Nether-

lands Red Cross Blood Transfusion Service, Amsterdam) as described recently (Gergely et al., 1970).

Hydrolysis with trypsin (twice crystallized, SIGMA) was carried out in 0.1 M Tris-HCl buffer, pH 8.0, containing 0.02 M $CaCl_2$ at an enzyme to protein ratio of 1 : 50 (w/w). When indicated the mixture contained also 0.01 M cysteine or 0.1 M 2-mercaptoethanol. Digestion was continued for 16 hours and terminated by the addition of soybean trypsin inhibitor at a trypsin to inhibitor ratio of 1 : 2 (w/w).

Papain hydrolysis was performed as described by Gergely et al. (1967). The protein was dissolved in 0.075 M phosphate buffer, pH 7.0, containing 0.075 M NaCl. EDTA was added at a final concentration of 0.002 M. Enzyme to protein ratio was 1 : 100. When indicated the mixture contained 0.01 M cysteine or 0.1 M 2-mercaptoethanol, too. Digestion was continued for 4 hours and stopped by addition of iodoacetamide at a final concentration of 0.01 M or at a concentration twice as high as that of the -SH compound present in the mixture.

The digests were analyzed by immunoelectrophoresis using either a rabbit anti human globulin serum precipitating all the Fab, Fc and Fc' fragments, or specific swine anti Fab and anti Fc + Fc' sera obtained from the SEVAC Institute, Prague.

Tryptic digests were fractionated by filtration through a 2.9×100 cm Sephadex G-75 column in 0.25 M NH_4HCO_3 at a flow rate of 40 ml/hr. Fractions were concentrated by freeze-drying or by negative pressure dialysis.

Results

1. *Tryptic fragmentation of normal human γG globulins*

Filtration of tryptic digests of normal human γG globulins through Sephadex G-75 resulted in three fractions, containing proteins precipitable by anti γG globulin sera. The fractions were analyzed by immunoelectrophoresis (Fig. 1) and the molecular weight of each fraction was estimated by thin layer gel filtration (Fig. 2 and Table 1).

Table 1

Molecular weight values of fractions of trypsin-digested normal human γG globulins as estimated by thin layer gel filtration

Molecular weight estimation was carried out by the use of a log M.wt. versus migration distance plot employing proteins with known molecular weight as standards

Fraction	Hydrolysis	
	without cysteine M.wt.	with 0.01 M cysteine M.wt.
I	92 000	88 000
II	52 000	48 000
III	28 000	27 000

The main component of fraction I was a fragment with a molecular weight intermediate between those of the intact protein and fragment Fab or Fc. This fragment gave only one precipitation line against anti human globulin serum. It was precipitated by both anti Fab and anti Fc sera (Fig. 1).

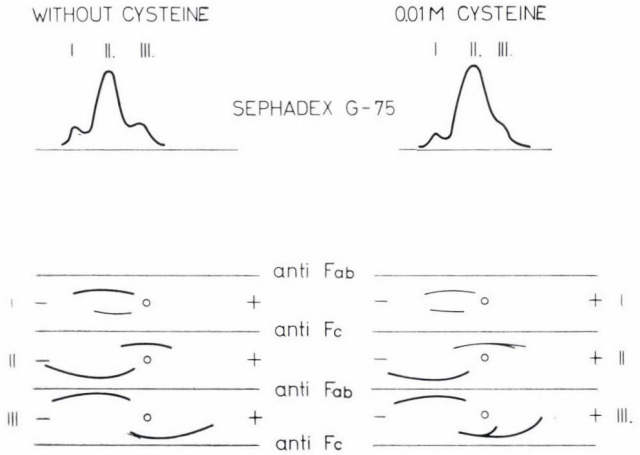

Fig. 1. Gel filtration of tryptic digests of normal human γG globulins and immunoelectrophoretic analysis of the fractions (schematic representation)

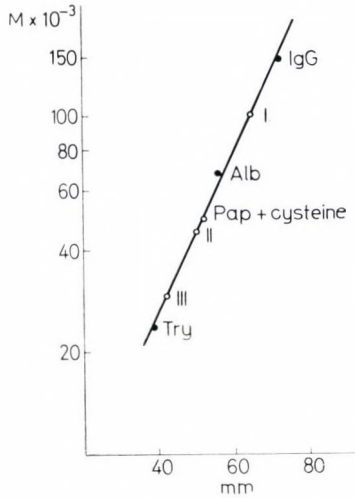

Fig. 2. Molecular weight versus migration distance plot of the three fractions obtained by gel filtration of a tryptic digest of normal human γG globulins. Thin layer gel filtration was performed on Sephadex G-150 (Superfine) layer. IgG, normal human γG globulins; Alb, human albumin; Pap + cysteine, normal human γG globulins digested by papain in the presence of 0.01 M cysteine; Try, trypsin

Estimation of the molecular weight of fraction II yielded a figure near the molecular weight of fragments Fab and Fc. Immunoelectrophoresis revealed two components. the slower was precipitable by anti Fab but not by anti Fc, while the faster one was precipitable by anti Fc, but not by anti Fab serum. Hereafter the slower, Fab-related fragment is termed tFab, while the fast Fc related one is termed tFc. Both tFab and tFc fragments were antigenically identical with

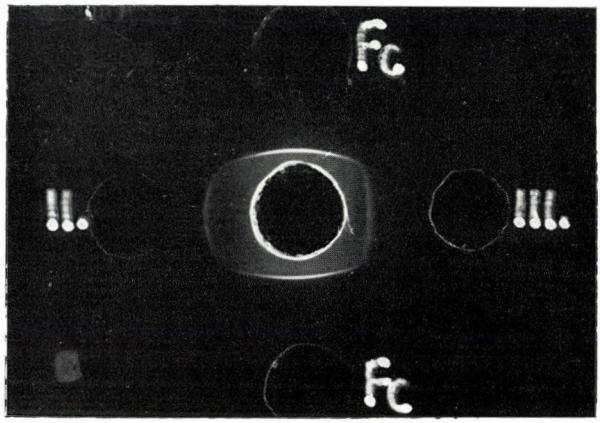

Fig. 3. Comparative immunodiffusion analysis of fractions II and III of a tryptic digest of normal human γG globulins. Antiserum: swine anti Fc + Fc'

Table 2

Molecular weight values of fractions of trypsin-digested myeloma γG globulins as estimated by thin layer gel filtration

Molecular weight estimation was carried out by the use of a log M.wt. versus migration distance plot employing proteins with known molecular weight as standards

Protein	Hydrolysis	
	without cysteine M.wt. $\times 10^{-3}$	with 0.01 M cysteine M.wt. $\times 10^{-3}$
Su, G1, Gm(4)	90; 51; 28	48; 27
Kgy, G1, Gm(4)	92; 52; 27	42; 27
Se, G1, Gm(4)	89; 45; 26	42; 24
Kof, G1, Gm(1, 2)	102; 48; 29	45; 26
Ba, G1, Gm(1, 2)	98; 48; 28	48; 28
Jas, G2, Gm(23)	140; 105; 44	47
Wih, G2, Gm(−23)	150; 98; 48	50*
Pa, G3, Gm(5)	56	52
Pre, G4	102; 49	45
66 G4	100; 47	47

* Hydrolysis was carried out in the presence of 0.1 M 2-mercaptoethanol.

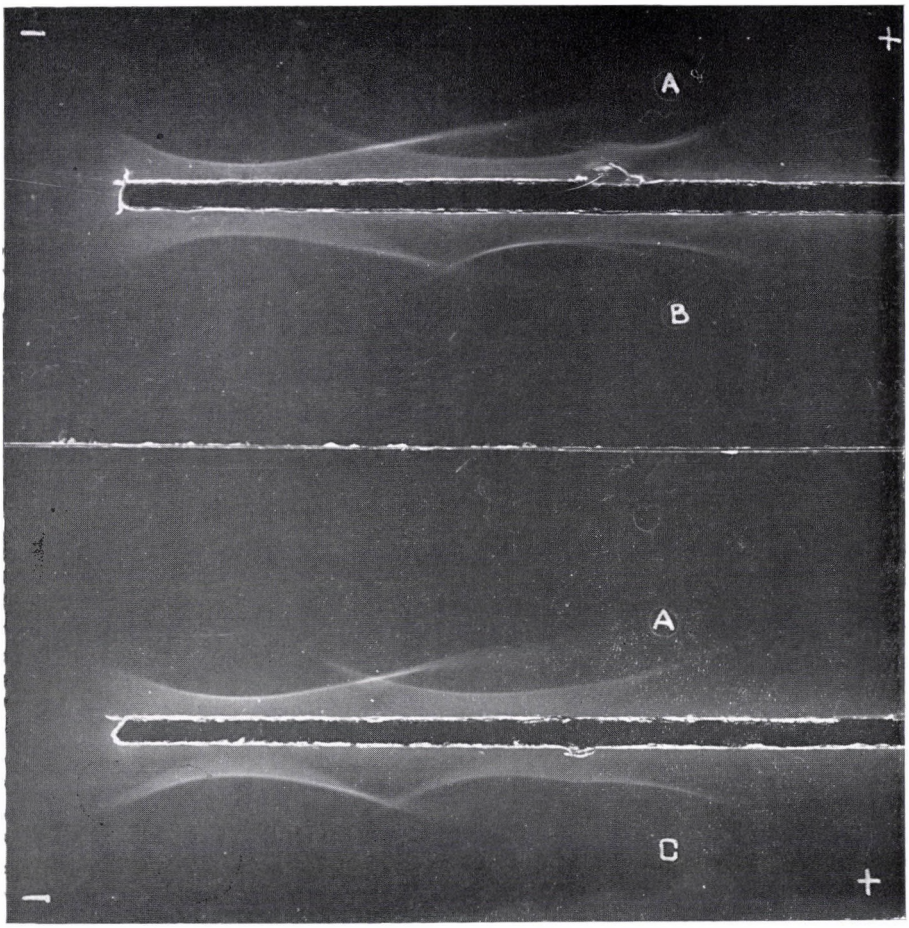

Fig. 4. Immunoelectrophoresis of tryptic digests of γG3 myeloma protein Pa. A, hydrolysis with trypsin without -SH compound; B, hydrolysis with trypsin in the presence of 0.01 M cysteine; C, hydrolysis with papain in the presence of 0.01 M cysteine (20 minutes)

the corresponding fragment obtained by papain proteolysis, i.e. Fab and Fc, respectively.

Like fraction II, fraction III contained two components precipitable by anti human globulin serum; the slower is related to fragment Fab, the faster to fragment Fc. The estimated molecular weight of this fraction is much lower than that of fraction II and of the papain-produced fragments Fab and Fc. These fragments are termed therefore tFab' and tFc', respectively. Double diffusion tests revealed antigenic deficiency of fraction III compared with Fc (Fig. 3).

The presence of 0.01 M cysteine in the digestion mixture led to a decrease of the amount of fraction I and to an increase in the amount of fraction II. Each fraction contained fragments antigenically identical with the corresponding fraction of the digest obtained in the absence of cysteine (Fig. 1).

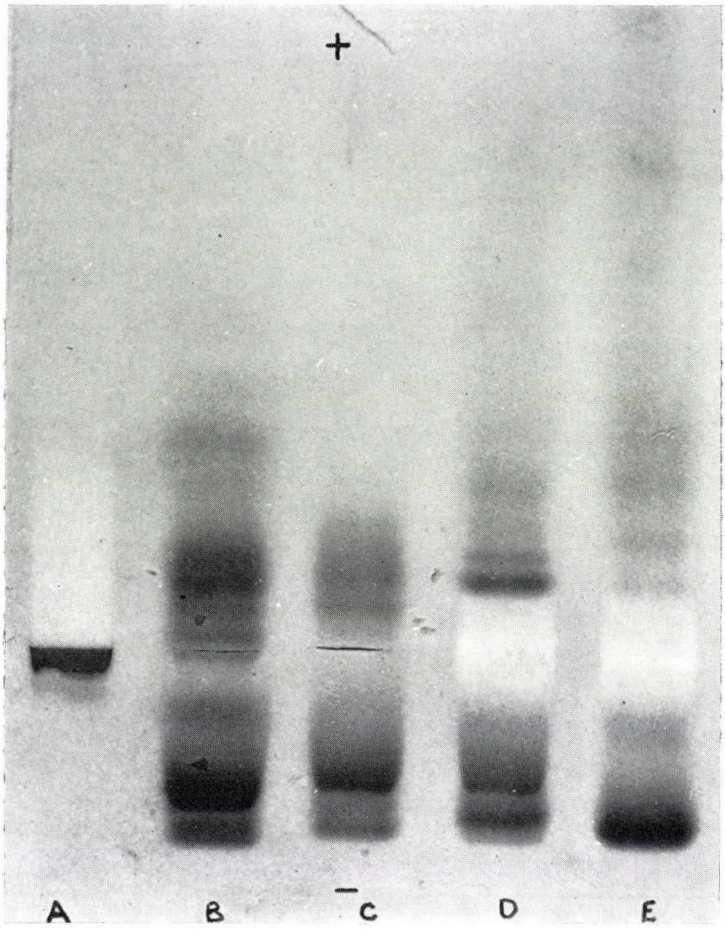

Fig. 5. Starch gel electrophoresis (Tris-borate buffer, pH 8.6) of γG3 myeloma protein Pa and its digests. A, untreated protein; B, digested with papain without cysteine; C, digested with papain in the presence of 0.01 M cysteine; D, digested with trypsin; E, digested with trypsin in the presence of 0.01 M cysteine

2. *Tryptic fragmentation of γG myeloma proteins*

Tryptic digests of ten γG myeloma proteins were analyzed. Their isotypic and allotypic characteristics are listed in Table 2.

The γG1 proteins were regularly cleaved into tFab, tFc, tFab' and tFc' fragments by trypsin without adding any -SH compound to the digestion mixture. A small amount of fragments larger than tFab and tFc was also isolated by gel filtration. These fragments were found to possess antigenic determinants of both fragments Fab and Fc like fraction I of the trypic digest of normal γG globulins.

The γG3 protein investigated was degraded into fragments with molecular weights near those of the papain produced Fab and Fc fragments. The digests gave two precipitation lines when examined in immunoelectrophoresis, an Fab-related slower and an Fc-related faster one. After digestion in the presence of 0.01 M cysteine a splitting of the Fc line was observed (Fig. 4). Comparison by starch gel electrophoresis of tryptic digests obtained in the absence and in the presence of cysteine revealed marked differences (Fig. 5).

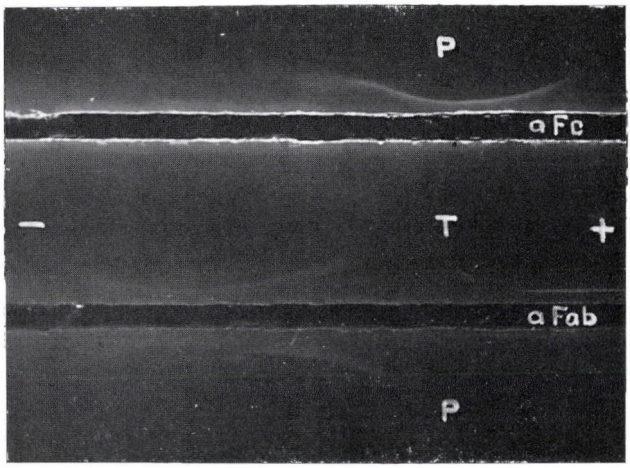

Fig. 6. Immunoelectrophoresis of tryptic (T) and papain + cysteine (P) digests of G4 myeloma protein Pre using swine anti Fc + Fc' (aFc) and anti Fab (aFab) sera

Immunoelectrophoresis of tryptic digests of γG4 myeloma proteins against anti human globulin serum resulted in a single precipitation band. This band was also revealed by anti Fab, but not by anti Fc serum (Fig. 6). Thin layer gel filtration revealed two components when hydrolysis was performed without cysteine, one with a molecular weight close to that of fragments Fab and Fc and a heavier one. Only the lighter component was present in the digests obtained in the presence of 0.01 M cysteine. A faint precipitation line indicating an Fc-related fragment was detected after 10–20 minutes of digestion. At that time the majority of the protein was still unsplit. After one hour of digestion no component reacting with anti Fc serum was observed.

Digestion of γG2 globulins in the absence of -SH compounds left most of the protein unsplit. A small amount of a fragment resembling Fc was formed, together with a heavy fragment precipitable by anti Fab serum (Fig. 7). Splitting one of the γG2 globulins studied (Jas) into fragments tFab and tFc was observed when digested in the presence of 0.01 M cysteine, while the other (Wih) was cleaved only in the presence of 0.1 M 2-mercaptoethanol. Likewise, protein Jas was split into fragments Fab and Fc by papain in the presence of 0.01 M cysteine, while the presence of 0.1 M 2-mercaptoethanol was necessary for the cleavage of protein Wih.

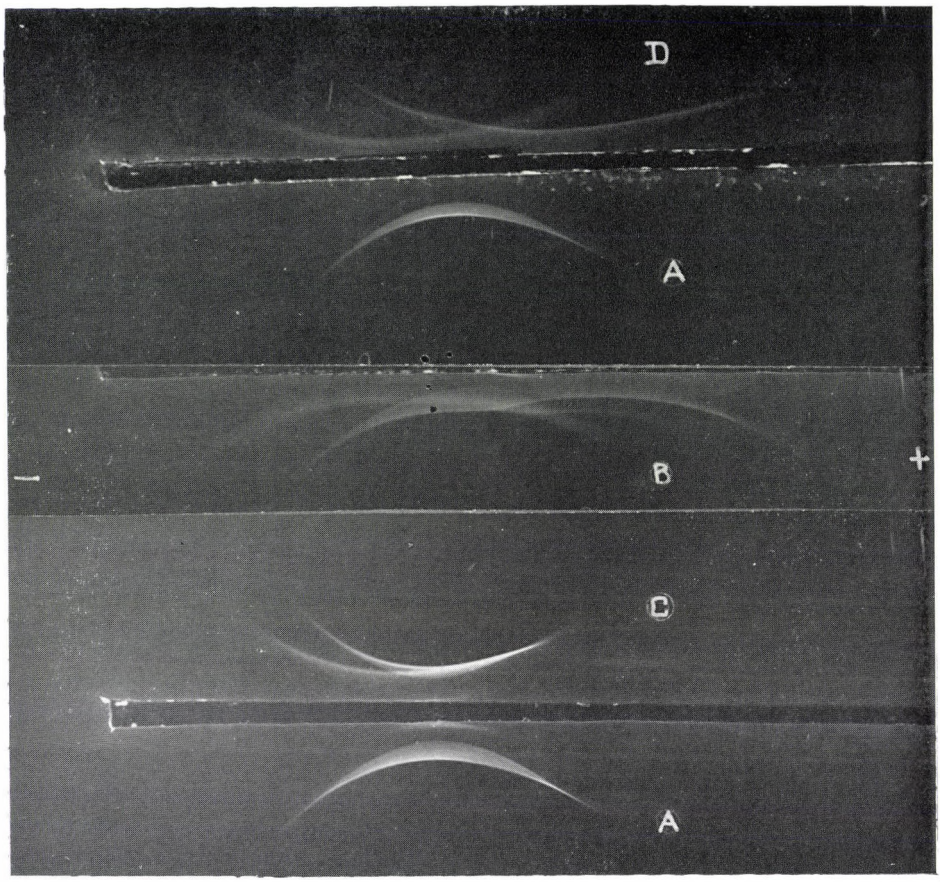

Fig. 7. Immunoelectrophoresis of tryptic and papain digests of γ G2 myeloma protein Wih. A, untreated protein; B, tryptic digest obtained in the presence of 0.1 M 2-mercaptoethanol; C, tryptic digest obtained without additional -SH compound; D, papain digest obtained in the presence of 0.1 M 2-mercaptoethanol

Discussion

Tryptic fragmentation of normal human γG globulins led to results which are in accordance with those of Hanson and Johansson (1963). Fraction I contained a component which resembled the intermediate fragment of rabbit γG globulins, first found by Nelson (1964) in a papain digest of this protein. Similar products have already been found in tryptic hydrolyzates of a γG2a type mouse myeloma protein (Gorini et al., 1969) and in tryptic digests of aggregated human γG globulins (Mattews, Stanworth, 1971). Fractions tFab and tFc resemble the papain products Fab and Fc, respectively. Both fragments, however, differ from their counterpart in electrophoretic mobility. Fragment tFab' is related to Fab, but its molecular weight is considerably lower than that of Fab. Fragment tFc'

should be a part of Fc, since it is related to this fragment antigenically, but it is deficient in comparison with the latter. Similar fragments have been isolated from peptic digests of γG1 (pFc') and from papain digests when further splitting of the Fc part occurred (Fc'). Both fragments consist of the C-terminal half of the Fc part, but pFc' contains a longer piece of γ1 chain than Fc' (Turner, Bennich, 1968). The Gm(1) allotypic antigen is retained by pFc' but almost completely destroyed on conversion of the latter into Fc' (Turner et al., 1969). Since the Gm(1) activity was detected on tFc' (unpublished observations), tFc' seems to be longer than Fc'.

The data presented here demonstrate the heterogeneity of human γG globulins with respect to sensitivity to tryptic fragmentation. This heterogeneity seems to be correlated with the heavy chain subclasses. The papain-sensitive subclasses γG1 and γG3 are likewise susceptible to trypsin. Both enzymes presumably act on peptide bonds located between the N-terminus and the inter-heavy chain disulphide bonds. Should the cleavage occur at the other side of these disulphide bridges, dimers of tFab should have been formed.

A remarkable difference was found between papain and trypsin in the degradation of the Fc portion into smaller fragments. The Fc part of γG3 globulins is known to be split into smaller fragments, not precipitated by anti γG globulin sera when the protein is treated with papain in the presence of 0.01 M cysteine. Papain without cysteine and trypsin does not degrade the Fc of the γG3 proteins (Poulik, Shuster, 1964). The present investigations demonstrate that fragment tFc is present in the tryptic digest of a γG3 protein even after 16 hours digestion in the presence of 0.01 M cysteine. On the other hand, the Fc part of G4 proteins was found to be extremely sensitive to trypsin while fragment Fc is present in the papain (with cysteine) digests of these proteins.

The observed differences in the tryptic susceptibility of different heavy chain subclasses of γG globulins may permit the typing of a given γG myeloma protein by immunoelectrophoretic analysis of a tryptic digest. If tFab, tFc and tFc' fragments can be distinguished in the digest, the protein is γG1. If only tFab and tFc are revealed, it is probably γG3. If there are only small amounts of fragments and the majority of the protein is apparently unsplit, it is γG2. Finally, if immunoelectrophoresis of the digest gives only one precipitation line which can be revealed only by anti Fab, but not by anti Fc sera, the protein is γG4.

The authors are indebted to Dr D. R. Stanworth (Department of Experimental Pathology, University Medical School, Birmingham) for informing them about his unpublished results. Thanks are due to Dr E. Horváth (National Institute of Haematology and Blood Transfusion, Budapest) for preparing potent anti human globulin sera. The skillful technical assistance of Mrs Éva Hetényi and Miss Clara Messel is gratefully acknowledged.

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Revised Amide Location for Porcine and Human Adrenocorticotropic Hormone

(Preliminary Communication)

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(Received August 30, 1971.)

Some results of our studies concerning the deamidation of various polypeptide hormones have already been reported (Gráf, 1971; Gráf et al., 1970a, b). The influence of the primary structure on this reaction was investigated in detail in case of bovine prolactin (Gráf et al., 1971). From these studies the conclusion was drawn that in alkaline medium asparagine residues undergo preferential deamidation as compared to glutamine residues. For the alkaline deamidation of peptide-bound asparagines a general mechanism has been proposed, according to which hydrolysis of the amide group proceeds through a succinimide ring system formed by the asparaginyl side chain and the neighbouring C-terminal amide nitrogen of the polypeptide chain. Opening of this succinimide ring by hydroxyl anions results in the formation of α- and β-aspartyl peptides (transpeptidation). The rate of ring formation is primarily determined by the steric hindrance of the side chain of the amino acid residue acylated by the asparaginyl residue. This mechanism explains the unusually rapid deamidation of asparagine at position 6 followed by a glycine in the sequence of bovine prolactin (Gráf et al., 1970b).

In light of these results the similarly rapid alkaline deamidation of a glutamine residue of porcine ACTH* at position 30 (Shepherd et al., 1956) seemed to be rather unexpected.

Porcine ACTH A_1** was isolated in a homogeneous and fully active form from a side fraction of the procedure described for the preparation of lipotropic hormones (Gráf, Cseh, 1968).

Figure 1 illustrates the conversion of ACTH A_1 to its deamidated derivative (D ACTH A_1) in 0.1 M ammonia solution, pH 10, at 37 °C. The apparent first order rate constant of this conversion was determined by density measurements of the discs and was found to be 4.8×10^{-3} min^{-1}. This value practically equals the rate constant of deamidation of an asparagine at position 6 in the reduced and carboxymethylated derivative of the NH_2-terminal tryptic fragment of bovine prolactin (3.2×10^{-3} min^{-1}) and of the synthetic tetrapeptide Pro-Asn-Gly-Pro (3.3×10^{-3} min^{-1}), measured under identical conditions (Gráf et al., 1971).

Both this analogy and the resistance of synthetic human ACTH (Bajusz et al., 1967) to alkaline deamidation strongly suggested that at position 25 of porcine

* ACTH, adrenocorticotropic hormone.

** Dixon and Stack-Dunne (1955) denoted the most basic component of ACTH as A_1.

ACTH A_1 an asparagine is present instead of the aspartic acid proposed by Shepherd et al. (1956). This revision would result in a Pro-Asn-Gly structure (positions 24 to 26), which was shown to be favourable for deamidation in bovine prolactin.

This suggestion is supported by the following experiments:

1. The COOH-terminal tryptic fragment of porcine ACTH A_1 (positions 22 to 39) was isolated. Deamidation of this fragment proceeds with a rate com-

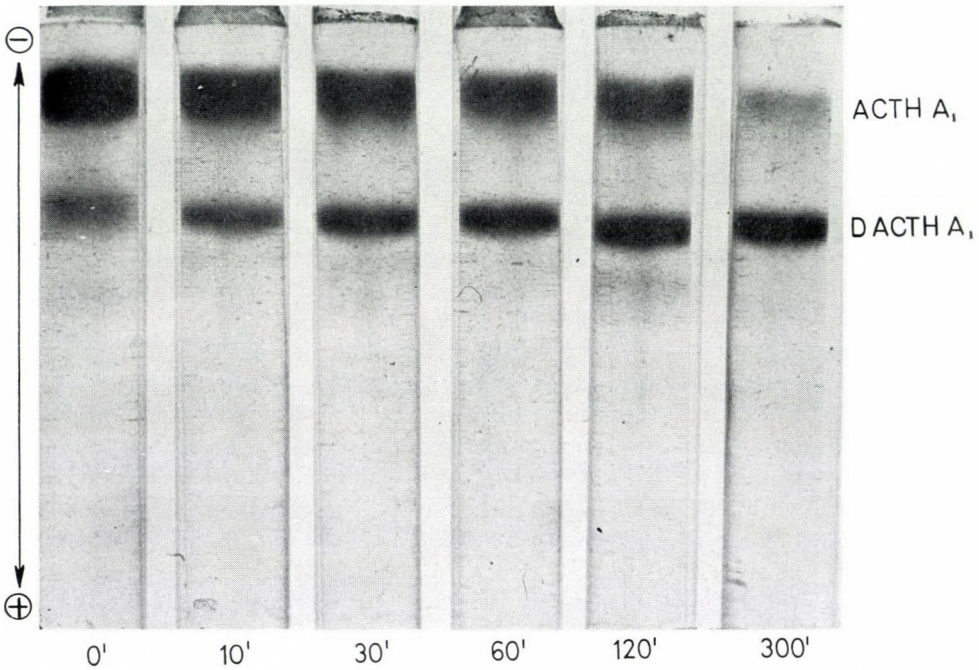

Fig. 1. Disc electrophoresis of porcine ACTH A_1 after 0, 10, 30, 60, 120 and 300 minutes of incubation in 0.1 M ammonia solution at 37 °C. Electrophoresis was performed at pH 9 in 8% polyacrylamide gel. Samples contained 150 μg of protein

parable to that of ACTH A_1 (as measured by the change of the electrophoretic mobilities). The intact and completely deamidated fragments were analyzed by the Edman-dansyl procedure (Gray, 1967). The analysis of the intact fragment gave the following sequence: Val-Tyr-Pro-Asx-Gly-Ala-Glx-. Asx at position 25 in the deamidated fragment could only partly be removed in the form of phenylthiohydantoin derivative and the residual aspartic acid appeared as an additional end group after each Edman step. This type of heterogeneity (Naughton et al., 1960) indicates that at position 25 transpeptidation has occurred. This phenomenon may be considered as an indirect evidence for the amidated state of this aspartic acid in ACTH A_1 (Gráf et al., 1971).

2. From the COOH-terminal tryptic fragment of ACTH A_1 a shorter fragment (positions 27 to 39) was produced by five cycles of Edman degradation. The exposure of this peptide to 0.1 M ammonia did not change its mobility, as checked by paper electrophoresis. Consequently the labile amide group must be located on the single carboxyl side chain of the removed amino acid residues, i.e. on aspartic acid at position 25.

3. The supposed Asn-Gly bond (positions 25–26) could be specifically cleaved with 2 M hydroxylamine at pH 9. The sensitivity to hydroxylamine of this bond also proves the presence of an asparagine residue instead of an aspartic acid at position 25 (Bornstein, 1969; Bornstein, Balian, 1970).

On the basis of the above data the amidated state of aspartic acid at position 25 of porcine ACTH A_1 seems to be unequivocal.

According to this revision the synthetic ACTH's produced till now ought to have an additional free carboxyl group as compared to the natural porcine ACTH A_1. On the other hand, the gel-electrophoretic mobilities of porcine ACTH A_1 and of the synthetic human ACTH were found to be identical at pH 9. This contradiction prompted us to re-examine the complete COOH-terminal sequence of porcine ACTH A_1.

The COOH-terminal tryptic fragment of porcine ACTH A_1 was digested with papain and 11 subfragments were isolated by paper electrophoretic and chromatographic techniques: residues 22–26, 22–28, 22–32, 27–32, 27–28, 29–33, 31–33, 33–34, 33–38, 34–38 and 35–38. The sequence analysis of these fragments was performed by the Edman-dansyl method. For the detection of asparagine and glutamine residues the critical fragments were completely hydrolyzed with aminopeptidase M or carboxypeptidase A and the digests were subjected to amino acid analysis. From these data the following sequence was obtained for the COOH-terminal tryptic fragment of porcine ACTH A_1 (α_p-ACTH*):

Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Leu-Ala-Glu-Ala-Phe-Pro-
22 23 24 25 26 27 28 29 30 31 32 33 34 35 36

-Leu-Glu-Phe
37 38 39

This sequence differs from that proposed by Shepherd et al. (1956) only in the position of the single amide group.

By comparing the fingerprints of papain digests of fragment 22–39 from natural human and porcine ACTH's the same amide location was proved, which is in accordance with the results of Lee et al. (1961).

The rate of deamidation of human ACTH is comparable to that of porcine ACTH A_1. Considering the structural basis of this effect the identity of the primary structures around the Asn–25 seems probable. The analysis of this portion of the sequence of human ACTH is in progress.

* Nomenclature proposed by Li (1959).

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The Submicroscopic Transversal Structure of Striated Fibril

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The transversal surfaces of fracture of fibril segments obtained by homogenization of striated muscle fibre in fixed and native state, as well as the transversal surfaces of fracture of fresh muscle fibres broken under deep-frozen condition were examined. The result showing these surfaces of fracture to display a straight line in the I and A bands supports the conclusion drawn from previous data, i.e. that there are also transversal connections in the fibril beside longitudinal elements.

Introduction

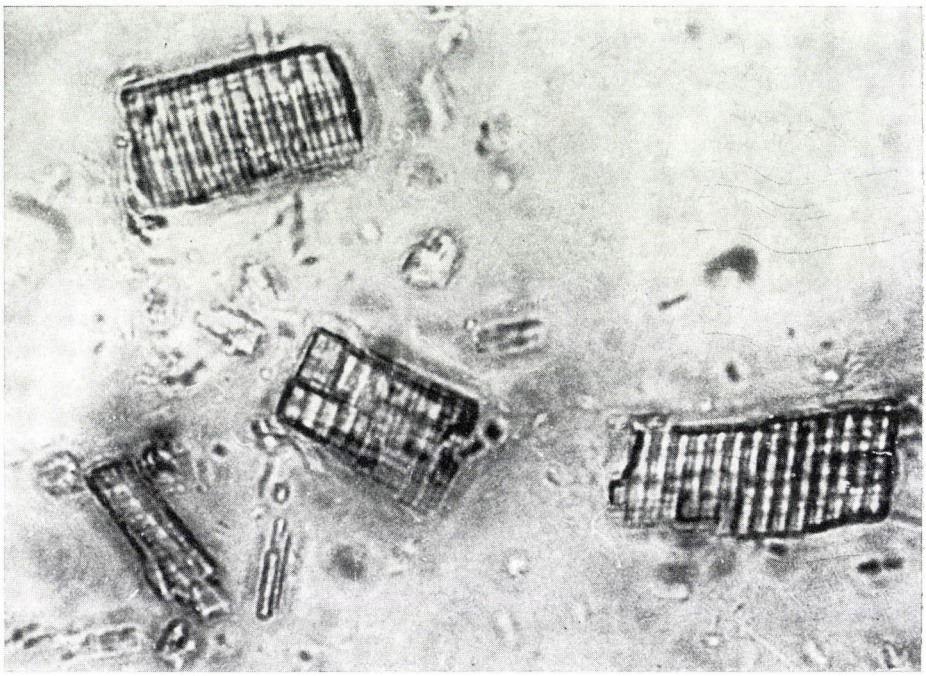
A transversal periodicity of about 400 Å was observed on striated fibrils by several authors (Draper, Hodge, 1949; Huxley, 1953; Sjöstrand, 1962; Ernst et al. 1956; Ernst, 1963). On the basis of further observation of these data (Ernst et al., 1969) it was assumed that the submicroscopic structure of native striated fibril consists of a net of longitudinal and transversal elements. We also were of the opinion (Trombitás, 1969) that this transversal periodicity indicates real structural elements, the elements of which connect the filaments transversally. Furthermore it was pointed out (Trombitás, 1970) that the surfaces of fracture of fibril segments obtained by homogenization of fixed muscle follow these transversal connections in a straight line, especially in the I band. The question was raised whether the transversal lines of break of fibril segments produced by homogenization of native muscle follow the assumed transversal connections; our experiments answered this question in the affirmative.

Materials and method

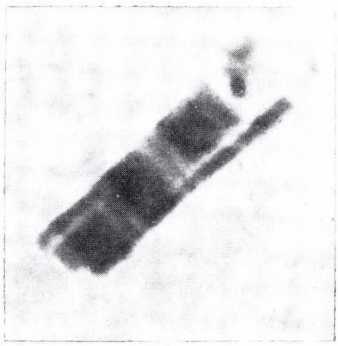
The sartorius muscle of frog (Rana esculenta) was used in our experiments.

a) Homogenization of fixed muscle: the sartorius was slightly stretched on a glass rod and then fixed. A 6.5 per cent glutaraldehyde fixative containing also 0.4 M glucose was applied in a 0.1 M sodium cacodylate buffer at pH 7.4. The time of fixation was 24 to 48 hours at 4°C. After fixing the muscle, it was minced to small pieces and then homogenized by hand with a Potter homogenizer for one hour. b) Homogenization of native muscle: the fresh muscle was homogenized with a waring blendor at a temperature under 4°C for 5×20 seconds in the generally used physiologic solutions. This was followed by homogenization with ultrasound in each case. c) Breaking of native muscle by deep-frezing: the sarto-

rius was slightly stretched on a glass rod, then dropped into a freezing mixture (acetone poured on dry ice of $-70°C$) (Ernst, Kellner, 1936). The muscle, frozen as hard as a bone, was broken and fixed. The fixative contained 6.5 per cent of glutaraldehyde and 0.1 M of sodium cacodylate buffered at pH 7.0. The time of fixing was 4 hours. The dehydration was performed in a row of alcohols of ascending concentration, and the substance was embedded into CIBA araldite. The muscle prepared in this way was checked in cases a) and b) with a phase contrast microscope before embedding. In case c) the blocks were trimmed in



a



b

Fig. 1. Phase contrast microscopic picture of muscle homogenized after fixing. The transversal fracture surfaces are regular. a) Fibre segments $\times 800$, b) one single fibril. $\times 2700$

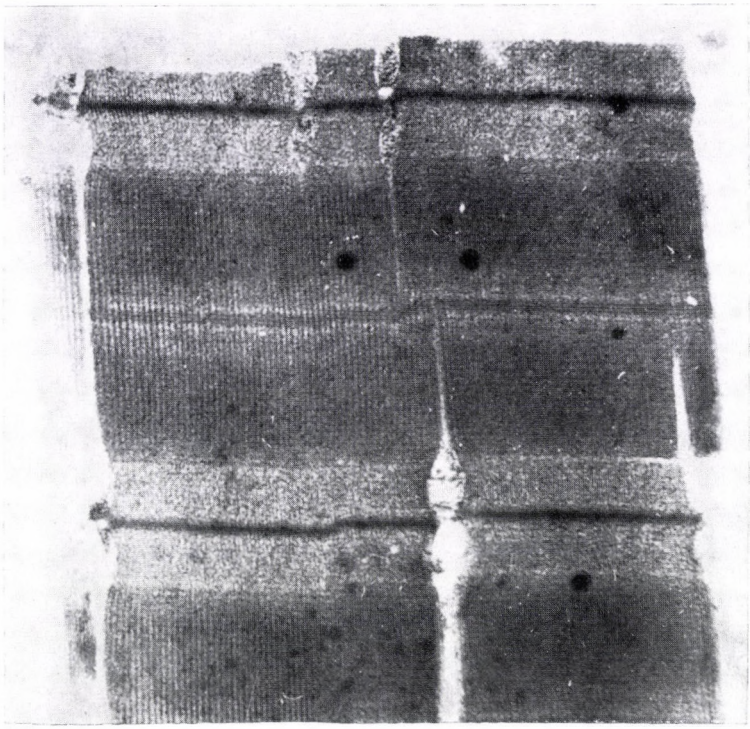


Fig. 2. Electron microscopic picture of fibrils homogenized after fixing. Submicroscopic transversal line-system in both the I and A bands. Fractures along the transversal lines in the I band. ×5000

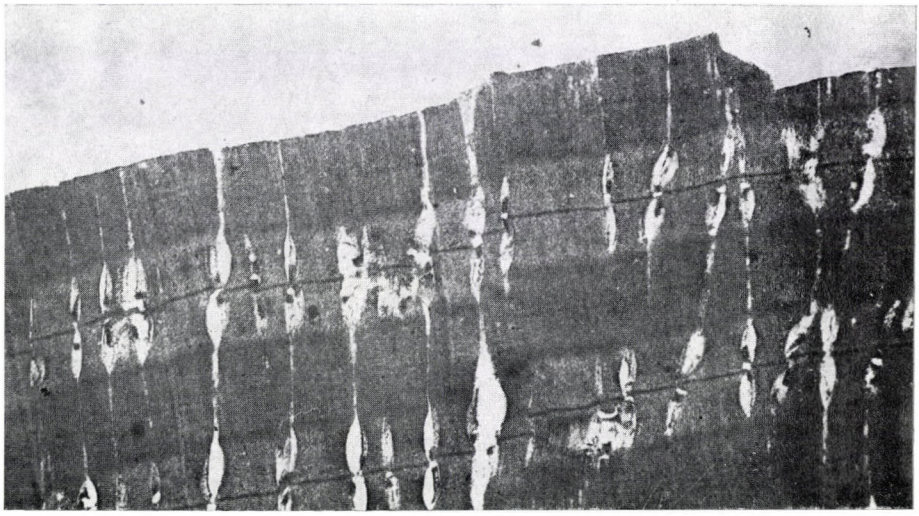


Fig. 3. Fibrils deep-frozen and broken after fixing. Regular transversal fractures in the A band. ×24 000

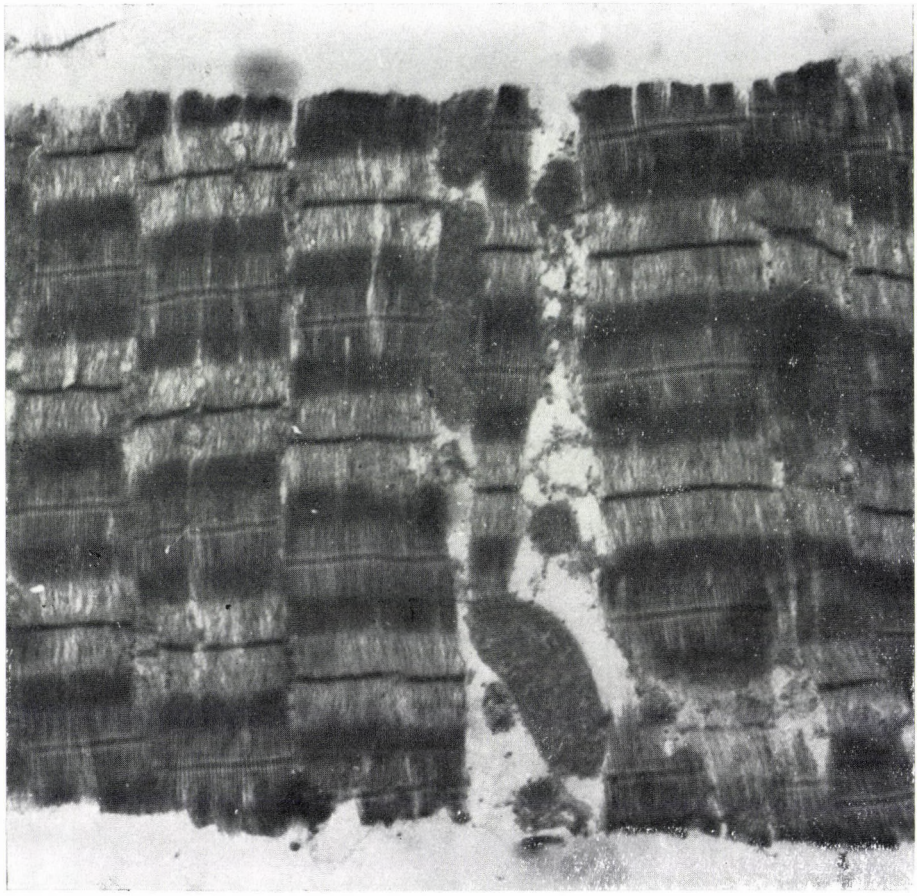


Fig. 4. Fibril segment deep-frozen and broken in native state. Regular lines of break in the A band. × 25 000

such a way that the surface of fracture of the muscle fibre lay in the middle of the section in a longitudinal direction when cutting it. The sections were made with an LKB ultramicrotome. The electronmicroscopic sections were contrasted with uranyl acetate and lead citrate (Reynolds, 1963). The recordings were made with a TESLA BS 513–613 type electronmicroscope with an accelerating voltage of 80 KV.

Results

a) Phase-contrast microscopic investigation of the muscle homogenized after fixing showed fibre segments containing 10 to 15 sarcomeres; their transversal fracture surfaces were regular almost without exception (Fig. 1a). Fig 1b shows a fibril segment, the fracture surfaces of which are all regular, perpendic-

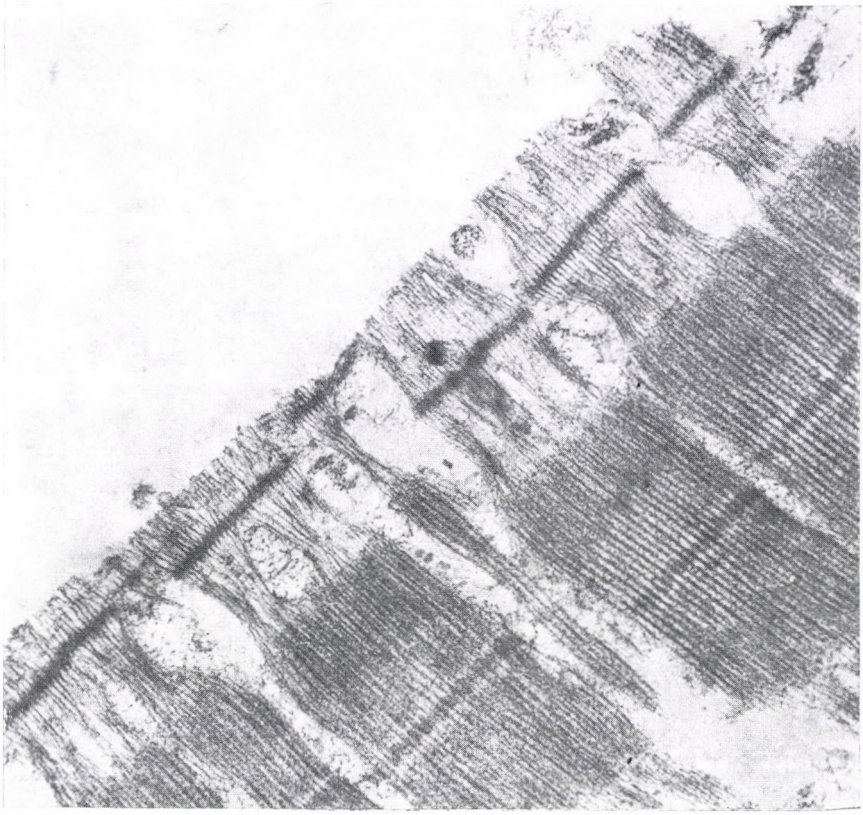


Fig. 5. Muscle treated as the former one. Regular lines of break in the I band. ×40 000

ular to the longitudinal direction. The fracture surfaces are situated in the I band and in the I–A border, respectively. Fig. 2 shows the electronmicroscopic picture of the homogenized muscle investigated above with phase contrast microscope. The fibrils broke at different heights in the I band. The lines of break are bordered with a border-line system of about 400 Å. In the fibril on the right side a sharp line of break starting along the I–A border can be seen. We obtained similar results when the muscles treated in the same way as the former ones, were frozen and broken. In this case the fracture surfaces were more regular and they became more frequent also in the A band (Fig. 3).

b) The mincing by homogenization of native muscle destroyed the structure of the fibrils; since our previous results concerning the fracture of fixed muscle were not yet reproducible on native muscle with the usual methods of homogenization, we changed over to breaking the muscles by deep-freezing.

c) The following figures show our results obtained by breaking the muscle with the aid of deep-freezing.

The electronmicroscopic picture of Fig. 4 shows a group of fibrils broken in deep-frozen condition immediately after preparation, with a fracture surface

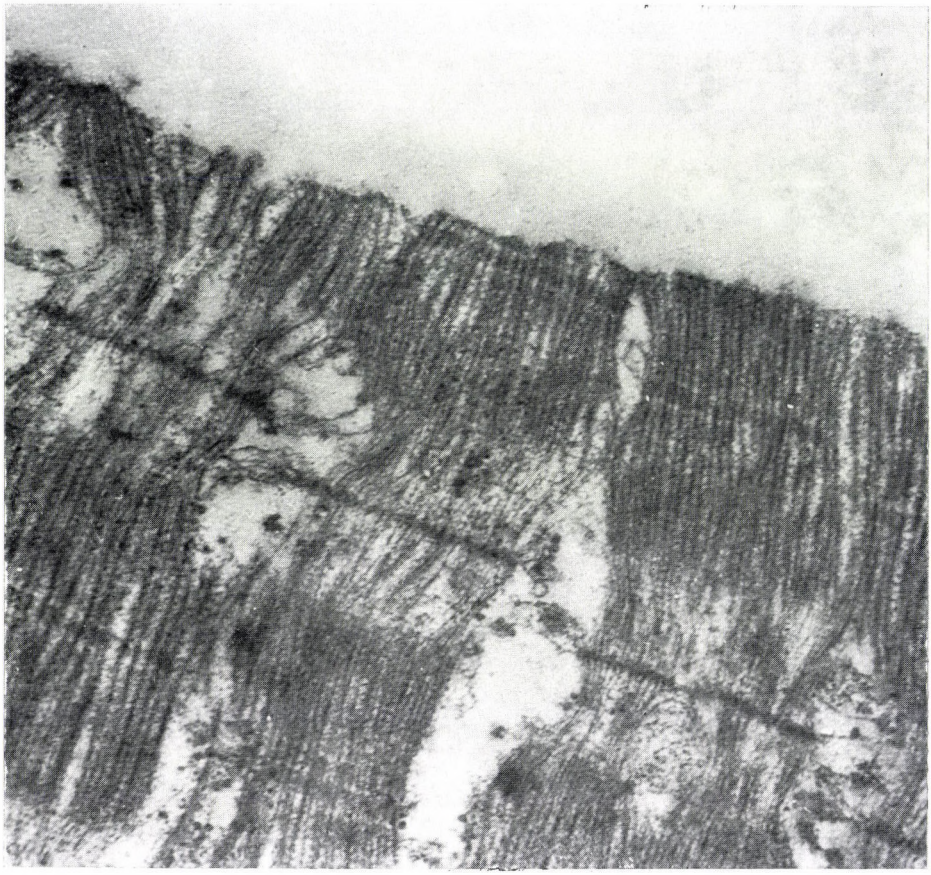


Fig. 6. Muscle treated as the former one. Regular fractures in the A band and in the H zone. × 48 000

on both edges. It can be observed that the line of break of certain A bands is a regular straight line. Fig. 5 shows the fracture of I band at different heights, where the fractures are bordered with transversals. Fig. 6 shows transversal fractures in the A band and in the H zone. In Fig. 7 we can see regular lines of break in the I band and on the I–A border; the fracture surfaces are bordered by the elements of the border-line system.

Discussion

In the case of native or glycerine extracted fibrils several authors have already presented light-microscopic photographs, where a straight transversal fracture surface of fibrils can be seen (Hodge, 1955, 1956; Ernst et al., 1956; Hanson, 1956; Pepe, 1965; Stephens, 1965). The photographs of Aronson (1965)

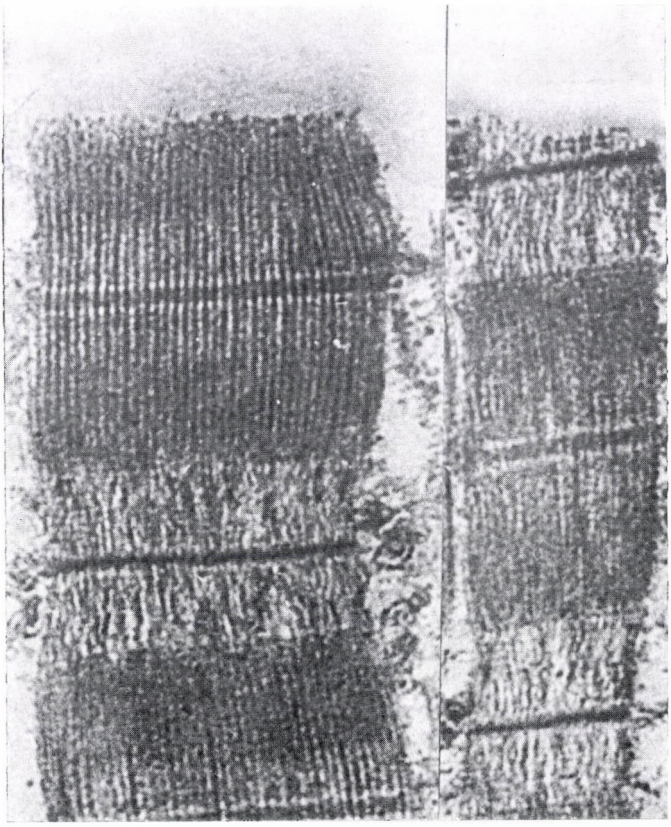


Fig. 7. Muscle treated as the former one. Regular fractures in the I band and on the I—A border. × 40 000

are especially beautiful. It was found in our examinations that the homogenization of the fixed muscle or the breaking of native muscle with deep-freezing may result in a regular transversal fracture surface of the fibrils. These fracture surfaces can develop in both the I and A bands, frequently on the I–A border and along the Z stripe. It seems that the system of transversal lines of about 400 Å has an important role in the development of fracture. The fractures are usually formed along the transversal line. One can often see a step-wise form of the fracture which follows the periodicity of the transversal line structure (Figs 2 and 3; Trombitás, 1970). We think that we have obtained an answer in the affirmative to the question raised at the beginning of our work: the transversal lines of fracture developed in the native muscle follow the supposed transversal connections just like the fracture of fixed muscle does. Thus an obvious explanation of the phenomenon can be the assumption that the native muscle has a submicroscopic transversal structure, the elements of which connect the filaments in a transversal direction and have a role in several processes of the activity of fibrils.

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Dielectric Dispersion of Phosphorylase *b*

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Dielectric properties of phosphorylase *b* have been examined. The relaxation time, characteristic for the rotational diffusion, and the calculated Stokes radius showed a good agreement with data obtained by other methods. There was no good correlation between the Perrin factors calculated from electronmicroscopic data found in the literature and those calculated from our results. Preparation procedures of the samples which were necessary for the different methods might have caused the deviations.

Introduction

Muscle phosphorylase (E.C.2.4.1.1. α-1,4-glucan; orthophosphate glucosyltransferase) plays an important role in carbohydrate metabolism. Two forms of phosphorylases are known: phosphorylase *a* is a tetramer (Mw 370 000), phosphorylase *b* usually shows a dimeric structure (Mw 185 000). According to electronmicroscopic results of Valentine and Chignell (1968; Chignell et al., 1968). the molecular dimensions of phosphorylase *b* are $110 \times 65 \times 55$ Å De Vincenzi and Hedrick (1967) calculated the Stokes radius of phosphorylase *b* by the aid of gel filtration techniques and got a value of 49.3 Å.

In the present paper we intend to get some informations on the molecular dimensions of hydrated phosphorylase *b* by the aid of dielectric dispersion curves.

Approaching the phosphorylase *b* by a sphere one can use the Stokes law for a rotating ball. The relaxation time can be obtained from the equation

$$\tau = \frac{4\pi\eta\, r^3}{kT} \tag{1}$$

where η, r, k, T are the viscosity of medium, the radius of the sphere, the Boltzmann constant and the absolut temperature, respectively. The molecule may also be approached by an ellipsoid of revolution having a volume $\frac{4}{3}\,\pi ab^2$, and in this case according to Perrin

$$\tau = \frac{4\pi\eta\, ab^2}{kT} f \tag{2}$$

where a and b are the half axes of the ellipsoid and f is the Perrin factor (Perrin, 1934). The relaxation time of rotational diffusion was obtained from the dielec-

tric dispersion frequency. The interrelationship between relaxation time and the f_c dispersion frequency is shown in Eq. (3):

$$\tau = \frac{1}{2\pi f_c} \tag{3}$$

The complex dielectric constant was determined from the Cole–Cole equation:

$$\varepsilon^* = \varepsilon_\infty + \frac{\varepsilon_0 - \varepsilon_\infty}{1 + (i\omega\tau)^{1-\alpha}}$$

where ε_0 and ε_∞ are the dielectric constants belonging to low and very high frequencies respectively, and α is the Cole–Cole parameter (Cole, Cole, 1941).

Materials and methods

Dielectric dispersion measurements have been carried out with an admittance bridge, constructed in our Department. The symmetric construction of admittance bridge is shown in Fig. 1. The bridge was supplied by an Orion 1132 wide range generator. The generator was connected to the bridge through a double

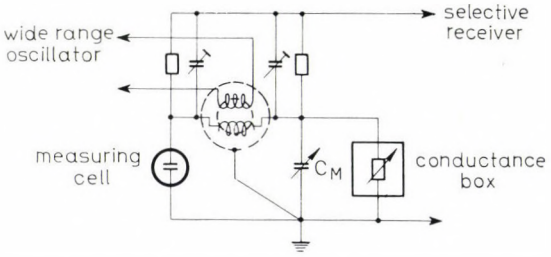

Fig. 1. Schedule of admittance bridge

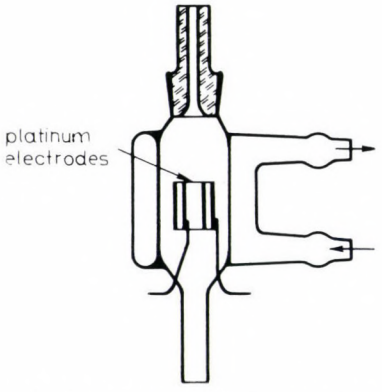

Fig. 2. Cross section of the measuring cell

shielded thoroid transformer. A Telmes TT 1302 type selective measuring receiver served to control the bridge-balance. ± 0.06 pF and 0.001 mS were the accuracy of capacity and conductivity determinations during the measurements. The measuring cell consisted of two concentrical cylindrical platinum electrodes put into double walled thermostable hard glass coat (Fig. 2). The electrodes for decreasing the polarization effect were black-platinized. $C_0 = 1.611$ pF cell

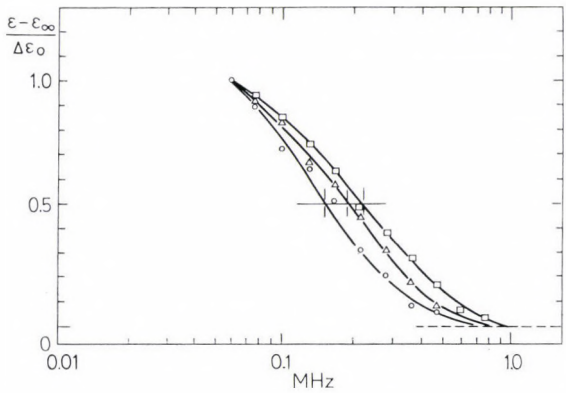

Fig. 3. Dielectric dispersion curves of phosphorylase *b*. ○ — ○ 291°K; △ — △ 298°K; □ — □ 308°K; pH = 7.25; *c* = 7.68 mg/ml

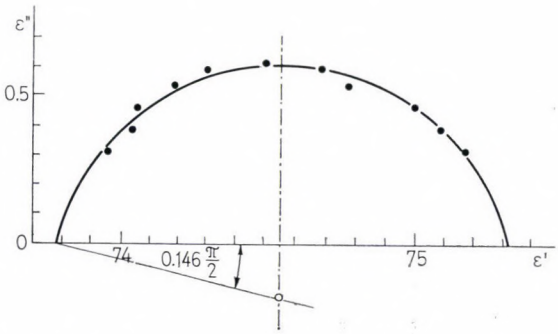

Fig. 4. The Cole–Cole plot (298°K; *c* = 7.68 mg/ml)

constant was obtained with deionized water ($\kappa = 10^{-6}$ ohm^{-1} cm^{-1}). 78.54 was chosen as dielectric constant of water at 25°C (Wyman, 1936). The glass coat was thermostated and the temperature was controlled by thermistor with an accuracy of ± 0.05°C.

Determining the dispersion curves logarithmic scale was used for the frequency. The frequency values were read on the scale of selective measuring receiver.

Rabbit skeletal muscle phosphorylase *b* was prepared and purified as described earlier (Damjanovich et al., 1967). The concentration of protein and the

absence of nucleotides were checked spectrophotometrically (Appleman et al., 1963). Cysteine and traces of 5'-adenosine monophosphoric acid were removed by dialysis for three hours before the experiments against ion free water. The ion free water was saturated with argon gas preventing the oxidation of protein. After dialysis the material was further deionized with Varion KS and Varion AD mixed bed resins to decrease the conductivity. Ion exchange resins were removed by centrifugation. 7.25–7.32 was the pH of solutions after deionization.

Ostwald viscosimeter was used to determine viscosity of solutions.

Activity assays after restoring the ionic strength of the medium showed that the enzyme has not been damaged irreversibly by the preparation procedure.

Results and discussion

The phosphorylase *b* was investigated in 33 vol. per cent glycerol. 7–8 mg/ml was the highest protein concentration attainable after deionization. Fig. 3 shows the dielectric dispersion curves in 10 kHz–1MHz interval at 18, 25 and 35°C. The Cole–Cole parameter calculated from the empirical Cole–Cole plot (Fig. 4) had a value $\alpha = 0.146$ which confirmed that relaxation time showed a distribution (Cole, Cole, 1941).

Table 1

Dispersion frequencies of phosphorylase b at different temperatures

t °C	f_c (kHz)	$\tau \times 10^7$ (sec)	η (P)	r (Å)
18.0	167	9.52	0.03171	45.9
25.0	190	8.36	0.02508	47.8
35.0	216	7.35	0.01776	51.9

(pH = 7.30)

Table 1 shows the dispersion frequencies, the relaxation times, viscosity values and the calculated Stokes radii belonging to the different temperatures.

The Stokes radius showed a good agreement with the data of De Vincenzi and Hedrick (1967), obtained by the gel filtration method, at 25°C.

The volume of molecule calculated from electronmicroscopic data resulted in 205 900 $Å^3$ taking the molecule as an ellipsoid of revolution, where the half axes $a = 55$ Å and $b = 30$ Å (Valentine, Chignell, 1968). Relying upon the above data and the viscosity in 33 per cent glycerol at 25°C, 5.55×10^{-7} sec relaxation time was calculated from Eq. (2). 8.36×10^{-7} sec was the measured relaxation time at 25°C. 2.58 value of the Perrin factor was gained from the relaxation time using measured data and molecular dimensions. The Perrin factor calculated from rates of axes ($b/a = 0.546$) was either 1.45 or 1.50 depending on which of the axes was fixed during rotation.

Proton fluctuations were not taken into account which might explain the differences between measured and calculated values.

Dipole moment of phosphorylase *b* was taken from Oncley's equation recommended for proteins:

$$\mu^2 = \frac{9\,000\,kT}{4\pi Nh} \cdot \frac{\Delta\varepsilon_0 M_w}{c}$$

where $\Delta\varepsilon_0 = \varepsilon_0 - \varepsilon_\infty$, M_w, N, c and h denot the dielectric increment, the molecular weight, Avogadro's number, concentration and an empirical constant,

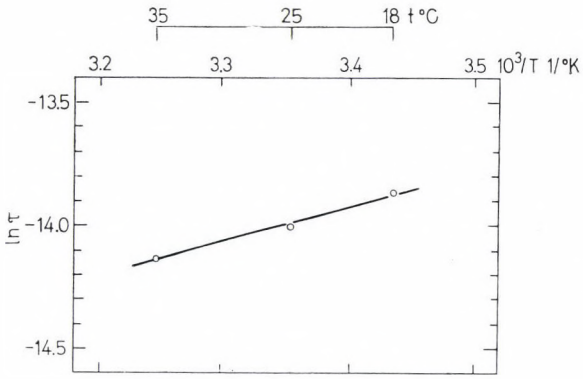

Fig. 5. Relaxation time-temperature diagram

respectively (Oncley, 1943). $\frac{\Delta\varepsilon_0}{c} = 0.20$ was obtained for the dielectric increment from the Cole–Cole plot. Choosing $h = 8.5$ the dipole moment of phosphorylase *b* is $\mu = 450$ debye units (Edsall, Wyman, 1958). Assuming that the relaxation temperature dependence follows the Eyring theory the activation energy for molecular rotation was calculated from the relaxation time-temperature diagram ln τ plotted against $1/T$ in Fig. 5.

$\Delta H^{\ddagger} = 3.29$ kcal/mole was calculated for the phosphorylase *b* from

$$\tau = \frac{h}{kT} \exp \frac{\Delta F^{\ddagger}}{RT} \quad \text{and} \quad \Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}.$$

The $\Delta H^{\ddagger} = 5.04$ kcal/mole calculated from the viscosity temperature diagram of glycerol was slightly different.

Comparing the dielectric properties of phosphorylase *b* determined by us with data obtained by other proteins one can establish that phosphorylase *b* had dielectric properties very similar to other globular proteins.

The good agreement between Stokes radii calculated from gel filtration by De Vincenzi and Hedrick and from dielectric dispersion by us provided further evidences that the latter method was a powerful tool in studying the structure of macromolecules.

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Dosimetry in the Reconstructed Biological Irradiation Channel of the Hungarian WWR-SM Reactor

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Dosimetry has been performed in an irradiation cavity by using solid state dosimeters, threshold detectors and computation. TL dosimeters (BeO and LiF) were used for gamma dosimetry. Neutron spectra were computed with a multi-group code and used in combination with threshold detector measurements to calculate the free-in-air kerma rate. The paper also deals with the estimation of absorbed dose in mice.

This paper deals with a few dosimetrical problems of mouse irradiation in the Biological Irradiation Facility of a WWR-SM reactor. The design, construction and preliminary results are discussed elsewhere (Makra et al., 1970).

Fig. 1 is a diagram of the reactor core facing from a distance of 150 cm away the Biological Irradiation Facility. The homogeneity of the irradiation is increased by using a rotation equipment (Fig. 2).

Method

Activation technique was used for neutron monitoring. The neutron dose was determined by means of the measured activities and appropriate calculations.

The neutron beam spectrum was calculated by using a multigroup aldebo code (Vértes, 1970). The reflector and shielding materials were idealized as infinite slabs. The theoretical cavity spectra were calculated from the beam spectrum by using the exponential attenuation law.

The effective neutron flux and spectrum were measured by the threshold reactions Al, P, S(n, p), ^{115}In(n, n') and Al(n, α), and thermal flux was measured with Au foils. The σ_{eff} values used agreed to ± 15 percent with the values calculated from the theoretical spectrum. Because no measurements were made below $E_{\text{eff}} = 1$ MeV, a correction was made on the basis of the theoretical spectrum.

The neutron kerma was calculated from the neutron spectrum (ICRU 13, 1969)

$$K = \int \frac{d\,\phi\,(E)}{\mathrm{d}E}\,(\mu_{\kappa}/\varrho)\,E\mathrm{d}E$$

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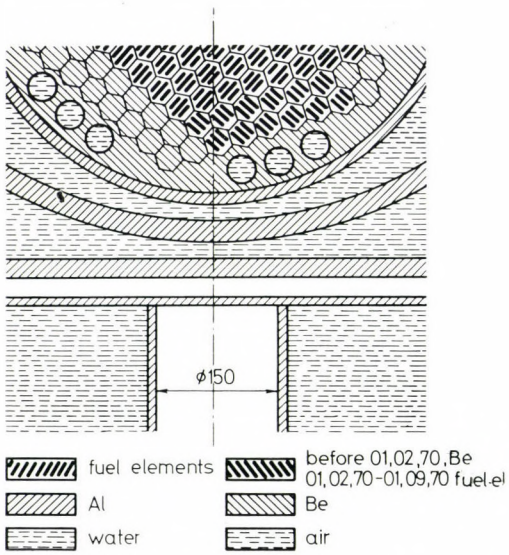

Fig. 1. Diagram of the reactor core facing the Biological Irradiation Facility

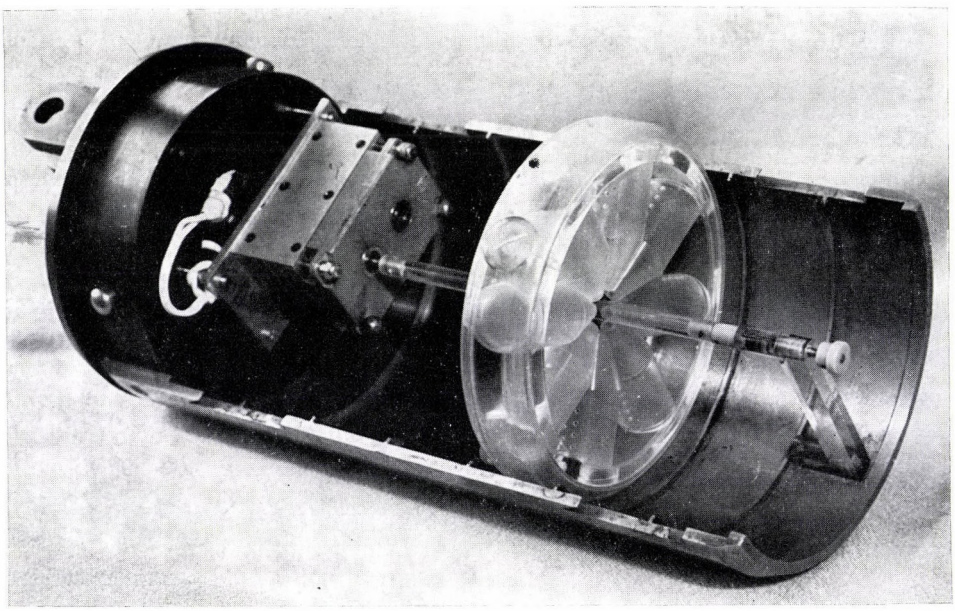

Fig. 2. Biological irradiation cavity with mouse cage and rotation equipment

where $\frac{d\phi(E)}{dE}$ is the neutron flux, E the neutron energy and μ_κ/ϱ the mass-energy transfer coefficient. Instead of the energy dependent quantity μ_κ/ϱ, an acceptable approximation (Lamberieaux, 1963) was used.

An attempt was made to compute the spectrum and dose from the measured activities by using a slight modification of the computer code RFO7 (Turi, Fischer, 1970).

In view of the mixed neutron – gamma field, dosimeters with low thermal and fast neutron sensitivity are most suitable for the gamma dose measurements. For these purposes the TLD 700 LiF-Teflon dosimeter (Con-Rad) and BeO ceramics were used (Tochilin et al., 1969). Great care was taken to work with dosimeters of the same thermal and irradiational history. Sensitivity was measured regularly by using dosimeters irradiated with ^{60}Co gamma rays.

Results

The specific activities induced in the foils from the threshold reaction are given in Table 1. The ϕ_{eff} values in Table 2 were calculated with the σ_{eff} values compiled in the same table. The foils were irradiated 17 cm from the front side of the cavity. The last two lines of Table 2 consist of the exposure and free-in-air

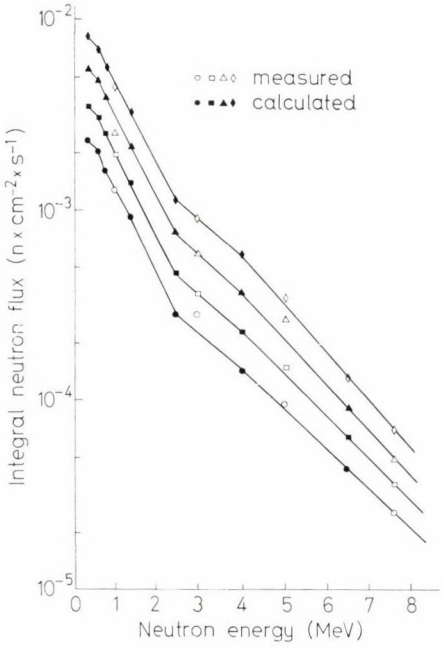

Fig. 3. Comparison of the measured and calculated integral fast neutron spectra normalized at an energy of 7.6 MeV filters: 70 Bi + 10 B_4C (◊); 95 Bi + 10 B_4C (△); 120 Bi + 10 B_4C (□) and 145 Bi + 10 B_4C (○)

kerma rates for different filters (measured between February and September, 1970).

The four graphs in Fig. 3 are the assumed integral spectra calculated from the "assumed spectra" normalized with the measured values at an energy of 7.6 MeV. The agreement in the energy interval 1 – 10 MeV is satisfactory. The measured flux values are obtained from Table 1.

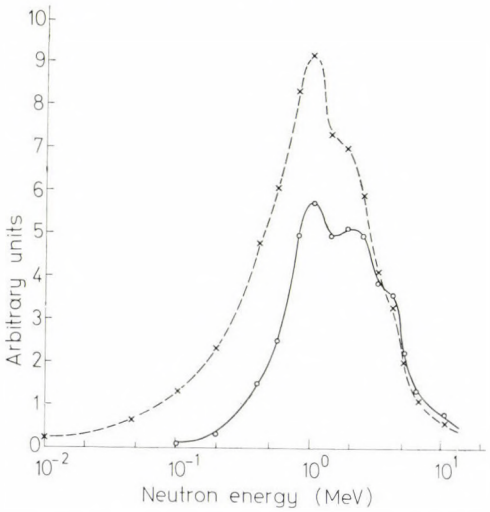

Fig. 4. Log spectra of the product of flux and energy (×), and that of kerma and energy in tissue (○) in the biological irradiation cavity

Fig. 4 shows the logarithmic spectra of the product of flux and energy, and that of kerma and energy in tissue (Lamberieaux-approximation) plotted against energy. These spectra are calculated for 95 Bi + 10 B_4C filters. The kerma spectrum justifies neglecting the contribution to the kerma of neutrons of energy less than 0.01 MeV.

Table 1

Specific activities of the neutron detectors irradiated in the biological irradiation cavity

Reaction	Composition of filters in mm			
	70 Bi + 10 B_4C	95 Bi + 10 B_4C	120 Bi + 10 B_4C	145 Bi + 10 B_4C
	spec. activity (decay/atom)			
^{115}In (n, n') ^{115m}In	2.16×10^{-16}	1.5×10^{-16}	1.2×10^{-16}	8.1×10^{-17}
^{32}S (n, p) ^{32}P	7.5×10^{-17}	5.7×10^{-17}	3.8×10^{-17}	3.0×10^{-17}
^{27}Al (n, p) ^{27}Mg	7.84×10^{-18}	—	—	2.7×10^{-18}
^{27}Al (n, α) ^{24}Na	1.9×10^{-18}	1.6×10^{-18}	1.3×10^{-18}	9.2×10^{-19}

Table 2

Neutron fluxes, free-in-air kerma rates and gamma exposure rates in the biological irradiation facility

Reaction	E_{eff} (MeV)	σ_{eff} (mb)	Composition of filters (mm)			
			70 Bi + 10 B_4C	95 Bi + 10 B_4C	120 Bi + 10 B_4C	145 Bi + 10 B_4C
			Φ_{eff} (n · cm^{-2} s^{-1})			
^{115}In (n, n') ^{115m}In	1.0	180	1.2×10^{9}	8.3×10^{8}	6.8×10^{8}	4.5×10^{8}
^{32}S (n, p) ^{32}P	3.0	300	2.5×10^{8}	1.9×10^{8}	1.3×10^{8}	1.0×10^{8}
^{27}Al (n, p) ^{27}Mg	5.0	80	9.8×10^{7}	—	—	3.4×10^{7}
^{27}Al (n, α) ^{24}Na	7.6	100	1.9×10^{7}	1.6×10^{7}	1.3×10^{7}	9.2×10^{6}
^{197}Au (n, γ) ^{198}Au	therm	98×10^{3}	2.05×10^{7}	—	—	1.4×10^{7}
gamma exposure rate (R/s)			2.4	1.25	0.7	0.35
free-in-air kerma due to neutrons (rad/s)			4.35	3.2	2.53	1.75

Discussion

It is sufficient to measure the neutron spectrum at one point only of the cavity, as it may be assumed to be uniform over this relatively small volume: therefore the kerma rate at every other point of the cavity can be determined by means of a single detector.

Between 90 and 95 per cent of the neutron kerma is imparted to hydrogen. As conversion factors from neutron fluence to kerma have been calculated not for mice the small difference in the hydrogen content of the standard man and that of mice (Storer et al., 1957) was taken into consideration.

In accordance with the measurements of Davids et al. (1969) and the calculations of Neary at al. (1957) and Auxier (1967), the absorberd neutron dose in the midline of a mouse is assumed to be 15 per cent less than the free-in-air kerma.

The part of the absorbed dose due to gamma rays was calculated from the exposure values by using a conversion factor of 0.97 rad/R assuming 5 per cent attenuation in 1.25 cm tissue (the front-midline distance in a mouse), the spectrum of a WWR-SM reactor being hard.

A contradiction appears between our present and previous results (Zarand et al., 1971) but this can easily be explained by the different configuration of the reactor core (see Fig. 1) and the difference between the positions of the measuring points.

Conclusion

The dosimetry described above is adequate for radiobiological measurements involving mixed neutron and gamma fields. Further improvement is expected by employing ion chambers and fission detectors.

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Biology and Mathematics

IV. Biology and Mathematical Isomorphism*

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1. By means of logarithm arithmetic operations can be reduced to their elements, e.g. multiplication to addition, exponentiation to multiplication; if in $y = \phi(x)$ one of the variables, e.g. y, varies according to arithmetic progression, while the other (x) shows geometric progression, then $y = k \log x$. 2. Likewise logarithm lends itself to reducing the numerosity of uncertainty in connection with a system; a case in point is the solution of Pfeffer's problem according to information theory in the form of $I = \mathrm{ld}\ H$, or the entropy formula of Boltzmann's problem, $S = K \log w$. 3. Some disputable statements based on such "mathematical isomorphism" and 4. their comparison with Wiener's cybernetic concept. 5. Several phenomena in Biology demonstrate that the living system is able to reduce elements of vast numerosity to categories of much smaller numerosity, with which it then operates. 6. There are different material systems each containing some elements the relations or ratios of which are systematized by us using certain time honoured methods; occasional similar relations in those different systems can be described with the same formula ("mathematical isomorphism") but the different systems need not therefore be included in some "whole".

In accordance with the present state of affairs, when speaking about the relationship between Biology and Mathematics, we wish to emphasize three points of view: (1) Biology can only be transformed into an exact science by using mathematical formulation as a general method, similarly to the other exact sciences. (2) A biological problem requires the application of an *adequate* mathematical method. (3) Mathematical formulation serves to describe *biological facts and assumptions* precisely and does not hypertrophy into some "branch of science" having a purpose in itself and detaching itself from biological reality.

The demand in point (1) is generally recognized, therefore it is superfluous to discuss it. The second demand was dealt with at the first conference of the present series; then I pointed out (Ernst, 1969) that Rashevsky and Monnier (later also Hill) expressed the relationship between stimulus and sensation in a differential equation. This, however, cannot be regarded as an adequate mathematical solution because neither stimulus nor sensation work with infinitesimal limit values and neither can be regarded as a continuum. Consequently the mathe-

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matical principle of the differential quotient

$$\lim_{\Delta x \to 0} \frac{\Delta y}{\Delta x} = \frac{\mathrm{d}y}{\mathrm{d}x}$$

cannot be applied to this process. It is probably due to this inadequate formulation that during the past four decades or so the question has not developed along this line.

The third demand divides into several factors, of which I have already dealt with (Ernst, 1971a) the problem of "Biology and Probability", and further (Ernst, 1971b), under the title of "Biology Heading for the Exact Sciences", with the question of how, in spite of the considerable developments in the last century, Biology has come to lag behind on the road of creating quantitative laws.

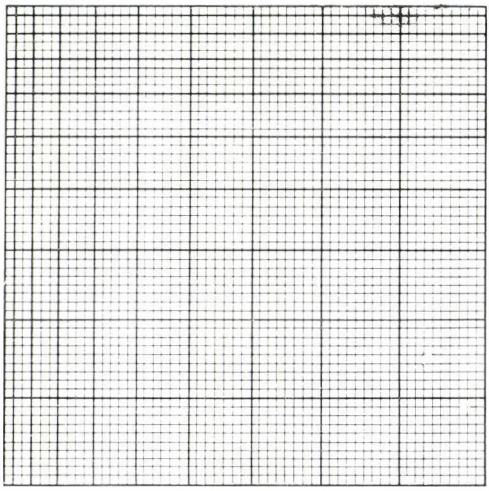

Fig. 1. Multiplication table

At present I am trying to analyse the question of whether the circumstance that we use "*isomorphic mathematical*" formulas to describe certain relationships common to different material systems is really indicative of an intrinsic identity of these systems.

1. The origin of computation is to be looked for in the endeavour to put in order the things experienced in our environment; but when passing on to computation itself without the material elements to be counted man encountered a great mental difficulty even some thousand years ago. This is indicated, among other things, by the fact that elementary *multiplication* was solved through *addition* with the help of suitable tables (Hogben, 1949), as can be seen from Fig. 1.

In connection with his thesis about the moving pulley-block, Archimedes arrived at the reduction of the multiplication of powers to addition of the expo-

nents, e.g.

$$2^2 \cdot 2^4 = 2^{2+4} = 2^6.$$

In the fourteenth century Oresmus generalized the operation by reducing division to subtraction, e.g.

$$\frac{2^6}{2^4} = 2^{6-4} = 2^2.$$

As further continuation in the sixteenth–seventeenth centuries, chiefly through the work of Napier and Briggs, logarithm becomes a method which reduces arithmetic operations; e.g. multiplication to addition, and exponentiation to multiplication:

$$\log 2^2 \cdot 2^4 = \log 2^2 + \log 2^4 = 2 \log 2 + 4 \log 2 = (2 + 4) \log 2.$$

As further examples for manifold applicability of logarithm let us view the data of the Archimedean pulley-block: if the independent variable (n = the number of moving pulleys) shows arithmetic progression, the dependent variable $\left(\frac{q}{p} = \frac{\text{load}}{\text{force}}\right)$ will change according to geometric progression; i.e. in the Archimedean function of $\frac{q}{p} = \phi(n)$

if $n = 1, 2, 3, 4, \ldots$, then

$\frac{q}{p} = 2, 2^2, 2^3, 2^4 \ldots$; in general

$$\frac{q}{p} = 2^n, \text{ therefore } n = k \log \frac{q}{p} \tag{1}$$

Thus the logarithm of the variable showing geometric progression will be in direct proportion to the variable showing arithmetic progression.

A similar result is arrived at in the question of so-called organic growth; viz. in the function $b = \phi(t)$, where time (t) is the independent variable, if t changes according to arithmetic progression, b will increase according to geometric progression, i.e.

$$b = 2^t \quad \text{and} \quad t = k \log b \tag{2}$$

The following case leads to a similar result: according to Weber's experimental results (and to Fechner) the relation of the independent variable stimulus (irritation = i) and the dependent variable sensation (s) can be formulated as

$$s = k \cdot \log i \tag{3}$$

This formula proved applicable (and thus approximately correct) by the fact that in (the medical practice of) audiometry the formula for the sensation of oudness,

$$s = k \log \frac{i}{i_0} \tag{4}$$

works well.*

It is very interesting that Wiener and Rosenblueth (Wiener, 1948) related the number of clonic oscillations (o) of the cat's quadriceps extensor femoris, as the dependent variable, to the spike-frequency (f) of the nerve (activating the muscle), as the independent variable; according to their results (p. 28) the explicit form of $o = \phi(f)$ can be expressed in the formula

$$o = k \log f \tag{5}$$

According to the last two examples natural processes are, on the one hand, able to reduce the numerosity of the stimuli in the sensation or of the spike-frequency of the nerve in the clonic oscillations of the muscle, and these activities can be described well with logarithm, on the other.

2. The formula of logarithm is suitable for describing certain relationships of reality; continuously, we wish to discuss how the numerosity of the uncertainty due to the large cardinal of the elements of a set will be reduced. This investigation can find application in a much wider field, and with an excellent result; e.g. we conjure up Pfeffer's discovery made in 1888, viz. that the spermatozoids of fern are attracted by malic acid. He had a set of shelves containing organic

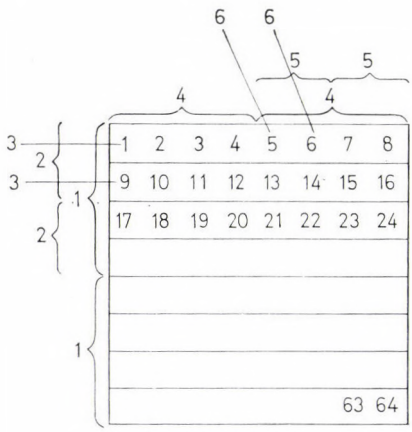

Fig. 2. Pfeffer's set of shelves

* This does not require the use of the differential-integral method employed by Fechner, which cannot be regarded here as adequate since, as mentioned above, the process of stimulus-sensation is not a continuum; but see Stevens, 1958.

compounds and assumed that one of them attracted the spermatozoids. To simplify the question, let us suppose that there were eight shelves and eight bottles on each shelf; i.e. let the cardinal of the elements of his organic compounds be 64; the question is, which of them attracts the spermatozoids. He used the following method to solve the question (Fig. 2). 1. He took the bottles of one half of the 8 shelves, poured the solutions together and did the same with the other half, thus establishing which of the two was efficacious. 2. After that he again took one half of the efficacious 4 shelves, and the other, to see which was efficacious; 3. again he took one of the two (remaining) shelves and the other,

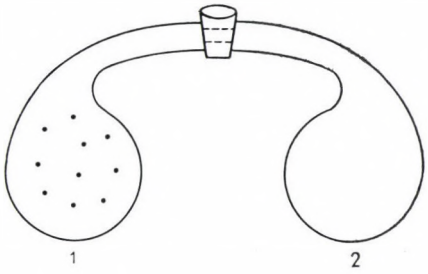

Fig. 3. The two-compartment vessel containing the 10 molecules

so it turned out which of them was efficacious. That is to say: with three trials he established on which of the 8 shelves the efficacious substance was. Afterwards he examined one half of the bottles on this shelf and then the other half (4), then (5) each of the two halves of the 4 efficacious solutions and at the end nothing remained (6) but to decide whether one or the other solution was efficacious; that is how he found malic acid. Thus he halved six times, which corresponds to the fact that

$$2^6 = 64, \quad \text{i.e.} \quad {}^2\log\ 64 = 6.$$

This is in full agreement with Hartley's statement made 40 years later, which gave the formula for information content (I) and amount of information (H) as

$$I = {}^2\log\ H \tag{6}$$

Accordingly, Pfeffer, *without knowing of information theory*, reduced the uncertainty of 64 equiprobable events: with 6 acts he eliminated the uncertainty numbering 64. And now, we describe this result with the method of logarithm.

Another example: Boltzmann investigated Clausius' function of state, entropy, in connection with the distribution of the molecules of an ideal gas. Simplifying matters again let us assume that one half of a two-compartment vessel (Fig. 3) contains 10 molecules at the beginning. The molecules 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 can be distributed between the two compartments in the following manner (see Table 1).

Table 1

The macrostates (M_j) of the 10 molecules and the microstates ($C_m^j = w_j$) realizing the former

No.	Number of molecules in the 1st part	Number of molecules in the 2nd part	Number of microstates* = w	
1	$j = 10$	0	$C_{10}^0 = C_{10}^{10}$	1
2	0	10		1
3	9	1	$C_{10}^1 = C_{10}^9$	10
4	1	9		10
5	8	2	$C_{10}^2 = C_{10}^8$	45
6	2	8		45
7	7	3	$C_{10}^3 = C_{10}^7$	120
8	3	7		120
9	6	4	$C_{10}^4 = C_{10}^6$	210
10	4	6		210
11	5	5	C_{10}^5	252
$11 = M_a$			Sum	$2^{10} = 1024$

* Disregarding the permutations of the molecules contained in a compartment and considering only the combinations of them $C_m^j = C_m^{m-j}$ ($j = 0, 1, 2, \ldots, m$).

The cardinal of all possible distributions (d) figures as the dependent variable against the number of molecules (m) as the independent variable. In the function $d = \phi(m)$, if

$m = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10$, then
$d = 2^1, 2^2, 2^3, 2^4, 2^5, 2^6, 2^7, 2^8, 2^9, 2^{10}$; i.e. $d = 2^m$ and $m = k \log d$ (7)

Here d stands for the number of all combinations without repetition of the number of molecules

$$d = \sum_{j=0}^{m} C_m^j \qquad (C_m^j = C_m^{m-j}) \tag{8}$$

Expounded further: the number of all macrostates

$$M_a = r \log d,$$

where $r = \dfrac{m+1}{m}$, if log = *ld.** The number of the microstates ($C_m^j = C_m^{m-j}$) realizing the macrostate M_j delivers the thermodynamic probability of this macrostate; $C_m^j = w_j$; e.g. the macrostate M_{6-4} can be realized by 210 microstates, i.e. its thermodynamic probability $w_{6-4} = 210$. Consequently – following Boltzmann's train of thoughts but avoiding his mathematical apparatus – it stands to reason to consider the uncertainty of the macrostate M_j characterized by w_j to be identical with that of the entropy (S), the state-function after Clausius, also characterized by w_j; therefore in the case of M_j

$$S = K \log w,$$

where K is the Boltzmann constant (log = ln).

3. Summing up the last two examples: the great number of the uncertainties of the organic compounds or the microstates realizing a macrostate was reduced to a much smaller numerosity, or eliminated, namely corresponding to the function of logarithm. However, identity of the mathematical method does not say anything about the material contents of the two sets. Viz. the dimension or unit of the value obtained was cal/degree in Boltzmann's case and, corresponding to the Hartley formula, the dimensionless bit in Pfeffer's. Thus *it does not seem to be motivated to apply the term entropy to the mathematical method of solving Pfeffer's problem, or to Hartley's formula.***

However, here we have to do not only with conflicting opinions about what term to choose but with a fundamental, objective question. Why, it was at the III[rd] Systems Symposium (Mesarovič, 1968), which was attended, among others, by Rosenblith from the MIT and Anochin of Moscow, that Waterman repeated the statement (p. 10) that

$$1 \text{ bit} = 10^{-23} \text{ cal/mol degree}***$$

However, it seems hardly justifiable that the "mathematical isomorphism" apparent in the use of logarithm should lead to the above equation of bit. By entropy a magnitude of the dimension of energy/degree was meant, originally too, and by

* $M_a = m + 1$.

** (Yokey, 1956; p. 21) Quastler . . .: ". . . I prefer not to use the word, 'entropy' as synonymous with 'information'."

*** A footnote, on the other hand, also says: "10^9 bits is approximately the information in a 1000-page . . . encyclopedia."

information content, either in Hartley's formula or in Shannon's

$$I = \sum_i p_i \log \frac{1}{p_i},$$

theoretically a numerical value without dimension.*

The unification of different systems on the basis of the "mathematical isomorphism" goes even much further. To demonstrate this I quote a textbook of biophysics (Beier, 1968) in which facts are especially emphasized, e.g. that the decay of radioactivity and the decrease of the weight due to starvation can be described by the formula e^{-kt}. According to such mathematical isomorphisms (p. 31) "the isomorphic laws observed in quite different fields" support the opinion which regards "the principle of dynamic *whole*** as the basis of the modern conception of the world". At any rate, the question arises: what actually is to be meant by "dynamic whole", which should include among the above examples the Archimedean pulley-block and the cell population or the radioactive decay and the loss of weight due to starvation. On the other hand, the question may also be raised whether there is a relationship, and what relationship, between the intellectual horizon of this "dynamic whole" and Driesch's conception of "the whole" in the sphere of his "modern" vitalism (Driesch, 1905).

4. Against the statements criticized above and in accordance with the purpose of my lecture indicated by its title, I wish to emphasize again that the use of identical mathematical methods for the treatment of similar relations in different material systems does not say anything about the belonging of these systems to some "dynamic whole". Let a quite primitive example follow: similarly to the law of gases: pv = constant, the relationship between the number of workers (M) and working time (t), the formula Mt = constant is borne out by experience. But in this case "mathematical isomorphism" will hardly lead to the view that the gas molecules and the workers should be included in some "dynamic whole". This similarity means nothing but *the use of an identical, time-honoured mathematical method for the formulation of similar relationships of the elements in different systems.*

A similar opinion is reflected already in the subtitle of Wiener's book entitled Cybernetics: "or Control and Communication in the Animal and the Machine". Namely, he emphasized the identity of *mathematical treatment of the problem* in animal and machine; this, however, is fundamentally different from including these two, on the basis of this similarity, in the conception of some

* A different question also expounded by myself (Ernst, 1962) in one of my earlier articles (p. 338): "The entropy of the system is decreased by a bit of information in comparison with a situation in which the same system is in action without any information; . . . biological information decreases the possibilities of the realization of a biological action, irrespective of the information taking its course in a nervous or a chemical way." However, unifying information with entropy on this or another basis would be the same procedure as is identifying the order to fire a volley, with firing a volley.

** Underlined by the author.

"dynamic whole". Wiener's formulation should follow here (p. 100): "Information is information, not matter or energy." It is from this angle that one may view cybernetics and biocybernetics, which are sure to be at least one of the main channels of the process through which Biology will become an exact science.

5. However, even though Biology is expectant with cybernetics we must clearly see that the *living systems* did not wait till the advent of cybernetics, but *much earlier produced functions similar to those revealed by the mathematical methods of our scientific analysis.*

We have mentioned before that the uncertainty showed by the cardinal of the elements of a set can be reduced with the method of logarithm, and thus the uncertainty of great numerosity is eliminated. An act corresponding to this takes place in the biological process of stimulus-sensation: I recall on the one hand the afore-mentioned formula, $s = k \log \frac{i}{i_0}$, by means of which the vast extent of sound intensity amounting to 10^{13} can be reduced with logarithm (ld $10^{13} \sim 43$), and, on the other hand, that the number resulting in this way corresponds in order of magnitude to *the really existing grades in the sensation of loudness* in man.

The situation is similar in the case of coloured vision. The immense number of frequencies (4.10^{14}) perceptible to the human eye *is reduced by our visual sensation to a relatively small number of distinguishable colours.* Even though this is more than the 7 colours of the spectrum given in text-books, and more than the about 50 shades of colour computed with logarithm, yet the number of shades of colour distinguished by man falls somewhere in this order of magnitude.

It is perhaps even more striking how the biological ability of this kind manifests itself in the field of human intellectual activity,* *in language or speech.* Owing to the scantiness of my knowledge I can only assert of the Greek and Hebrew languages that as early as several thousand years ago they used at least ten thousand word-symbols; but even in the primitive society the number of single words (expressed as sounds or letters) must have amounted to several thousands. *The vast number of those symbols has been reduced to about 30 sounds or letters.***

As a non-expert allow me to work out oversimplified averages: assuming that today 3000 languages, each with a vocabulary of 10 000, are being used, this would amount to 3×10^7 words used on Earth. By reducing this number in the usual manner the result is obtained that this requires 25 letters or sounds.***

Let us further assume that the sounds or letters in use average 30, and let us take into account that they may be repeated in the same word, and let the words consist of an average of 7 sounds or letters. Then the number of words to be made up of 30 letters (in the order of magnitude) is given by variation with repetition

$$V_{30}^{7i} = 30^7 \sim 10^{10}$$

as against the 3×10^7 being used today; i.e. the alphabet of 30 letters will also in the millennia to come be enough to meet the increasing demand of languages for forming new words.

* See: Pfeffer's problem.

** Disregarding e.g. the several thousand basic signs of the Chinese language.

*** Disregarding the different frequencies of the different letters.

6. The reduction of the countless word-symbols to 30 sounds or letters was achieved by the human race without scientists or science; likewise, the function of the sense-organs is independent of the method of our scientific description when perceiving and evaluating the constant stream of stimuli pouring on them from the environment; they reduce these terribly great numbers. Why, the appearance of these faculties can be measured with at least millennia, but our present mathematical method used to describe these biological facts has developed only during the past few centuries.

The reasoning and concept-forming operation of the human mind, with which man has so successfully brought the various fields of his environment within the bounds of his knowledge can also be traced back to thousands of years. When applied, however, e.g. to beauty and e.g. to solidity the formation of these notions with the same method, sc. likewise through abstraction, did not lead to a principal unification of the two different systems from which those notions had been abstracted. Likewise, the "mathematical isomorphisms" produced by the operation of our intellect do not say more than certain mathematical methods are suitable for describing similar proportions or relations found in different parts of our world.

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On the π Electron Structure of Some Compounds of Biological Interest

(Preliminary Communication)

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Several years ago Pullman called the attention to the importance of electron donor-acceptor properties of some biologically active molecules (Pullman, 1964). Ernst used this concept for a possible explanation of the effects of some compounds acting on the nerves (Ernst, 1968). For this reason the π electron structure of the following biologically active compounds have been calculated with the aid of the standard Pariser—Parr—Pople (P-P-P) (Pariser, Parr, 1953; Pople, 1953) method: novocaine (I), aneurin (II), chloromycetin (III) and streptomycin (IV). In Fig. 1 we show the chemical formulae of those parts of the mentioned compounds which contain delocalized π electron systems.

In the calculations we used the Mataga—Nishimoto (Mataga, Nishimoto, 1957) approximation for the $\gamma_{r,s}$ Coulomb integrals, and took the valence state ionization potentials and electron affinities from Hinze and Jaffé (1962) and the necessary geometrical data from Sutton (1958). In Table 1 we give the values of the used $\beta_{r,s}$ core integrals.

The calculations have been performed on the ICL 1903A computer of the Institute for Industrial Economics and Plant Organisation of the Ministry of Heavy Industry. To reach self consistency in three decimals of the charge-bond matrix elements $P_{r,s}$, usually we needed only 5—6 iteration steps.

In Fig. 1 we show denoted by asterisks the diagonal elements of the chargebond order matrix (the π electron charges)

$$\mathbf{P} = 2 \sum_{j=1}^{[n} \mathbf{C}_j \mathbf{C}_j^+ \tag{1}$$

(n^* is the number of filled MO-s) and the bond orders for the chemical bonds (the appropriate off-diagonal elements of **P**). In Table 2 we give the HOMO and LEMO energies.

We can see from the charge distributions given in Fig. 1 that we have in all compounds $P_{r,r}$ values for the carbon atoms around 1 if they have not more electronegative neighbours. The more electronegative nitrogen and oxygen atoms of course pull away charge from the carbon atoms decreasing on them the $P_{r,r}$ values to 0.95, 0.90 in the rings. Correspondingly the charges on the N and

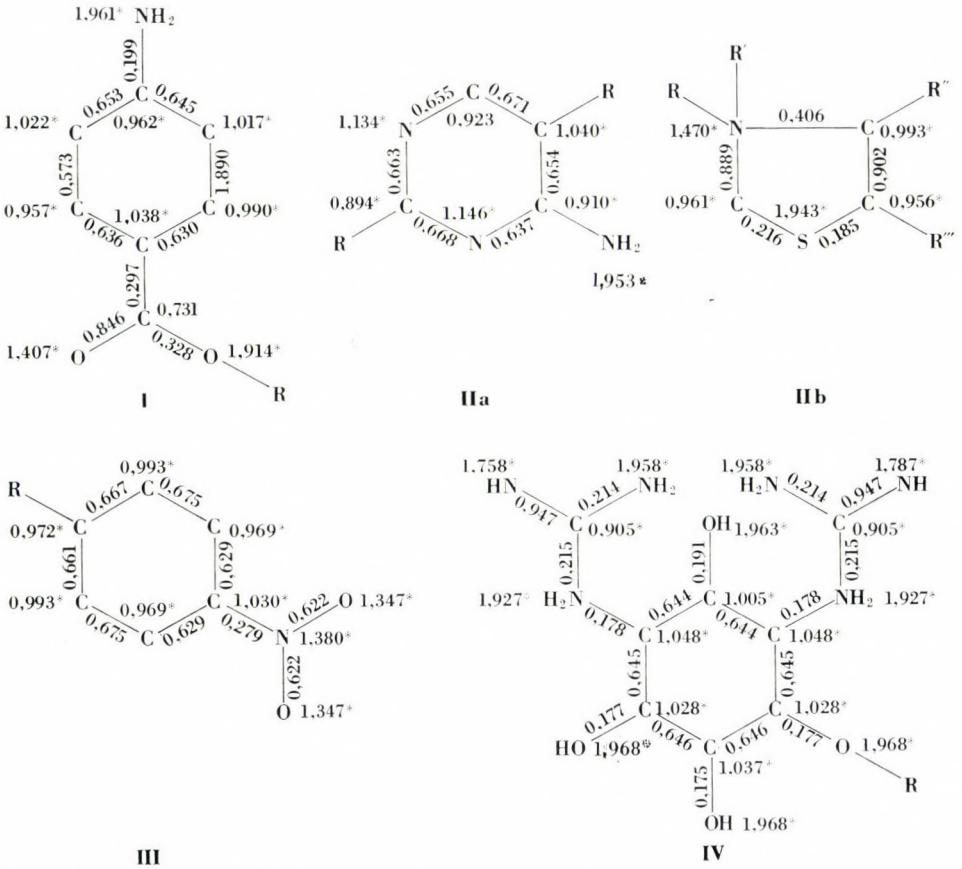

Fig. 1. The chemical formulae of those parts of the compounds I—IV which contain a delocalized π electron system. The numbers designed by asterisks at the atoms indicate the π electron charge densities, the numbers on the bonds the π electron bond orders (see below). In the case of aneurin, IIa and IIb indicate the two parts of the molecule, isolated by a saturated CH_2 group which possess delocalized π systems

O atoms increase from 1 to 1.15 and 1.35 to 1.45, respectively. The $P_{r,r}$ values on nitrogen (−N−) and oxygen (−O−) providing 2π electrons to the delocalized π systems are around 1.95. All these charge density values are in agreement with PPP results obtained for many other compounds which indicates that our parametrization was correct.

Looking now into Table 2 we can see that all compounds have rather low lying energy levels on their highest occupied MO-s. At the same time the LEMO energies are rather high. The energy difference between them is (with the exception of chloromycetin) $3.5-3.8\,\beta = 8.4-9.1$ eV which makes improbable

that they can act as electron donors or acceptors. For instance the difference of the HOMO of the IIb part of aneurin and the LEMO energy of novocaine is $(4.31-0.87)\,\beta = 3.44\,\beta = 8.22$ eV which does not indicate an electron donor-acceptor interaction between them.

Table 1

The values of the used $\beta_{r,s}$ integrals ($\beta = -2.39$ eV units)

Bond	$\beta_{r,s}$	Bond	$\beta_{r,s}$
C–C	1.00	C–N[c]	1.00
C=N	0.90[a]	N=O[c]	1.90
C–N	0.80		
O			
C	1.35[b]		
O			
C–O–	0.95		

[a] In the ring [b] For both bonds [c] In C(=O)–N(=O)=O

Table 2

The energies of the highest filled (HOMO) and lowest unfilled (LEMO) MO-s (in $\beta = -2.39$ eV units)

	HOMO	LEMO
Novocaine	4.391	0.866
Aneurin IIa	4.435	0.676
IIb	4.309	0.882
Chloromycetin	4.686	1.625
Streptomycin	4.040	0.313

The highest occupied level of streptomycin lies at $4.04\,\beta$, while the lowest empty one of chloromycetin at $1.63\,\beta$. Thus the energy difference between them is only $2.41\,\beta = 5.76$ eV which is, however, still too large to expect electron donor-acceptor interactions between streptomycin and chloromycetin.

It should be emphasized, however, that all these results are true only in the P-P-P approximation. There is a possibility that the planned all valence electron (extended Hückel or CNDO) calculations will change the picture.

We should like to express our gratitude to Academician E. Ernst for suggesting the problem and for helping our work with his advices. We are further indebted to the Institute for Industrial Economics and Plant Organization of the Ministry of Heavy Industry for making possible to perform the calculations on their ICL 1903/A computer.

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Book Review

Analog Computer in Biologie und Medicin. By Wolfgang A. Knorre, VEB Gustav Fischer Verlag, Jena 1971. 208 pages

For the past decade it has been repeatedly declared that computers will be of enormous usefulness in biology and medical practice. The successful application of computer technology requires at one side some fundamental knowledge of the physical and mathematical principles involved in computer operation and, on the other hand, in the computer era biological and medical education implies acquisition of knowledge that relates to new techniques that promote an improvement in research work. The book written by W. A. Knorre is a useful tool for those — students, biologists and physicians having some mathematical and physical qualifications — who are interested in practical details or who wish to get an introduction about the analog computer application in biology. The book consists of five chapters.

After a short introduction (Chapter 1), where the definitions of biological system, module diagram, model and computer simulation are given. The difference between digital and analog computers, the hybrid computers are outlined in Chapter 2 and a brief survey is offered about the possible practical applications of analog computers in chemistry, biochemistry, molecular biology and physiology.

In the third chapter the construction and operation of the analog computer are described. Separate parts are given to the different modules as coefficient potentiometer, operational amplifier, inverter, addition module, differentiation and integration circuits, function generators, analog storage device. The last part of this chapter deals with the construction and the guide of the analog computer and with the input/output terminals. All the modules and the construction are specified to the analog computer MEDA-T.

In Chapter 4 an introduction is given about the programming of analog computers (programming of algebraical and differential equations, normalization), and some special wiring diagrams to model biological processes are discussed.

For biologists the most interesting part of the book is the last chapter, where some special problems as chemical reaction-rate analysis, enzyme kinetics, metabolic regulation, pharmacokinetics, tracer kinetics are detailed.

The different models, programmings, wiring diagrams and results presented with a number of illustrations make easier to gain informations and, therefore, the book is a powerful tool for understanding the analog computer technique and it offers the possibilities of alternate means of research work in biology and medicine.

The book is completed with references related to special topics in the analog computer application.

J. BELÁGYI

Proceedings of the Sixth Conference on Biophysics Budapest, 1971

Abstracts

Contents

The Role of Radiobiological Investigations in the Protection of Man's Environment (Biosphere)

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Man has gradually changed the natural state of the biosphere through utilizing the results of science and through civilization. This change has been rapidly accelerating in the recent decades, and is dangerous for living beings, first of all, for the man. Among the harmful factors of the biosphere ionizing radiations play an increasingly significant part (medical applications of radiation sources, wide-spread utilization of radioactive isotopes, atomic energetics, etc.). It was science that rendered the utilization of ionizing radiations and atomic energy possible, and it is the task of the science to minimize the risk of it. This task falls first of all on radiation biology. Radiation biology is a branch of science of the borderlands: it involves radiation physics, radiochemistry, etc. Its experimental results are utilized in the practice by radiation hygiene, radiation protection. Radiation biological researches have to clarify the interactions of ionizing radiations with biologically important macromolecules, the biophysical and biochemical mechanisms of the radiation effect, the physiological and morphological effect of ionizing radiations. Without the knowledge of these basic mechanisms and effects the extent of the risk cannot be estimated or scientifically based and no reasonable method or procedure can be worked out for protection. The task of radiation biology is to investigate the possibility of the biological and chemical protection. In the investigation of the biological effects of incorporated radionuclides not only radiation effects but also the consequences of their transmutation have to be taken into account; nor can the mass of certain radionuclides different from that of their stable isotopes be unconsidered. On the basis of the results of the experimental research it becomes possible to evaluate biologically the radiation loading of the whole population and its smaller groups, respectively. Radio-oecology has a significant role in the research of the radioactive impurity of biosphere.

The review sets forth the suggested problems, touching upon the results, tasks and possibilities in this country.

Determination of Whole-Body Potassium in Man with in vivo Gamma Spectrometry

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The fat-free body weight, as a useful reference basis for different physiological parameters can be determined knowing the whole-body potassium content.

It is important to know the whole-body potassium content also from a clinical point of view. The change of potassium in human organism can be detected more correctly with the aid of in vivo gamma spectrometry than with the determination of the serum potassium level.

The potassium content of the human body has been directly measured with our low background whole-body counter on the basis of the detection of the gamma radiation of the ^{40}K-isotope, naturally present in the organism.

The structure and technical parameters of our whole-body counter are described. The results of the potassium calibration of the apparatus obtained with phantom and the sources of error are discussed.

Whole-body potassium values determined by in vivo gamma spectrometry in a group of healthy people and in some patients suffering from potassium deficiency are reported.

Determination of ^{210}Po(RaF), a Natural Radioactive Element from Cigarette, and Detection of the Isotope Inhaled by Smoking in the Blood

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The ^{210}Po content of the tobacco of the most frequently smoked Hungarian cigarettes (Kossuth, Fecske, Munkás) was measured. Our results were compared with similar measurements of other countries in order to obtain relative information concerning the dose loading the bronchial system. This radioactive material can influence — also according to other authors — the neoplasm developing in the bronchial system. It was demonstrated by an apparatus imitating a man that about 25 per cent of ^{210}Po, already completely sublimating at 500°C, can get in the respiratory tract. We counted the value of the dose reaching the lungs of a person in the case of smoking 2 packets of Kossuth cigarettes on the average. Parallel with this examination blood samples of smoking and not smoking persons were analyzed for ^{210}Po. It was found that a significant increase of concentration appears in the blood samples of smoking persons, which proves that ^{210}Po gets into the metabolism of the person from the lungs.

Radiosensitivity of in vivo Cultured Reproductive Cells (Mouse Ascites Lymphoma)

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The postirradiation period of reproductive cells is characterized by the failure of division and by the increased destruction of cells. In our experiments the connection between the size and seriousness of inhibitory and decay processes, respectively, and the state of cell division at the time of irradiation were studied in vivo.

The division cycle of Németh and Kellner ascites lymphoma cells in the phase of logarithmic growth has been previously determined with the labelled mitosis method. A value

of 6 hours was obtained for G_1, 10.6 hours for S, 3 hours for G_2, 0.7 hours for M, and 20 to 21 hours for a complete cycle of division.

In parallel experiments the cells were in vivo labelled with a radioactive DNS precursor (with ^{14}C and/or ^{3}H thymidine) in the host animal. After the radioactive labelling and in vitro ^{60}Co irradiation the cells were implanted into untreated CFL/P male white mice. A double labelling was applied in a part of the experiments: with ^{14}C and ^{3}H-TdR administered at 3 hour intervals cell groups of different labelling were obtained. G_2 cells contained ^{14}C, those in the 2nd half of S phase were labelled by both isotopes. Cells in the 1st part of S phase incorporated only ^{3}H-TdR. The inactive group of cells was represented by cells in the G_1 state. In another case the so called "window" technique was applied in the radiosensitivity examination of cells at different stages of division. The radiosensitivity of a given group of cells was characterized by the lifetime of cells and the frequency of cytopathologic transformations. The evaluation was done with radioactive measurement (liquid scintillation method) and with light-microscopic autoradiography. According to our results the inhibition of division after the irradiation causes an inhibition of cell growth. This is followed by an increasing destruction of the cells, which happens periodically. These processes are in connection with the state of cell division at the time of irradiation.

The Connection of Structure and Function in the Radiation Injury of T7 Phages

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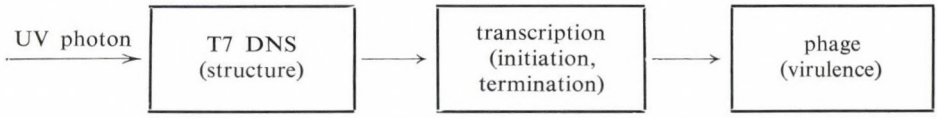
The process of radiation injury was analyzed from the absorption of UV photon up to the biological manifestation with the aid of our radiation kinetic model developed previously in connection with the UV radiation injury of T7 phages and with the aid of the data in the literature concerning the molecular characteristics of T7 phage. It has been pointed out indirectly during the analysis that the damageable sites supposed in our model correspond to the number of cytosine clusters of T7-DNA. These are the initiation and termination sites of the transcription, respectively. The single-hit kinetics is based on the information content of T7 genes containing nonlysogene functions. The radiation damage of T7 phage expressed in the biological function can be summarized in the following scheme:



Examination of the Effect of Ultraviolet Irradiation on MS2 Phages

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The effect of $\lambda = 254$ nm UV light was studied on MS2 phages and a dose effect curve of inactivation taken up. In contrast with previous data in the literature a concave curve was found. The analysis of the initial part of the dose effect curve was performed

with the aid of our radiation kinetic model developed for T7 phages, and a value of $\alpha_{MS2} = 3 \times 10^{-4}$ was obtained for the probability of inactivation by UV photons. This value, as to the order of magnitude, agrees with the $\alpha_{T7} = 1.7 \times 10^{-4}$ probability obtained for T7 phages, which indicates that, at the beginning of irradiation, the photochemical processes resulting in inactivation of the MS2 phage containing RNA as well as of the T7 phage containing double stranded DNA are of similar character. The concave shape of the dose effect curve is explained by the partial restoration of radiation injury. Supposing the value of probability of direct photoreversion in MS2 to equal that determined previously in the case of T7 phages ($\gamma = 2 \times 10^{-5}$) the comparison of the two dose effect curves enables to follow the kinetics of the production of irreversible photoproducts.

Biophysical Problems with Extracorporal Blood Irradiations

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The blood of a patient can be irradiated by ionizing radiation by passing the blood through an irradiator in an external circuit. So the irradiation of the patient's body, especially that of the bone marrow, can be avoided.

The first problem was to assure that, during the extracorporal irradiation of the blood with very high doses, the whole body of the patient should be entirely protected from the primary and secondary radiations. By the use of our specially constructed cobalt unit (GRAVICERT), it was necessary to provide for the pumping of the blood through the about 12 m long external circuit from the patient in the control room to the coil placed around the cobalt source (1600 Ci) before the treatment head, and back to the patient. On account of the many biophysical difficulties of such an appropriate pumping, we constructed another apparatus using the beta radiation of the ^{90}Sr. As a consequence of the much more higher specific dose rate of the beta radiation, it was enough to use ^{90}Sr with activity of 20 Ci only.

The ^{90}Sr was plated on 4 metal plates with an active size of 50×25 mm. Between this plates were run the 5 threads of the plastic pipe coil. The Bremsstrahlung of the irradiator was absorbed by lead shielding of 11 cm thickness. So the patient could be placed close to the irradiator and no extra pumping was necessary.

The last problem was the exact measurement of the absorbed dose in the blood running through the coil of the irradiator.

Measurement of the Discharge Speed of ^{32}P Coli-Endotoxin in Mouse by Whole-Body Counting

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^{32}P Coli-endotoxin of high specific activity was produced by biosynthesis. The beta activity of the endotoxin suspension obtained in this way was 5×10^5 cpm/ml.

The effectiveness of the whole-body counting measuring set was checked. Further on, the smallest activity detectable concerning the braking radiation induced by the beta particles of ^{32}P in a water-equivalent medium was determined.

Unlabelled endotoxin was given to a group of white female mice at two concentrations 6 times. Thereafter both groups were treated with ^{32}P-labelled endotoxin. The control group was given only labelled endotoxin. The discharge speed was determined with the already calibrated whole-body counter after the administration of the tracer endotoxin.

Our results were compared with values of discharge speed determined with another method.

Radiation Protection and Radiation Hygienic Problems in Modern Diagnostic Examinations with Radioisotopes

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The application of radioisotopes by physicians has been advancing rapidly in the recent years. One of the stages of development was the appearance of isotope generators. The short half-life daughter elements of isotope generators allow to use a large quantity of radioactive material in each examination, which raises new problems in the radiation hygiene. The paper touches upon the following problems in connection with the application of isotope generators:

1) Radiation protection of isotope "production" (eluation).
2) Problems of radioactive contamination.
3) Radiation loading of different isotope diagnostic examinations performed with isotopes obtained from generators.

Beside the problems connected with the generator the paper also deals with the radiation loading involved in double tracer techniques.

A Biological Evaluation of Surface-Dose Distribution Obtained by Large Size Film-Dosimeter during X-ray Examinations

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During X-ray diagnostic examinations the exposures to different parts of the body — thus also to the critical organs — are not the same. In the case of irradiation of non-uniform surface dose distribution it does not seem to be enough for the biological evaluation of radiation burden to measure the exposition in R at certain points of the irradiated body surface. In order to discuss the exposure received inevitably or gratuitously by the different parts of the body, the isodose curves of the non-uniform surface dose distribution obtained

during different X-ray examinations were determined. The isodose curves were adopted on the basis of the data of large size film-dosimeter exposed during X-ray investigations; a computer programme of ALGOL language was worked out for this purpose. The surface dose distribution which can be considered characteristic of each type of X-ray investigation is presented.

In evaluating the isodose curves from the point of view of radiation burden to the particular organs the zones prominent from the point of view of radiosensitiveness and surface dose centers, resp., are emphasized.

The results obtained are also in favour of the importance of knowing the dose distribution for calculating the integral dose, especially when the body surface has a non-uniform dose distribution.

Measurement of Surface Dose for Determination of Medical Radiation Burden Considering Dose Distribution and Integral Dose

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The radiation exposure obtained during X-ray diagnostic examinations represents an important part in the exposure level to the individual and to the population. The X-ray examination involves the irradiation of a relatively large surface of the body, i.e. it goes together with a significant integral dose. Among the methods measuring the diagnostic exposure the measurement of the surface incident dose ($R \cdot cm^2$) is considered the most appropriate, because thus the most important parameters for determination of the integral dose can be taken into account. These are chiefly: the exposition (R), the size of the irradiated surface and the dose distribution.

Calculating the integral dose the surface dose is given by the double integral of the dose distribution function. The dose distribution was determined and represented by the method of large size film dosimeter at different types of diagnostic examinations. The problem of calculating the surface dose and the integral dose was expressed mathematically for the film-method at uniform and non-uniform surface dose distributions. An ALGOL computer programme carried out the calculation of the surface incident dose using the large size film-dosimeter. A method of successive approaches was applied for calculating the double integral. The surface doses obtained by different X-ray examinations were determined.

Technical and Physical Problems in Radiobiological Experiments with Atomic Reactor

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A neutron generator or nuclear reactor can be used as radiation source for the study of the biological effect of neutron radiation. The latter can be a special reactor made for radiophysical and radiobiological purposes; or an irradiation channel shaped into a

reactor for universal research. The different solutions are compared from the point of view of the dose rate, the proportion of neutron and gamma radiation, the homogeneity of the space and other characteristics.

The different dose measuring techniques such as the ionization chamber method, calorimetric, and radioactivation methods are compared. Finally we report on the construction of the biological irradiation channel built into the Hungarian atomic reactor, and our methods of dose measurement. In our apparatus four kinds of dose intensity and neutron/gamma rate can be adjusted with the aid of bismuth filters that can be changed automatically. With the irradiation of mice the homogeneity is increased by rotating the cage. For the constant checking of dose rate, a monitor with GM tube and a double ionization chamber are used. Our methods of calculation and measurement of neutron-spectrum are reported in another paper.

Dosimetry of Mixed Neutron–Gamma Radiation in the Biological Irradiation Facility of the CRIP

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The paper deals with some dosimetric problems of an apparatus appropriate for mixed (neutron—gamma) irradiation, the dose rates attainable, and some possibilities of its utilization.

According to the construction reported in another paper, a system most appropriate for the dosimetry of mixed radiation consists of threshold detectors insensitive for gamma radiation and of solid state dosimeters of low sensitivity to neutrons. When measuring the neutron kerma value the real spectrum is determined from the theoretically calculated neutron spectrum by making use of the measured activities with the aid of a computer fitting, and the dose rate is determined from it. The solid state detectors used for gamma dosimetry are LiF and BeO. Though to a small degree they are sensitive for neutrons too; this must be taken into account during the evaluation.

In CRIP the core of a thermal (WWR-Sm) reactor is reconstructed in about every 6 months because of burning out, which changes also the neutron flux and the neutron to gamma ratio. By considering this fact and applying different filters the dose rate can be changed between 1 and 25 rad/s and the neutron/gamma ratio between 1 and 5.

The apparatus is appropriate for the irradiation of tissue cultures, blood samples, plant seeds and small animals (mouse, rat, guinea pig).

Ionic Background of the Post Irradiation Changes in Muscle Excitation

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It has been demonstrated in our laboratory that relatively small doses of ionizing radiation can cause a very significant biopositive change in the excitability of the cross striated muscles. Investigating the origin of this phenomenon, we studied the ionic changes of the muscle and the electrical properties of muscle-membrane systematically. We found that both

the K efflux and the Na influx decrease in the irradiated muscles when compared with the control, non-irradiated ones. The Ca content increases because of the elevation of Ca influx. The electrical resistance measured transversally does not change, but the longitudinal one increases. We never found an increase in membrane permeability after moderate dose of irradiation. According to our measurements the basic factor of the changes in the muscle excitation should be the modification of the electrical structure, and the shift of the calcium content.

The Effect of Small-Dose Beta and Gamma Irradiation on the Ion Content of Striated Muscle

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The changes caused by beta and gamma irradiation in the K content of the striated muscle (m. sartorius of Rana esculenta) were examined as a primary irradiation reaction.

Isolated sartorius muscles were incubated at temperatures of 2°C and 20°C, respectively and, after irradiation with ^{3}H of some thousand rads or ^{60}Co of 4 to 500 rads, the K content in the ash of the muscles was determined with a flame photometer. In our experiments this value was relatively higher than that of the controls incubated under otherwise similar conditions. The effect can be modified with different metabolic inhibitors (2,4-dinitrofluorobenzene, monoiodoacetic acid). The latter observation may have a relation to the data of Kaach, who reported on a temporary increase of O_2 utilization in nerve tissue under similar conditions. Our experiments supply new data on the biphasic radiation reactions of excitable tissues, and offer a basis for supposing that general metabolic factors have to be looked for in their background.

E.p.r. Studies on Frog Nerves Incubated in ^{24}Na, ^{42}K and ^{32}P Ringer Solution, I

J. BELÁGYI, A. NIEDETZKY

Biophysical Institute and Central Laboratory, Medical University, Pécs

The validity of the condition described earlier by Hevesy et al. that in the tracer method, the system is not measurably influenced by the tracer substance, can be controlled by using e.p.r. method to monitor the changes in e.p.r. spectra that may occur in frog nerves during incubation in Ringer solution containing isotopes of sodium, potassium or phosphorus.

N. ischiadicus of the frog (R. esculenta) were immersed in ^{24}Na, ^{42}K, or ^{32}P Ringer solution of 4°C for 24 and 48 hours. The electron resonance spectra were taken at room temperature using a Zeiss ER 9 spectrometer operating at 9370 MHz.

It was found that in all cases the incubated nerves gave a well defined singlette spectrum of 7 G line width the g-value of which did not show any significant deviation from the g-factor of organic free radicals. The free radical concentration in incubated nerves was estimated to be about twice as high as that in the control ones.

E.p.r. Studies on Frog Nerves Incubated in ^{24}Na, ^{42}K and ^{32}P Ringer Solution, II

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The free radical concentration was examined with the e.p.r. method on frog nerves (N. ischiadicus) incubated for 24 hours in isotonic solution containing ^{24}Na, ^{42}K and ^{32}P radioactive isotopes. It was compared with the free radical concentration of the nerve from the same animal, incubated in an inactive solution of the same composition. The free radical concentration of nerves incubated in the radioactive solution was 7×10^{14} on an average, and that in the control nerve 3×10^{14} calculated for 1 g of wet material.

With these experiments we wanted to prove that a radioactive element behaves in a way different from the inactive one as opposed to the basic method of the tracer systems; so the biological system does differentiate between the isotopes of the same element.

The Migration of Electron Excitation Energy between Chlorophylls and Other Molecules in Solutions

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The transfer of electron excitation energy from chlorophyll-b to chlorophyll-a depends on the exciting wave length; in the anti-Stokes range the frequency of transfer decreases. This phenomenon appears also in the photosynthetic productivity; the dry material content of tomato plant grown under interference filters is higher at wave lengths for which a higher frequency of energy transfer from chlorophyll-b to chlorophyll-a was found in solutions.

The dependence of energy transfer on concentration indicates that the Förster theory of energy migration is not valid for mixed solutions containing chlorophyll-b and chlorophyll-a. Because of the strong overlapping of the absorption spectra of the two pigments an exciton type interaction can also occur, especially at higher concentrations; therefore, the transfer of energy takes place according to a distance law between $1/R^6$ and $1/R^3$.

The energy migration between mixed dimeres of chlorophyll-a and chlorophyll-b as well as between carotenes and chlorophylls has found experimental proof in in vitro systems.

These phenomena are important also in the energy migration within the chloroplast.

Energy Migration in Dye-Detergent Systems

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The study of the complex mechanism of light absorption and energy transfer in in vivo systems is difficult for even chemically identical molecules can exist in different physical states in the system. The presence of associates and bonds to the parts of chloroplast result

in the formation of absorption bands different from the bands of in vitro systems. The situation becomes more complicated due to the fact that in vivo systems are very sensitive to light, temperature and experimental conditions.

The experimental difficulties can be partly avoided, if the experiments are performed in model systems well approximating the structure and properties of the photosynthetizing system. In our investigations solutions of detergent containing micellae were used as models.

This paper deals with the formation of micella systems, the energy transfer in them, the absorption properties of dye-detergent systems, and the effect of temperature on the systems. The connection between the absorption and fluorescence spectra of these systems as well as the dependence of the effective temperature of the excited molecules and the lifetime of the excited state are also discussed.

Migration of Electron Excitation Energy in Mixed Solutions of Beta-Carotene and Chlorophyll-a

J. SZABAD

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The absorption, fluorescence and fluorescence excitation spectra of equimolar solutions of beta-carotene and chlorophyll-a mixtures were determined at room temperature in polar and non-polar solvents of 10^{-5} to 10^{-3} M.

The transfer of the energy of electron excitation from beta-carotene to chlorophyll-a was found in a range from 380 to 500 nm of exciting wavelength. The effectiveness of the transfer depends on the wavelength and the nature of the solvent. From the concentration dependence of the transfer the existence of an exciton interaction is concluded; however, the energy transfer can be attributed also to the appearance of mixed dimers.

On the Method of Investigation of Protein Fluorescence

Z. VÁRKONYI

Biophysical Institute, József Attila University, Szeged

The inner, genuine fluorescence and the outer fluorescence of proteins labelled with fluorescent dyes can give informations on the structure and function of proteins. The report briefly summarizes and criticizes the results obtained up to now, and discusses the fluorescence method of investigation of proteins. Several examples demonstrate the exact evaluation of the experimental results to cause considerable changes in the characteristics of fluorescence.

Investigation of the Biological Isotope Effect on Yeast Cells

A. NIEDETZKY

Biophysical Institute, Medical University, Pécs

Yeast cells (Saccharomyces) were cultured at 30°C in BOAS fluid medium containing different concentrations of D_2O and the number of cells in volume unit of the fluid medium was determined every day. The multiplication of yeast cells was expressed in values relative

of the starting value. These values were compared with the number of yeast cells multiplying in a fluid medium of the same composition made of bidistilled water. It was found that in the case of a D_2O concentration over 40 per cent the multiplication of yeast cells decreases significantly and the effect is proportional to the D_2O concentration. With our experimental data we wished to support the validity of the isotope effect in biological systems.

Luminescence of Excitable Biological Tissues Caused by Cu^{2+} Ion

L. KUTAS

Biophysical Institute, Medical University, Pécs

According to our experiments a luminescence can be observed on the frog muscle (M. sartorius) and nerve (N. ischiadicus) incubated in Ringer's solution containing 5×10^{-6} to 10^{-3} M cupricions. The muscle or the nerve of the opposite side, kept in normal Ringer's solution, was used as a control. The incubation was performed at a temperature of 0°C and the optimum of incubation time was 96 to 120 hours. The light was detected with a photoelectron multiplier operating in impulse work, at a temperature of 20°C. At a concentration of 10^{-4} M the intensity of the luminescence was 10^5 photon cm^{-2} min^{-1} in order of magnitude. The intensity was 40, 10 and 0.5 per cent of the previous value at concentrations of 10^{-3}, 10^{-5} and 5×10^{-6} M, respectively. At a concentration of 5×10^{-6} M the copper content determined photometrically in the solution of ashes was 3 to 4 μg/g wet muscle. The copper content of controls was 1 μg/g, and they did not show any measurable luminescence.

On illuminating the muscle or nerve with a visible light, the intensity of light emission decreased remarkably, and returned to the original value in darkness with exponential-like characteristics in about one hour only. Procaine or veratrine added to the incubation medium in a concentration of 0.5 per cent reversibly lowered the luminescence in some hours.

Our result, which is a peculiar biological trace-element effect caused by copper-impurity especially essential in radiobiology, can be considered as an analogy to the activator impurity of inorganic luminophores and can contribute to the investigation of the role of trace elements and biological semiconductors in excitation.

E.p.r. Investigations on Nerve and Muscle Tissues Containing Copper Ions

J. BELÁGYI

Central Laboratory, Medical University, Pécs

It has been already pointed out that on nerve-muscle preparations of the frog (R. esculenta) perfused with Ringer solution containing 10^{-6} to 10^{-4} M Cu^{2+} ions a decrease in the stimulus threshold, and spontaneous series of action potentials can be observed. On the other hand, the scintillations occurring in muscle and nerve tissues showed

a significant increase as compared with the untreated nerves when the nerves and muscles were incubated for 48 to 120 hours in Ringer solution containing copper ions.

In order to obtain information about the role of copper ion, e.p.r. spectra of nerve and muscle tissues kept in Ringer solution containing 5×10^{-5} to 10^{-3} M copper chloride were recorded at room temperature in Carl Zeiss Model ER 9 spectrometer operating at 9560 Mc/s.

It is concluded that after an incubation at 4°C for 72 hours e.p.r. spectra appear referring to copper protein complex ($g_{II} = 2.25$; $g \sim 2.06$; $A_{II} = 0.017$ cm^{-1}).

Frequency Changes in Sensibilized and Illuminated Crayfish Ganglionic System under the Effect of Electrotonic Potentials

T. LAKATOS

Biophysical Institute, Medical University, Pécs

Previous experiments performed in this institute showed that in excitable tissues stained with a sensitizer action potentials were elicited by illumination. This phenomenon supports the assumption that electron processes also take part in the generation of excitation.

Recently, experiments were performed to investigate how the photodynamic excitation was influenced by electrotonic potentials.

In our experimental apparatus a pair of recording electrodes was placed close to the electrodes producing catelectrotone and anelectrotone; the effect of the two electrotones was observed simultaneously on the screen of a double beam oscilloscope. An increase in the spike frequency was found under the effect of catelectrotone, while anelectrotone reduced the frequency.

Our results are in good agreement with the hypothesis formed on the basis of previous experiments.

Semiconductor Property of Frog Muscle Fibre

L. NAGY

Biophysical Institute, Medical University, Pécs

Starting with the results obtained during investigating the semiconductor properties of excitable tissues in this institute, the photoelectric response of these tissues was examined.

In our experiments, the membrane potential of frog sartorius muscle fibre was measured with a microelectrode; the muscle was kept in normal Ringer's solution without any sensibilizing substance. Illumination with an intensive visible light of the environment of the microelectrode inserted into the fibre caused the injury potential to increase with an average of 500 μV; in the case of hyperpolarized fibre we observed an average increase

one order of magnitude higher than that of 2.5 mV. The time course of the phenomenon was also examined.

The operation of the semiconductor photodiode offered itself as the physical analogy to the greater photo effect found in the case of hyperpolarization, because, within given limits the processes taking place in photodiode can be adapted to the behaviour of the muscle fibre in our experiments.

Interaction between Nerve and Muscle Excitation

G. Biró

Biophysical Institute, Medical University, Pécs

The nerve and muscle action potentials generated by an indirect stimulus were simultaneously recorded, on one hand, on frog's nerve-muscle preparationf consisting of N. ischiadicus and M. gastrocnemius — where the middle part of the nerve was connected to the distal part of the muscle by means of a stripe of filter paper moistened with Ringer's solution — and, on the other, in Läwen–Trendelenburg preparation. In the case of both preparations also the muscle action potential appeared in the channel of the nerve record, in addition to the nerve action potential.

The fact that muscle action potential can be recorded from the nerve may draw attention to the following:

1) It is possible to record the nerve and muscle action potential by means of a single amplifier channel.

2) In the case of nerve record in "in situ" circumstances, muscle action potential can be recorded with larger amplitude than nerve action potential.

3) It is worth to examine in further experiments the effect of the presence of muslec action potential on nerve excitation both in the part of the nerve directly touching the muscle and in the part far from the muscle.

Digital Simulation Methodics for the Quantitative Analysis of Periodically Working Biological Automatisms

G. Széphalmi

Automatization Research Institute of the Hungarian Academy of Sciences, Budapest

After a short summary of the difficulties relative of the exact treatment method of biological control systems an integro-differential equation system is described, the mathematical representative of a hormonal control system consisting of an optional number of hormones.

During the discussion of the formula the notion of automatism as well as the feedback number of a control system will be defined in an abstract way.

The possibility of a digital simulation method — that can be applied in the case of periodically working biological automatisms — is brought up for treating the complex mathematical construction unsolvable with classical mathematical methods.

The merits of the recommended computer method are as follows: it removes the difficulties of computation technique without decreasing the complexity and at the same time the rightness of the mathematical control-theoretical model; it is able to "learn", which makes it suitable for finding the unknown parameters of the model-constant process and, at last, it can be used not only in a single concrete case, i.e. it can be considered as a method.

Finally we refer to the results of an application already realized.

About the Role of Receptor Adaptation in the Information Theory

A. TÖRÖK, I. GYŐRI, T. JÁRDÁNHÁZY

Institute of Physiology, Central Laboratory,
Brain Research Institute, Medical University, Szeged

The authors examined from a theoretical point of view the known neurophysiological fact that also receptors can show adaptation. More correctly: the two big groups of receptors generate action potential in a slowing rhythm, after a longer or shorter period of time, upon the effect of a stimulus of constant time intensity. This is an important fact for the living organism because it obtains the information about the state of the environment, among other things, through the intensity of the stimulus. The authors have further developed to some extent their recursive receptor model introduced at the congress of the Hungarian Biophysical Society at Szeged in 1968. Thus the relative information content can be dealt with, because the entrance of the information channel has a memory. Their establishments are presented in a mathematical connection.

Viscosity and Enzyme Kinetics

S. DAMJANOVICH

Biophysical Institute, Medical University, Debrecen

The vital processes are influenced by the temperature of the environment both quantitatively and qualitatively. Temperature can influence the kinetics of the events going on in a liquid medium, most easily by changing diffusion and viscosity, respectively. Taking the relationships

$$\overline{x^2} = \frac{2kT}{6\pi\eta\varrho} \cdot t, \quad \overline{\Theta^2} = \frac{2kT}{8\pi\eta\varrho^3} \cdot t$$

worked out by Einstein and Stokes into consideration, diffusion and viscosity, respectively — not considering the effect of the opposite direction — can be used as synonyms in a given system.

The processes going on in the cells take place in the protoplasm and in the nucleus of cell in a medium of very high viscosity (~ poise). The question is whether the different parameters of reaction kinetics — which are determined in the case of macromolecules usually in thin diluted solutions with a low viscosity (~ centipoise) — can be influenced by the viscosity of the environment. If the answer to this question is in the affirmative, the data of kinetics obtained in vitro can be used for explaining in vivo processes only if appropriate controls are used.

In our present paper we examine the relationship between the viscosity of medium and certain parameters of kinetics, first of all enzyme kinetics. We also deal with biophysical problems arising from the sketchiness of the kinetics of liquids.

Comparative Examination of K^+, Rb^+ and Cs^+ Transport Kinetics in Erythrocytes

S. GYÖRGYI

Biophysical Institute, Semmelweis Medical University, Budapest

The characteristic parameters of K^+, Rb^+ and Cs^+ transport were compared in order to study the ion transport mechanism of erythrocytes. ^{42}K, ^{86}Rb and ^{137}Cs isotopes, respectively, were added to freshly taken rat blood of 37°C and the time dependence of the increase of the activity of erythrocytes was determined. In order to evaluate the experimental results a two-compartment tracer kinetic model was applied. Fitting the function obtained on the basis of the model to the points of measurements we calculated the transfer coefficients characteristic of the probability of the two-direction transport of ions. By comparing them with each other we can state that the probability of the influx of Rb^+ is nearly the same as that of K^+, and that of Cs^+ is smaller; at the same time the sequence of the probabilities of efflux is: Cs^+ K^+ Rb^+. These results can also support the experience that the mechanisms of the inward and outward transport differ from each other.

Three-Compartment System Model for Investigation of K^+, Rb^+ and Cs^+ Transport in Erythrocytes

B. KANYÁR

Biophysical Institute, Semmelweis Medical University, Budapest

The tracer kinetic results obtained by examining the K^+, Rb^+ and Cs^+ transport of rat erythrocytes were evaluated with an analogue computer. For sake of this evaluation a three-compartment model (plasma, membrane, erythrocyte) was supposed which can be described with a linear differential equation system. The transport constants were determined with the aid of the model in the case of all the three ions. Furthermore it became possible

to register the activity of the membrane and the erythrocyte separately, while this separation cannot be made experimentally.

By applying a curve-fitting method the results of measurements were also evaluated in a digital computer, and the transport constants obtained in this way were compared to the values obtained with the analogue computer.

Erythrocyte—Ion Transport and a Comparison of the Physical Parameters of Ions

I. SUGÁR, S. GYÖRGYI

Biophysical Institute, Semmelweis Medical University, Budapest

In connection with the membrane K^+, Rb^+ and Cs^+ transport of erythrocytes an attempt has been made to interpret the differences in the transport of these ions at a molecular level. It was assumed that ions can pass through the membrane at the same places and, that the probabilities of transport are determined in the first place by the probability of the transfer through the lipid layer. So, from a mathematical point of view, the membrane was treated as a homogeneous dielectric medium.

In the case of the above membrane model the ion-membrane interactions were examined in function of the parameters characteristic of the ions. These interactions were separately studied as to the surface and the inside of the membrane. The dependence of the mobility of the cations in the membrane on the ion parameters was determined. The results obtained from our model were compared with the kinetic parameters obtained during the experiments, on the basis of which we can state that the possibilities of transport of cations are principally determined by the interactions proceeding on the surface of the membrane.

Reaction Kinetic Approximation of K^+, Rb^+ and Cs^+ Transport in Erythrocytes

K. BLASKÓ, K. G. BARTHA

Biophysical Institute, Semmelweis Medical University, Budapest

The interaction of K^+, Rb^+ and Cs^+ ions in the process of active transport was examined in the case of rat erythrocytes. Supposing that the three ions get into the erythrocyte by linking to the same place of the membrane, the saturation kinetic model was applied for the quantitative characterization of interactions. Adding inactive alkali ions in different quantities (5 to 30 meq/l plasma) to freshly taken blood of 37°C the speed of the ion uptake of the erythrocytes was determined with the aid of ^{42}K, ^{86}Rb and ^{137}Cs isotopes. In the case of a given ion the other two acted as inhibitor substances. The speed of uptake was also measured with different inhibitor concentrations.

The constants characteristic of the transport of the three ions were determined from the parameters of the straight lines fitted to the data of measurements with the method of the smallest squares.

Comparison of Penetration Characteristics and Bactericidal Effect of Antibiotics

M. SZŐGYI, GY. TAMÁS

Biophysical Institute, Semmelweis Medical University Budapest

The uptake of penicillin, oxacillin, meticillin and ampicillin by Staphylococcus aureus cells was examined. The penetration constants of the enumerated antibiotics were determined. For these same microbes also the bactericidal effect of the above-mentioned antibiotics was measured. On the basis of the comparison of penetration constants and bactericidal effect it can be supposed that the differences in the antibacterial effect of penicillins are in connection with the penetration characteristics of these antibiotics.

Phage Distributions in Liquid Two-Phase Systems

J. FIDY, A. KARCZAG

Biophysical Institute, Semmelweis Medical University, Budapest

For investigation of the structure of bacteriophages with any arbitrary physical method it is previously necessary to purify and concentrate the phage in question. For solving this problem, distributions in two-phase liquid polymer systems can be used. The distribution of ΦX174, λ and MS2 phages and the parameters influencing their distribution were examined in systems of the same type. The applied two-phase systems consisted of the water solutions of sodium dextran sulphate and polyethylene glycol in each case. The systems contained the salts of the M9 synthetic medium in different concentrations, in addition to these basic constituents. We succeeded in selecting a special system in which the phages concentrated in the boundary surface of the two phases in the case of all the three phages. Thus an increase in concentration of 1.5 to 2 orders of magnitude can be reached in one step.

Experiences in E. Coli Continuous Cultures

S. GÁSPÁR, GY. RONTÓ

Biophysical Institute, Semmelweis Medical University, Budapest

One of the greatest advantages of continuous bacterial cultures is that they produce for a long period of time, a physiologically constant bacterial culture. The use in various experiments of such stable cultures is necessary for making the biological investigations quantitative. On the basis of the data in the literature available — and after solving some particular technical problems — a chemostat working with synthetic M9 medium was developed. The characteristics of the apparatus are as follows: a culture vessel of 55 ml; the time of generation can be varied between 0.5 and 10 hours; the maximum concentration

of microbes available in the case of E. coli B and E. coli C 3000 strains is about 3 to 5×10^8/ml. The connection between the adjusted generation time and the phage-microbe interaction was examined in our chemostat. Our apparatus served as a model for a chemostat of larger capacity.

An Indirect Method for Obtaining the Volume-Pressure Curve of Arteries of Extremities

L. DRAGANOV

Institute for Occupational Diseases, Sofia, Bulgaria

The volume-pressure curve of an artery shows the elasticity properties of its wall. Regarding the arterial volume oscillations according to the well-known problem of Laplace we have found a relationship between the changes of the arterial pressure, the arterial oscillogram and the pressure in the cuff. This allows to draw the volume-pressure curve using the data from the arterial oscillogram and from the arterial pressure measured directly or after Korotkov's method. After presenting the curves obtained in this way in dimensionless form, they show a correlation with the clinically established state of the arteries of the investigated persons.

A Molecular Enzyme Kinetic Model

B. SOMOGYI, S. DAMJANOVICH

Biophysical Institute, Medical University, Debrecen

Our present physical knowledge concerning the detailed molecular structure of liquids is far from being complete. As a consequence, the enzyme reactions going on in liquid medium cannot be treated but with some negligence.

It is the aim of this work to elaborate an enzyme kinetic model which gives a molecular meaning to each of the individual kinetic parameters.

On the basis of the proposed model, certain kinetic parameters can be calculated. These parameters are usually determined by fast reaction techniques.

Examination with Ultrasonic Energy of the Conformation Change of d-Glucose and d-Fructose

G. NAGY-GERECZE

Biophysical Institute, Medical University, Debrecen

A connection was found between the molecular structure of the medium conducting ultrasonic energy and the velocity of propagation of ultrasonic ray. This empirical fact was applied for the examination of the change of molecular structure. The time change of the

velocity of propagation of ultrasound of a frequency of 814 kHz was examined in freshly prepared aqueous solutions of d-glucose and d-fructose of different concentrations at a temperature of 23°C. The velocity of propagation was calculated on the basis of wavelength measurements with an acoustic interpherometer. The value of the velocity of propagation increased in function of the frequency until the open chain form changed into the closed ring form. Our results were compared with the time change of the optical activity characteristic of the conformation change of both materials.

The Electronic Structure of Glucose-1-Phosphate

R. GÁSPÁR JR.

Biophysical Institute, Medical University, Debrecen

Calculation was performed for the divalent anionic form of the α-D-glucose-1-phosphate molecule with the extended Hückel method. The charge distribution and the bond-orders were determined in the ground state and in the first excited state of the molecule. On the basis of the results obtained some favourable places in the molecule may be suggested for the enzyme action.

Dielectric Dispersion of Phosphorylase b

J. FITORI

Biophysical Institute, Medical University, Debrecen

The dielectric dispersion curves of rabbit skeletal muscle phosphorylase b were determined in deionized water, heavy water and glycerine solution between 10 kHz and 1 MHz. The measurements were done with an admittance bridge. The electrodes of the measuring cell were made from blackplatinized platinum in order to decrease electrode polarization. Experiments were carried out at 18, 25 and 35°C. The dipole moment and Stokes radius of phosphorylase b molecule were determined from the results obtained. By determining the latter from the relaxation time, a good agreement was obtained with the data measured with other methods. The Cole–Cole parameter calculated from the Cole-Cole plot indicated that the relaxation time showed a distribution. Taking the molecule as a rotation ellipsoid the Perrin factor of the molecule was calculated on the basis of data obtained by electron microscopic measurements. The relaxation time measured by us was greater than that calculated from the data of the molecule approximated with an ellipsoid. The difference can be probably attributed to the hydration of the protein molecule.

On the Problem of the Uptake and State of Water in Seeds

F. VETŐ

Biophysical Institute, Medical University, Pécs

One of the most intensively investigated biophysical problems of our days is the state and role of water in biological systems (e.g. Ernst, 1970; Tait and Franks, 1971). The water content of plant seeds — which is low even in natural state (about 10 to 13 per cent) —

seems to be appropriate for investigating the peculiar qualities of this water. In our experiments we measured the water uptake with swelling of native, living pea seeds and that of lifeless ones denatured by heat treatment, but containing the same amount of water. The change of vapour pressure ("suction force") was also measured.

Results: 1. During the typical water uptake with swelling there is no difference in the change of the relative water content (i) of living and lifeless seeds. Approximately: $i = \frac{127\,t}{t + 3.58} + 11$; where t is the time of swelling in hours (in an interval of time from 0 to 40 hours). 2. The loss in the dry-matter content of living seeds is only a half of that of denatured seeds. The permeability of the denatured ones is higher. 3. In native, living seeds the vapour pressure of water in the first 2 to 3 hours of swelling is significantly lower than that of the denatured seeds containing the same amount of water. So the water of living seeds is less volatile (bound, "polymer"?) than that of the lifeless seeds containing the same amount of water. The destruction of the living seed goes together with the re-arrangement of water structure, the decrease of water binding and the increase of permeability.

Bound Water in Plant Sections

S. PÓCSIK

Biophysical Institute, Medical University, Pécs

Thin slices of plant roots (potato) were made and gradually dried. A gradual increase in the density of plant water and in that of the plant slice was found during the gradual drying just as is the case with muscles. From the experiments we concluded to a gradually increasing water binding of the plant.

Some Data on the Question of the Stretch Induced Crystallization of Myosin

N. GARAMVÖLGYI

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The main question of the discussion going on at present in respect to the structure of smooth muscle is whether myosin filaments will be formed in the course of contraction only or they represent regular constituents of smooth muscle, too. According to our opinion (Garamvölgyi, Vizi, Knoll, 1971) the condition of the establishment of myosin filaments is that the fixation of muscle should be carried out under a certain degree of mechanical tension. In our opinion the lack of myosin filaments in electronmicrographs is a mere artifact. This opinion is supported also by our observation made on bee wing muscle; lack of myosin filaments could have been brought about as an obvious embedding artifact. These phenomena may be interpreted in terms of the crystallization of myosin induced by stretch (Ernst, 1963, 1970). Our observations indicate also that myosin filaments of the resting inactive muscle are subject to mechanical tension as well.

On the Transversal Structure of Myofibril

I. ACHÁTZ

Biophysical Institute, Medical University, Pécs

The existence of a longitudinal structure of muscle fibril has been unambiguously proved by former examinations, but that of the transversal one has remained questionable. During the examinations of this problem the fibril was stretched in a transversal direction with a micromanipulator. After cessation of the stretch the fibril roughly regains its original form and the stretched longitudinal bundles shorten again. Model experiments performed with rubber threads can prove that this phenomenon arises not only from the longitudinal elasticity; the fibril returning to its original form has also transversal connections as shown by other experiments.

Submicroscopic Transversal Structure of Striated Fibrils

K. TROMBITÁS

Central Laboratory, Medical University, Pécs

In the case of the homogenization of fixed fibrils to an extra degree the fibrils break with a regular fracture perpendicular to the longitudinal axis both in the I and the A bands, corresponding to the transversal periodicity of 400 Å noticeable in each case. We succeeded in producing this regular break of fibrils also in native substance in deep-frozen state. These data seem to support the opinion that the transversal periodicity of 400 Å is brought about by a transversal network actually present in the native state, which holds the longitudinal filaments together perpendicularly to their direction.

Localization of Potassium in Atrophied Muscle

N. KÁLLAY

Biophysical Institute, Medical University, Pécs

The intrafibrillar distribution of ^{42}K was examined in the previously denervated gastrocnemius muscle of the rat. About 3 hours after the administration of the isotonic KCl solution intraperitoneally, the animal was killed, bled, and 2 or 3 pieces of the gastrocnemius muscle were prepared for an electron microscopic examination. The sections placed on the grids were covered with a monolayer of Ilford L-4 emulsion. After a 72-hour exposure and photographic treatment the sections were examined in an electron microscope.

In our experiments a significant difference was found between the number of the grains in the denervated and normal muscle fibres. On the side of the normal muscle fibre

we found a grain number about 25 per cent higher than on the previously denervated side. At the same time the proportion of the distribution of the grains — among the parts of the fibril — was not changed by the atrophy.

This difference in the number of grains originating in ^{42}K is attributed to the atrophy-induced change in the state of bound K.

Muscle Excitation and Potassium Mobilization

P. VARGA-MÁNYI

Biophysical Institute, Medical University, Pécs

The effect of direct and indirect stimuli on the change of the K content of the muscle was examined in frog preparations (Rana esculenta). The experiments were performed on normal and anaesthetized preparations. Frogs were stimulated directly through the M. gastrocnemius and indirectly through the ischiadic plexus.

In our experiments a potassium loss of about 10 per cent was found with direct stimulation of the frogs both in normal and anaesthetized state while, in the case of indirect stimulation, no significant potassium difference could be demonstrated in the gastrocnemius of either normal or anaesthetized frogs when compared with the potassium content of the control muscle.

The Structure of Water and Ion Mobility in Polyelectrolyte Solutions

G. MASSZI

Biophysical Institute, Medical University, Pécs

According to previous investigations in this institute the physico-chemical state of muscle-water is changed by water binding with swelling to a significant degree. In connection with these experimental results the question is raised, how the water binding with swelling influences the ionic conductivity of muscle and to what degree are the diffusion processes decreased by it.

According to our microwave measurements performed in gelatine solutions the time of relaxation of the dipole rotation of water significantly increases with the increase of gelatine concentration. On the basis of the Debye equation the increase in the viscosity of water is proportional to the increase of the time of relaxation. Taking this effect into consideration the calculations concerning the ionic conductivity of NaCl and KCl agree well with the measured values. In the case of $CaCl_2$ the measured conductivity is smaller than the calculated ones, which finding can be presumably explained with the Ca binding characteristics of gelatine.

Our investigations indicate that with the aid of microwave measurements there arises a possibility for determining the "real" concentration of biological materials.

Ion-Dependent Change in the Structure of the DNP System of Cell Nuclei

M. KELLERMAYER, K. JOBST

Central Clinical Laboratory, Medical University, Pécs

K^+ and Na^+ ions have a significant role in the functional organization and submicroscopic arrangement of nucleoproteids. In the experiments the ion-dependent change in the structure of DNP of isolated cell nuclei was followed with painted polarization technique. According to our measurements the cell nuclei are isotropic under a cation concentration of 0.07 M and birefringent at higher cation concentrations. Parallel to the submicroscopic morphological examinations the K and Na content of isolated cell nuclei and the volume of the nucleus were determined. A relationship seemed to exist between the intranuclear electrolyte level and the submicroscopic change of the structure of DNP, i.e. the development of nucleus anisotropy.

The Time Relations of Initial Volume Decrease and Contraction in Frog Muscle

B. SCHÄFFER

Biophysical Institute, Medical University, Pécs

During indirect stimulation of unstretched frog gastrocnemius muscle we simultaneously examined the initial volume decrease and contraction of the muscle, and its action potential and contraction, respectively, in a temperature range from 4 to 24°C.

It was found that in the function of time, both the latency and the time passed up to reaching the maximum of mechanic activity differ markedly from the appropriate time data of the action potential and the volume decrease.

Relationship between Initial Volume Decrease and Action Potential in Frog Muscle

F. ARADI

Biophysical Institute, Medical University, Pécs

The initial volume decrease and the action potential were investigated simultaneously on ischiadicus and gastrocnemius preparations excised from frog (Rana esculenta).

During single twitches the changes of relative time data referring to volume decrease and action potential run parallel, in function of the temperature; moreover, the absolute value of the maximum volume decrease correlates to the descending branch of the action potential in the temperature range between 4 and 24°C.

The "dead time" of the volume decrease and the absolute refractory period of the action potential measured by applying two consecutive electric stimuli were identical. The minimum value of them was found smaller than 1 ms.

Diffusion Inhibition in a Gap Next to a Permeable Layer and the Balneotherapeutic Effect

L. HOMOLA

Ambulatory Clinic, Pécs

In a paper appeared in Acta Biochim. Biophys. Acad. Sci. Hung. 5 (3) p. 365 (1970) the author described that a stream of water comes about in the narrow gap between a layer permeable for the solute but permitting osmosis (e.g. parchment) and a not permeable layer hindering diffusion, if solved material and then water flow into the gap through the permeable layer, and that thin solution can flow out from the gap to a place of lower pressure. The flowing water carries along solutes to a degree permitted by the structure of the gap. Two such gaps can be connected into a system and the area of each gap can be different. In this case a water and solved material transport seeming "active" can come about in the case of the continuous production of an osmotically effective "driving" material hindered in its diffusion and adequate for the gap structure. Such a system seems to be utilized not only in the explanation of "active" transport processes but also of the mode of action of balneotherapeutic water. Chemical compounds seem to be able to increase the metabolism of the cell after getting through the structure of the surface of the cell. The diffusion of the agent out of the cell also exerts an effect after finishing the bath (bath-reaction). According to the experience some smaller material changing the gap-structure is also effective, e.g. fluorine and certain radiations, too, e.g. radioactive radiation.

High-Speed, Automatic Skin Resistance Measurement

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Research Institute of Physical Education
and Computation Center of the Ministry of Heavy Industry, Budapest

In the past years several papers appeared the authors of which attributed diagnostic significance to skin resistance maps. The task to solve was as follows: the skin resistance map is to be made on the basis of the many data of measurements obtained at different points of the skin surface. But at the time of measurement the value of skin resistance changes in consequence of the effect of several factors. If one works with the traditional technique, a measurement takes several seconds. Considering that the time constant of the resistance change falls within this order of magnitude, the accuracy of the results is limited. The electrode polarization, the skin galvanic reflex, the effect on the resistance, of the change in skin temperature, etc., can be mentioned as causes of the time dependence of skin resistance.

It is obvious from the above mentioned factors that reliable and reproducible results can only be reached by introducing methods with the aid of which the resistance measurement can be performed at several points of the skin surface within a fraction of a second. For the high-speed automatic measurement of skin resistance the following method has been developed by the authors. A control unit opens the gate circuit series connected with the electrode wire one after the other at equal periods of time. The voltage appearing between the electrode belonging to the open gate and the common electrode is proportional to the resistance of the skin surface under the electrode. The analogous sign obtained in

this way is recorded with the facsimile program of a multi-channel analyser. The ordinate of each point of the result-diagram corresponds to one of the resistance values. The method was further developed by the authors by eliminating the common electrode of large surface through a parallel connection of the electrodes belonging to the inactive gates.

Thermo-Osmotic Model Experiment on Egg

F. VETŐ

Biophysical Institute, Medical University, Pécs

According to previous communications from this institute temperature gradient is not a negligible factor in transport processes. Our present work corroborates this finding. It is commonly known that the shell of hard boiled egg can be easily separated if it is put into cold water while being still hot. As a consequence of temperature gradient some liquid gathers under the membrana testacea. The degree of concentration of this liquid was measured with a Hewlett-Packard vapour pressure osmometer and, in 46 measurements, it was found to be 180 ± 13 mOsm. In the same eggs the concentration of the pressed fluid of protein gel is 223 ± 24 mOsm. So the temperature gradient — corresponding to thermo-osmosis — produces a hypotonic liquid (4/5 conc.).

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