VOLUME 5
NUMBER 1
1970

Acta biochimica et biophysica

ACADEMIAE SCIENTIARUM HUNGARICAE

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Acta Biochimica et Biophysica

Academiae Scientiarum Hungaricae

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Volume 5

Akadémiai Kiadó, Budapest
1970

Acta Biochimica et Biophysica

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (1), pp. 1–8 (1970)

Nucleotide Antagonism in the Hormone-sensitive Lipolysis of Fat Tissue

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(Received July 4, 1969)

The changes of epinephrine-induced lipolysis in rat epididymal fat bodies incubated in albumin-free Krebs–Ringer phosphate buffer were studied after the addition of various nucleotides (c-AMP, AMP, and ATP). Lipolysis was followed by measuring the accumulation of fatty acids in fat tissue and the release of glycerol into the medium.

Our experiments confirmed the earlier finding that epinephrine-induced lipolysis is reduced upon the addition of either AMP or c-AMP. However, in the presence of theophylline c-AMP increased lipolysis, whereas the effect of AMP and ATP remained unchanged.

The mechanism of this phenomenon was studied with fat tissue incubated at a theophylline concentration which, by itself, did not enhance lipolysis. AMP appeared to inhibit the activating effect of c-AMP on hormone-sensitive lipase. The inhibition was proportional with the logarithm of AMP concentration and affected both free fatty acid and glycerol production. The inhibitory effect of AMP proved to be kinetically competitive with respect to both c-AMP and epinephrine.

The results of hormone-sensitive lipase activation are discussed on the basis of the hypothesis put forward by the Sutherland-group. However, in the interpretation of the control of hormone-sensitive lipolysis, emphasis is laid on the c-AMP—AMP equilibrium and the phosphodiesterase activity that plays some role in it.

From the point of view of the interactions between the living cell and its environment, those biochemical mechanisms which form a link between hormonal and cellular metabolism are of special interest. According to our present knowledge such a process is the formation of c-AMP, which is being regarded as the intracellular mediator substance for an increasing number of hormones (Haynes, 1958; Marsh et al., 1966; Sutherland, Robison, 1966). c-AMP seems to be involved also in the appearance in fat tissue of hormone-sensitive lipase activity (Butcher, et al., 1965). It is assumed that the nucleotide participates in the activating process of an enzyme present in the inactive form. We know very little about this process, but it appears to be certain that in addition to c-AMP other factors also play a role in it. c-AMP when added to fat tissue in vitro does not enhance lipolysis in contrast to e.g. the catecholamines, moreover, it decreases lipolysis induced by the latter (Vaughan, 1960; Dole, 1961). Its effect is similar to that of nucleosides and non-cyclic nucleotides (Dole, 1961; Kappeler, 1965). This inverse effect of exogenous c-AMP raises the idea that in the control of hormone-sensitive lipase activity, in addition to c-AMP, AMP may also participate. Since the actual ratio c-AMP/AMP might be considerably influenced by phosphodiesterase, the enzyme

that catalyses the conversion of c-AMP to AMP (Sutherland, Rall, 1958), in the present paper the effect of nucleotides on lipolysis was studied in fat tissue, in which phosphodiesterase activity had been inhibited with methylxanthine to various extents. In these investigations another nucleotide, ATP, which is known to take part in the formation of c-AMP (Rizack, 1964), was also included.

Materials and Methods

Male Wistar rats of 250 to 300 g weight were used in the experiments; the animals were not starved before use. After decapitation the epididymal fat pads were immediately removed and put into Krebs—Ringer phosphate buffer, pH 7.4. The fat pads were then cut into 30 to 50 mg pieces with a pair of scissors, and after blotting them with filter-paper 170 mg portions were weighed out, each portion put into a 25-ml conical flask which contained 1.7 ml of Krebs–Ringer phosphate buffer, pH 7.4. The substances to be tested were then added in a volume of 0.1 ml to the incubation mixtures. The final volume of the incubation mixture was 2.0 ml. The incubation was carried out at 37 °C with shaking (100 rev. per minute) for one hour. The gas phase was air.

The extent of lipolysis was determined by measuring the amount of free fatty acid accumulated in the fat tissue and of glycerol released into the medium (Rudman et al., 1964). For glycerol determination 0.2 ml samples were withdrawn from the incubation mixture, and the rest, together with the fat tissue, was homogenized in a Potter-Elvehjem-type tissue grinder after the addition of 10 ml of Dole extracting solution. The free fatty acid content of the homogenate was determined according to the method of Dole and Meinertz (1960). Glycerol was measured by the method of Korn (1955). The difference between the amounts of glycerol (expressed in micro-moles) and fatty acid (expressed in microequivalents), respectively, in the samples before and after incubation was extrapolated to 1 g of fat tissue (wet weight).

1-Epinephrine (Koch—Light Labs.) was dissolved in the hydrochloride form. Theophylline (Calbiochem), c-AMP (Calbiochem), AMP and ATP (Reanal, Budapest) were dissolved in water. Dilutions were made with distilled water.

Results

Epinephrine at concentrations higher than 6×10^{-8} M increases the lipolysis of rat epididymal fat cells in vitro (Weiss et al., 1966). We used 50 times higher concentrations; an average increase of 4.2 μeq in fatty acid accumulation and of 1.7 μmoles in glycerol release per g of wet fat tissue per hour was observed with 6 rats. When c-AMP, AMP or ATP was also added to the fat tissue, only about half of the above increase occurred (Table 1). The hormone-sensitive lipolysis was thus reduced by all three nucleotides.

When immediately before epinephrine theophylline was added to the incubation mixture, a further increase of fatty acid accumulation could be detected

in the fat tissue; in the presence of over 5×10^{-4} M theophylline there was a steep increase in lipolysis up to a maximum. (The same level could also be attained with theophylline alone, but only at a concentration one order of magnitude higher.) As shown in Fig. 1, c-AMP at various theophylline concentrations behaved as epinephrine, moreover, even at the lowest theophylline concentration applied it did

Table 1

Effect of epinephrine and nucleotides on lipolysis in fat tissue

Incubation mixture as described in Methods.
Epinephrine concentration 3×10^{-6}M, nucleotide concentration 5×10^{-4}M

Added substance	Lipolysis	
	Fatty acid μeq/g/hr	Glycerol μmoles/g/hr
—	0.7±0.2*	0.3±0.1
Epinephrine	4.9±0.7	2.0±0.4
Epinephrine+ATP	3.1±0.5	1.3±0.3
Epinephrine+AMP	2.3±0.3	1.2±0.2
Epinephrine+c-AMP	2.8±0.5	1.4±0.2

* Average value and standard deviation from 6 experiments

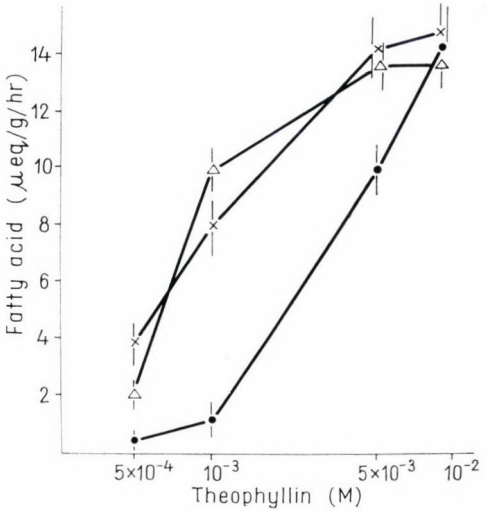

Fig. 1. Effect of epinephrine and c-AMP on lipolysis in the presence of various theophylline concentrations. The incubation mixture described in Methods was completed with theophylline (●) at various concentrations, and with 3×10^{-6} M epinephrine (x) or 5×10^{-4} M c-AMP (△). The points indicate the average values from 4 experiments, the vertical lines represent the deviation of the mean

not reduce lipolysis. From the curves one can choose a theophyllin concentration which by itself scarcely enhanced fatty acid accumulation, whereas it increased the effect of epinephrine or c-AMP several-fold. In our system this concentration proved to be 1×10^{-3} M. In the standard test for the further study of nucleotide interaction theophylline was added at this concentration to the incubation mixture described in Methods.

Table 2 shows the effect of nucleotides plus epinephrine on lipolysis in the standard test. It can be seen that in the presence of theophylline there was a difference in the effect of nucleotides: c-AMP was able to increase hormone-sensitive lipolysis even at 5×10^{-6} concentration whereas AMP or ATP decreased lipolysis, just as without theophylline (cf. Table 1).

Table 2

Effect of nucleotides on the hormone-sensitive lipolysis in fat tissue after the addition of theophylline

The incubation mixture described in Methods also contained 1×10^{-3}M theophylline and 3×10^{-6}M epinephrine

Added substance	Concentration M	Lipolysis	
		Fatty acid μeq/g/hr	Glycerol μmoles/g/hr
—		8.8±1.1*	3.6±0.7
ATP	5×10^{-4}	4.5±0.4	
AMP	5×10^{-4}	3.9±0.5	1.7±0.3
	5×10^{-5}	4.5±0.4	2.8±0.3
	5×10^{-6}	6.3±1.0	3.6±0.6
c-AMP	5×10^{-4}	12.7±1.4	5.0±0.7
	5×10^{-5}	10.3±0.9	
	5×10^{-6}	9.8±0.5	

* Average value and standard deviation from 6 experiments

Since in the standard test c-AMP proved to be an activator of lipolysis both with epinephrine and alone, whereas the non-cyclic nucleotides inhibited hormone-sensitive lipolysis, we examined the kinetic relationship, without added epinephrine, between diester and monoester nucleotides with respect to their influence on lipolysis.

The results of one of these experiments are shown in Fig. 2. The addition of 1×10^{-3} M theophylline and 1.25×10^{-4} M c-AMP induced 10.6 μeq/g/hr fatty acid production and 4.4 μmoles/gr/hr glycerol release in the fat tissue. These values did not change when AMP in 1/50 of the amount of c-AMP was added; however, higher amounts exhibited a reducing effect proportionally to the logarithm of concentration. Both fatty acid and glycerol production were inhibited;

this is also proved by the fact that at the various AMP concentrations tested the fatty acid/glycerol ratios were almost identical (2.70, 2.77, 2.50).

The kinetic aspects of the antagonism between c-AMP and AMP in lipolysis were also studied at various activator and inhibitor concentrations. The results

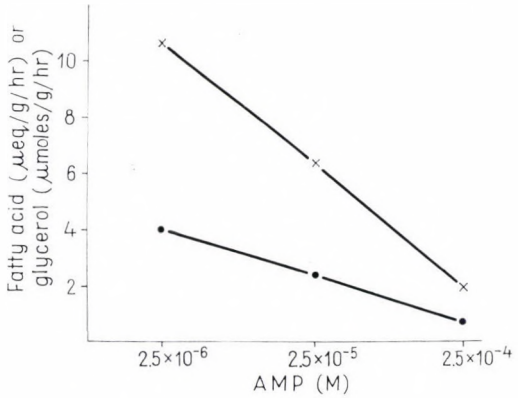

Fig. 2. Effect of AMP on the c-AMP-induced lipolysis of theophylline-treated fat tissue. The incubation mixture contained 1×10^{-3} M theophylline, 1.25×10^{-4} M c-AMP and various amounts of AMP. Free fatty acid content: x, glycerol release: ●

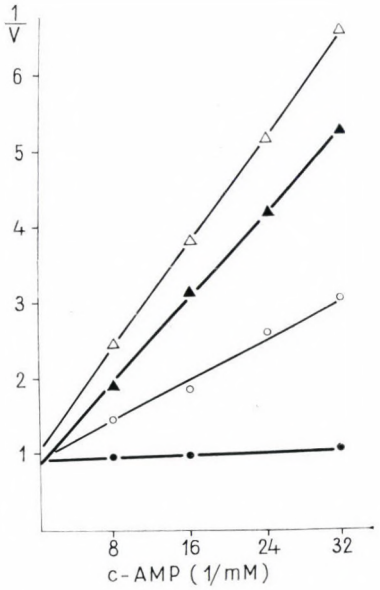

Fig. 3. Lineweaver—Burk plot of the inhibition by AMP of c-AMP-induced lipolysis. AMP concentrations: 0.0 M (●), 0.31×10^{-4} M (o), 0.62×10^{-4} M (▲), and 1.25×10^{-4} M (△). The ordinate shows the reciprocal of the change in free fatty acid content in μeq/g/hr, multiplied by 1000. Incubation was carried out in the standard test system

are presented in a double-reciprocal plot (Lineweaver, Burk, 1934) in Fig. 3. On the abscissa the reciprocal of c-AMP concentration is shown, whereas the ordinate gives the reciprocal of the rate of lipolysis in terms of fatty acid accumulation. The regression straight lines, each representing a different inhibitor (AMP) concentration, converge and have a common intercept on the ordinate. Consequently, the two nucleotides operate in a competitive manner in the control of lipolysis.

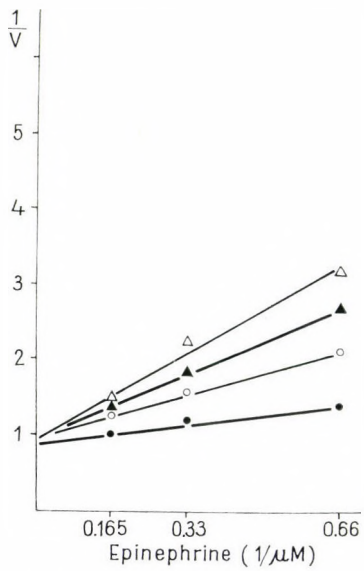

Fig. 4. Lineweaver—Burk plot of the inhibition by AMP of epinephrine-induced lipolysis. AMP concentration: 0.0 M (●), 0.31×10^{-4} M (o), 0.62×10^{-4} M (▲), and 1.25×10^{-4} M (△). The ordinate shows the reciprocal of the change in free fatty acid content in μeq/g/hr, multiplied by 1000. Incubation was carried out in the standard test system

The intercept on the ordinate gives the maximal effect, E_{max}: 12 μeq fatty acid/g/hr. This value agrees well with the direct measurements (cf. Fig. 1). A similar competitive pattern is obtained for AMP when epinephrine is used instead of c-AMP in the standard test (Fig. 4).

Discussion

Our results confirm the earlier observation (Dole, 1961; Kappeler, 1965) that monoester nucleotides inhibit the hormone-sensitive lipolysis in fat tissue. In addition, we have also found that there is a competition between monoester and cyclic diester nucleotides with respect to the above effect. This competition could be detected by using a methylxanthine derivative, theophylline.

It is known, that methylxanthines inhibit phosphodiesterase, the enzyme that inactivates c-AMP (Butcher, Sutherland, 1962). The reversal of the effect of

c-AMP following theophylline addition can also be attributed to the inhibition of phosphodiesterase. This inhibition is only partial: according to Weiss (Weiss et al., 1966) the theophylline concentration used in our standard test decreases phosphodiesterase activity only by 1/3. But even such a slight inhibition may induce exogenous c-AMP to act in the same way as the endogenous, intracellular accumulation of this nucleotide does. At the same concentration of coffeine epinephrine increases the c-AMP content of fat cells nearly 30-fold (Butcher, Sutherland, 1968). This is presumably connected with the specific membrane localization of the hormone-sensitive lipolytic system (Rizack, 1964).

Our results provide further support for the participation of c-AMP in hormone-sensitive lipolysis. The earlier experimental basis for this idea was the finding that the c-AMP content of the tissues changed parallel with lipolysis (Butcher et al., 1965), and that a nucleotide derivative, N^6-2'-0-dibutyryl-c-AMP, increased lipolysis (Posternak et al., 1962; Butcher et al., 1965). However, it has recently been shown that the effect of this nucleotide derivative is not identical with that of c-AMP (Goodman, 1969). In our standard test system we could demonstrate with c-AMP the following; 1. c-AMP not only increases the lipolytic effect of epinephrine, but also elicits it without epinephrine; 2. AMP reduces lipolysis induced by either epinephrine or c-AMP; 3. there is a competitive antagonism between c-AMP and AMP just as between epinephrine and AMP. These results also indicate that the mediator of epinephrine-induced lipolysis is c-AMP.

The present investigations allow us to draw some conclusions concerning the mechanism of action of AMP in the lipolysis in fat tissue. This has not been so far studied in detail. According to Dole the insulin-like effect of nucleosides and nucleotides is due to an enhancement of lipogenesis (Dole, 1962). Our experiments revealed an antagonism between cyclic diester- and monoester-nucleotides; this antagonism exists also in the absence of epinephrine. Thus it follows that the competition between the nucleotides takes place on the level of lipase rather than adenyl-cyclase. In this way AMP would participate in the so far unknown activating process of inactive lipase, by counteracting the effect of c-AMP. This role of AMP is fundamentally different from that of other substances reducing lipolysis. Alpha-adrenolytic agents act also on the level of lipase but not in a competitive manner; the other natural tsubstance that competitively inhibits lipolysis, prostglandin, competes with ATP on the level of adenylcyclase (Stock, Westermann, 1967). In our experiments ATP and AMP behaved in a similar manner; it can thus be assumed that exogenous ATP has undergone dephosphorylation and exerted its effect as AMP.

Our observations fit into the theory of the Sutherland-group about the mechanism of hormone-sensitive lipolysis (Butcher, Sutherland, 1968). However, in their scheme AMP is only a degradation product of c-AMP, whereas our experiments suggest that it is an active participant in the control of lipolysis. In our opinion the activity of lipase is determined by the concentration ratio of c-AMP and AMP prevailing in that part of the cell. Consequently much greater significance is to be attributed to phosphodiesterase in the control of hormone-sensitive lipolysis, since this enzyme plays an important role in the regulation of the concentration ratio of the two nucleotides.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (1), pp. 9–18 (1970)

The Influence of Allosteric Effectors on the Conversion of Phosphorylase-*b*

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(Received July 23, 1969)

The influence of allosteric effectors inducing a conformational change of phosphorylase-*b* was studied in the phosphorylase-*b* → phosphorylase-*a* reaction.

Glucose-6-P reduces the reaction rate of *b*-kinase. This inhibition increases with increasing glucose-6-P concentrations and decreases with increasing amounts of phosphorylase-*b*.

AMP alone has no effect on the reaction rate of *b*-kinase, but suppresses the inhibition caused by glucose-6-P. This suppressing effect of AMP decreases with increasing amounts of phosphorylase-*b* at identical glucose-6-P concentrations.

Glycogen increases the reaction rate of *b*-kinase. The degree of activation is directly proportional to the glycogen concentration and inversely proportional to the amount of phosphorylase-*b*. The activating effect of glycogen may be neutralized and even surpassed by the inhibitory effect of glucose-6-P. The activation curve of glycogen and the inhibition curve of glucose-6-P have a sigmoidal character.

From these observations it is concluded that the applied effectors (glucose-6-P, AMP, glycogen) most probably cause a conformational change of the phosphorylase-*b* which serves as substrate and that this change results in an inhibition or activation of the *b*-kinase reaction.

Phosphorylase (E.C.2.4.1.1: α-1,4-glucane: orthophosphate glucosyl transferase) is known to occur in the muscle in both the active form *a* and the inactive form *b*. The conversion of phosphorylase-*b* into phosphorylase-*a* is performed by phosphorylase-*b* kinase (E.C.2.7.1.38; ATP: phosphorylase phosphotransferase). The conversion rate highly depends on whether *b*-kinase* is in an activated or non-activated state. While non-activated *b*-kinase will promote conversion only at pH 8.2, the activated form is capable of effecting this process also at pH 6.8 (Krebs et al., 1959, 1964). There are several reports on the mechanism of action of *b*-kinase and on the activation of the non-activated form (Krebs et al., 1959; Krebs, Fischer, 1960; Krebs et al., 1964; Meyer et al., 1964; Krause, Wollenberger. 1967; DeLange et al., 1968; Riley et al., 1968; Mayer, Krebs, 1968; Walsh et al., 1968). However, only few papers refer to the influence of various intermediates and nucleotides on the conversion activity of *b*-kinase. Krebs et al. (1964) have reported that glucose, glucose-6-P and excess ATP will inhibit, while glycogen will

* Abbreviations used in the text: *b*-kinase = phosphorylase-*b* kinase; glucose-6-P = glucose-6-phosphate; glucose-1-P = glucose-1-phosphate; UDPG = uridine-diphosphate-glucose; AMP = adenosine-5'-phosphate.

enhance the rate of conversion of phosphorylase-*b*. Glucose-1-P, UDPG, AMP and NaF have no effect on conversion (Krebs et al., 1964; DeLange et al., 1968).

The possibility arises that the effectors which influence the reaction rate of *b*-kinase modify phosphorylase-*b* which serves as a substrate rather than *b*-kinase itself. This assumption is supported by the allosteric sensitivity of phosphorylase-*b*. The activity changes which are easily brought about under the influence of effectors point to this allosteric sensitivity. Such conformational changes affect, however, beside the activity also the convertibility and conversion rate of phosphorylase-*b*.

In the present communication it will be shown that the conversion reaction $b \rightarrow a$ is activated by glycogen and inhibited by glucose-6-P and that these effects depend on the quantity of phosphorylase-*b*. It will be further shown that the modifying effect of AMP on phosphorylase-*b* is involved in this reaction too, that AMP is capable of suppressing the inhibition caused by glucose-6-P, and that the degree of suppression also depends on the amount of phosphorylase-*b*.

Materials and Methods

Phosphorylase-b

Crystalline phosphorylase-*b* was prepared from the skeletal muscle of rabbit by the method of Fischer and Krebs (1962). The enzyme was first crystallized from a 0.05 M glycerophosphate – 0.05 M mercaptoethanol buffer (pH 6.8) containing 0.1 M magnesium acetate and 0.01 M AMP. For the second crystallization the same buffer was used, but without magnesium acetate and AMP. The nucleotides were removed from the preparation by treatment with Norit cellulose (Fischer, Krebs, 1958). The absorption of the phosphorylase preparation obtained by this procedure was measured at 260 and 280 mμ. The ratio $E_{260}/E_{280} = 0.59$ is in a good agreement with literary data which vary between 0.54 and 0.60 (DeVincenzi, Hedrick, 1967; Bresler, Firsov, 1968). Repeated treatment with Norit-cellulose did not alter the value of the above ratio.

Phosphorylase-b kinase

Phosphorylase-*b* kinase was prepared from rabbit muscle by the method of Krebs et al. (1964). The purified enzyme was converted in the presence of Ca^{++} into activated kinase (Krebs, Fischer, 1962). The product was stored without an activity loss in 0.05 M glycerophosphate – 0.002 M EDTA buffer (pH 7.0) in a frozen state.

Glycogen

Glycogen was prepared from rabbit liver by extraction with trichloroacetic acid and repeated precipitation with alcohol. The nucleotides were removed during preparation by Norit treatment (Helmreich, Cori, 1964).

Measurement of the activity of phosphorylase-b kinase

The activity of *b*-kinase was measured by the method of Krebs et al. (1964) in a slightly modified form. Various amounts of phosphorylase-*b* in 0.035 M glycerophosphate – 0.033 M mercaptoethanol buffer (pH 6.8) were added to the incubation medium. *b*-Kinase in 0.0025 M mercaptoethanol – 0.02 M Tris – 0.02 M glycerophosphate buffer (pH 6.8) was added in an amount which converts the 10 per cent of the phosphorylase-*b* added during incubation. Incubation was for five minutes at 30 °C and pH 6.8. The reaction was started by adding Mg^{++} (0.01 M) and ATP (0.003 M). After incubation the reaction was stopped by the addition of 0.04 M glycerophosphate – 0.02 M EDTA buffer (pH 6.8). The quantity of phosphorylase-*a* formed during the reaction was determined, after appropriate dilution, according to Illingworth and Cori (1953) by measuring the amount of inorganic P liberated from glucose-1-P. Phosphorus was determined by the method of Taussky and Shorr (1952).

The quantity of phosphorylase-*b* and phosphorylase-*a* was expressed in units. One unit is that amount of the enzyme which liberates 1 μmole inorganic P/min, with or without 1 mM AMP.

One unit of *b*-kinase corresponds to that amount of the enzyme which produces one unit of phosphorylase-*a* in one minute in the presence of about 200 U/ml of phosphorylase-*b*.

Protein content was determined by the method of Velick and Wicks (1951).

Results

The effect of glucose-6-P on the conversion of phosphorylase-b

As mentioned in the introduction the inhibitory effect of glucose-6-P on the activity of *b*-kinase has been pointed out by several authors (Krebs et al., 1964). This inhibition may be caused not only by any eventual modification of *b*-kinase, but also by some change in the conformation of phosphorylase-*b* which serves as a substrate. This question was studied by varying in the conversion reaction not only the concentration of the inhibitory agent, glucose-6-P, but also that of the substrate, phosphorylase-*b*. The results are shown in Fig. 1. Side "A" shows the changes in reaction rate, side "B" the inhibitory effect of glucose-6-P.

It can be seen in Fig. 1 (side "A") that the conversion rate decreased with increasing concentrations of glucose-6-P. The curves representing the reaction rate have a sigmoid shape for all three phosphorylase-*b* concentrations. Side "B" which shows percentage inhibition demonstrates the significant inhibition caused by as little as 1–2 mM glucose-6-P in the presence of small quantities (100 U) of phosphorylase-*b*. However, when the quantity of phosphorylase-*b* was higher (160 U and 860 U) the same quantity of glucose-6-P was ineffective and only higher concentrations caused inhibition. The sigmoid character of the rate curves and the latent period indicated by the percentage inhibition curves point to the allosteric nature of glucose-6-P inhibition whose degree will depend on the quantity of phosphorylase-*b*.

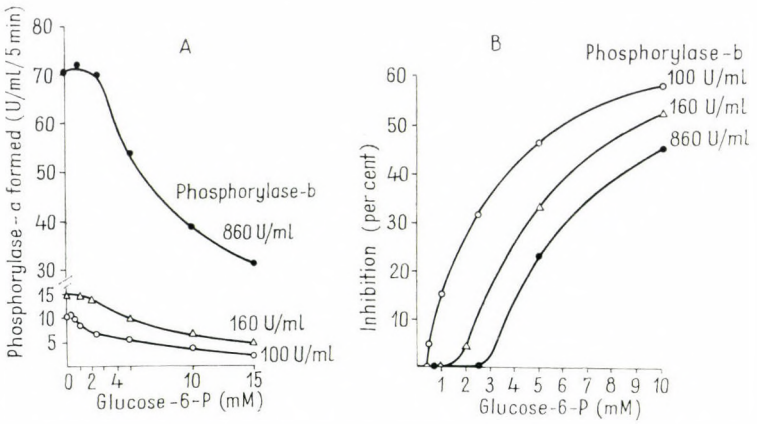

Fig. 1. The effect of glucose-6-P on the conversion reaction phosphorylase-*b* → phosphorylase-*a* in the presence of different amounts of phosphorylase-*b*. Composition of the incubation mixture: 100, 160 and 860 U/ml of phosphorylase-*b* in 0.033 M glycerophosphate — 0.033 M mercaptoethanol buffer (pH 6.8); 2.9 U/ml of *b*-kinase in 0.0025 M mercaptoethanol — 0.02 M Tris — 0.02 M glycerophosphate buffer (pH 6.8); ATP-Na 0.003 M, Mg-acetate 0.01 M, glucose-6-P in the concentrations indicated. The quantity of the phosphorylase-*a* formed was determined after a 1 : 20 dilution with 0.040 M glycerophosphate — 0.002 M EDTA (pH 6.8)

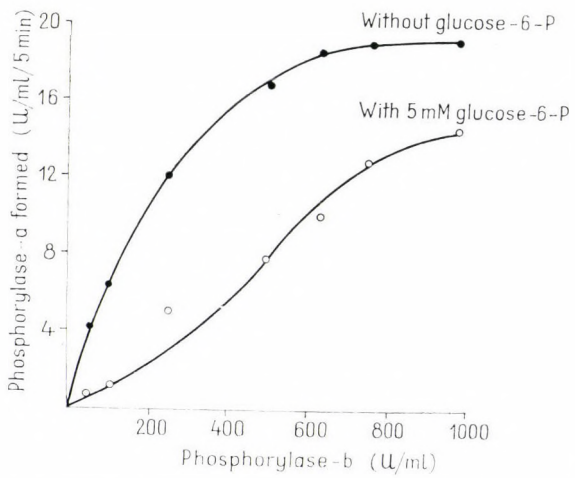

Fig. 2. The effect of glucose-6-P on the saturation of phosphorylase-*b*-kinase. Composition of the incubation mixture: 50—1000 U/ml of phosphorylase-*b* in 0.033 M glycerophosphate — 0.033 M mercaptoethanol buffer (pH 6.8); 2.4 U/ml of *b*-kinase in 0.0025 M mercaptoethanol — 0.02 M Tris — 0.02 M glycerophosphate buffer (pH 6.8); ATP-Na 0.003 M, Mg-acetate 0.01 M, glucose-6-P (if any) 0.005 M. The quantity of the phosphorylase-*a* formed was measured after dilution (1 : 20) with 0.040 M glycerophosphate — 0.002 M EDTA (pH 6.8)

For a further investigation of this phenomenon the saturation curve of *b*-kinase was plotted with and without the inhibitory agent, glucose-6-P. Fig. 2 shows the initial rate of the *b*-kinase reaction for increasing quantities of phosphorylase-*b*.

It is clear from Fig. 2 that with increasing quantities of phosphorylase-*b* the rate of its conversion increased hyperbolically when no inhibitory agent was present. In the presence of glucose-6-P, however, the saturation curve had a sigmoid shape. Accordingly, in the presence of small quantities of phosphorylase-*b* glucose-6-P had a marked inhibitory effect which later decreased as the quantity of phosphorylase-*b* was increased. The fact that the shape of the saturation curve takes a sigmoid character in the presence of the inhibitory agent points to an allosteric effect. Studies on the effect of AMP have indicated that this allosteric effect could be attributed to a conformational transformation of the substrate phosphorylase-*b*.

The effect of AMP on the conversion of phosphorylase-b and on the inhibitory action of glucose-6-P

In experiments aimed at suppressing the inhibitory effect of glucose-6-P by ATP or AMP it was found that a proportional rise in the concentration of ATP and Mg^{++} did not influence the inhibitory effect of glucose-6-P.

The effect of AMP was first studied on a *b*-kinase reaction without inhibitory agent. It may be supposed that AMP as the most powerful allosteric activator of phosphorylase-*b* will modify the reaction rate of *b*-kinase by influencing the substrate, phosphorylase-*b*. Our results, however, have shown that the reaction rate of *b*-kinase was the same both with and without AMP. Another effect was, however, observed when AMP was used in a reaction inhibited by glucose-6-P. In this case AMP greatly reduced the inhibitory effect of glucose-6-P. Fig. 3 shows the effect of AMP with equal amounts of phosphorylase-*b*.

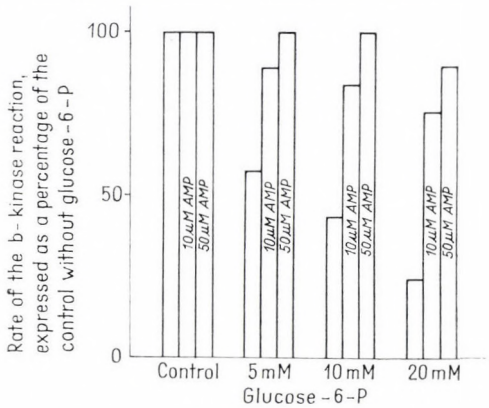

Fig. 3. The effect of AMP on the *b*-kinase reaction with and without glucose-6-P. Composition of the incubation mixture: 100 U/ml of phosphorylase-*b* in 0.033 M glycerophosphate — 0.033 M mercaptoethanol buffer (pH 6.8); 2.02 U/ml *b*-kinase in 0.0025 M mercaptoethanol — 0.02 M Tris — 0.02 M glycerophosphate buffer (pH 6.8); ATP-Na 0.003 M, Mg-acetate 0.01 M; glucose-6-P and AMP in the concentrations indicated

It is seen from Fig. 3 that AMP, without the inhibitory agent, had no effect on the reaction rate of *b*-kinase (control). In reactions inhibited with 5 and 10 mM of glucose-6-P 10 μM of AMP reduced inhibition and 50 μM of AMP completely suppressed it. The inhibition caused by 20 mM glucose-6-P was only reduced but not fully suppressed with both AMP concentrations. Hence AMP had no effect on the conversion reaction, which was not inhibited, but suppressed the inhibition of the inhibited reaction (liberator effect).

Furthermore, there was but a slight difference in the effects of the two AMP concentrations (10 and 50 μM). This, however, was only valid when the quantity of phosphorylase-*b* to be converted was the same. In the next experiments in

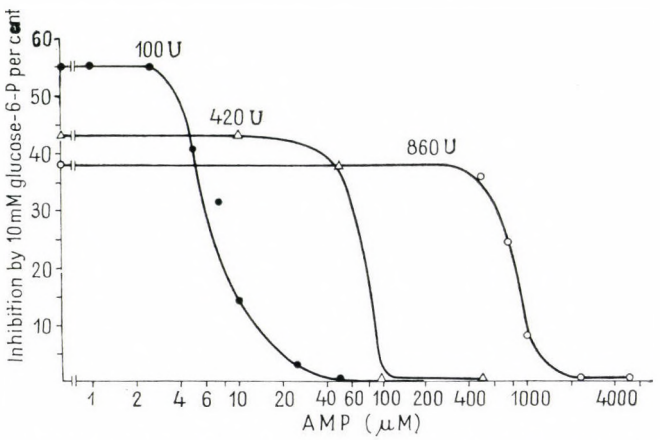

Fig. 4. The effect of AMP concentration and of the amount of phosphorylase-*b* on the inhibition by glucose-6-P. Composition of the incubation mixture: 100, 420 and 860 U/ml of phos phorylase-*b* in 0.033 M glycerophosphate — 0.033 M mercaptoethanol buffer (pH 6.8); 3.2 U/ml of *b*-kinase in 0.0025 M mercaptoethanol — 0.02 M Tris — 0.02 M glycerophosphate buffer (pH 6.8); ATP-Na 0.003, Mg-acetate 0.01 M, glucose-6-P 0.01 M, AMP in concentrations indicated

which the amount of phosphorylase-*b* was varied it became quite clear that the suppressing effect of AMP depended on the quantity of phosphorylase-*b* rather than on the concentration of glucose-6-P.

Next, the effect of phosphorylase-*b* concentration on the liberator effect of AMP was studied. Fig. 4 shows the inhibitory effect of identical glucose-6-P concentrations in the presence of different amounts of phosphorylase-*b* and the inhibition suppression caused by different concentrations of AMP.

Fig. 4 shows the effect of AMP in the presence of 100, 420 and 860 units of phosphorylase-*b*. AMP was shown to suppress the inhibitory effect of 10 mM glucose-6-P in concentrations of different orders of magnitude, depending on the quantity of phosphorylase-*b*. Thus, in the presence of 100 U of phosphorylase-*b* as little as 25 μM AMP was sufficient for the suppression of inhibition, but the same quantity of AMP was ineffective in the presence of 420 U of phosphorylase-*b*. This 420 U of phosphorylase-*b* was released from inhibition by 100 μM AMP,

which, in turn, was insufficient when 860 U of phosphorylase-*b* was present to reduce the inhibitory effect of glucose-6-P. In the presence of 860 U of phosphorylase-*b* more than 1000 μM AMP was necessary to suppress the inhibition caused by 10 mM glucose-6-P.

One of the possible explanations of the phenomenon is a modification by glucose-6-P of the substrate phosphorylase-*b* rather than that of *b*-kinase. This would explain that though in the presence of more phosphorylase-*b* glucose-6-P had a lesser inhibitory effect, nevertheless a higher concentration of AMP was needed to neutralize this effect.

The effect of glycogen

From the effects of glucose-6-P and AMP it could be supposed that glycogen, too, will increase the rate of conversion of phosphorylase-*b* to phosphorylase-*a* through a modification of phosphorylase-*b* and that the effect in this case, too, is

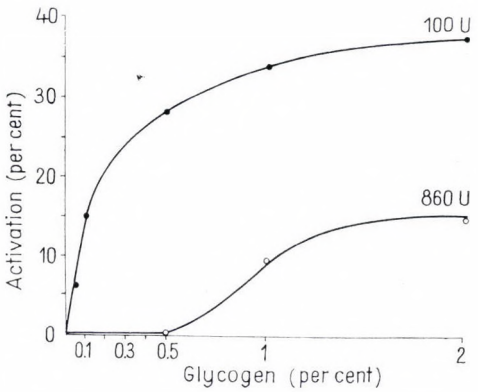

Fig. 5. The effect of glycogen on the conversion rate of phosphorylase-*b*. Composition of the incubation mixture: 100 and 860 U/ml of phosphorylase-*b* in 0.033 M glycerophosphate — 0.033 M mercaptoethanol buffer (pH 6.8); 3.6 U/ml of *b*-kinase in 0.0025 M mercaptoethanol — 0.02 M Tris — 0.02 M glycerophosphate buffer (pH 6.8); ATP-Na 0.003 M, Mg-acetate 0.01 M, glycogen in concentrations indicated

related to the amount of phosphorylase-*b*. Fig. 5 shows the effect of glycogen on the reaction rate of *b*-kinase for two different phosphorylase-*b* concentrations.

It is clearly indicated in Fig. 5 that when small quantities (100 U) of phosphorylase-*b* were applied as little as 0.1 per cent glycogen was sufficient to raise quite considerably the reaction rate of *b*-kinase. However, the conversion rate of higher amounts (860 U) of phosphorylase-*b* was not increased by even 0.5 per cent glycogen. Higher concentrations of glycogen proved to be effective activators. The activation curve has a sigmoid character. The correlation between the quantity of phosphorylase-*b* and the effect of glycogen to increase the conversion rate seems again to indicate that conformational changes in phosphorylase-*b*, the substrate, are responsible for the higher reaction rates of *b*-kinase.

Concerning the physiological regulation of glycogenolysis the interaction of the opposite effects of glycogen and glucose-6-P in the kinase reaction promised to be particularly interesting. Fig. 6 shows the effect of increasing concentrations of glucose-6-P on the transformation of phosphorylase-*b* with and without glycogen.

It can be seen from Fig. 6 that the activating effect of 1 per cent glycogen was not influenced by 1 – 3 mM of glucose-6-P in the presence of 100 U phosphory-

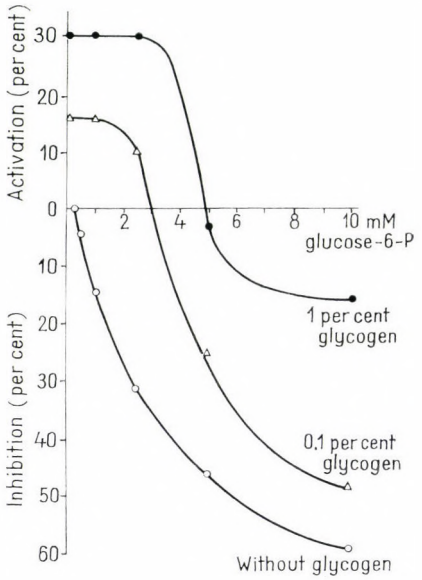

Fig. 6. Interaction of glycogen and glucose-6-P on the conversion of phosphorylase-*b*. Composition of the incubation mixture: 100 U/ml of phosphorylase-*b* in 0.033 M glycerophosphate – 0.033 M mercaptoethanol buffer (pH 6.8); 2.02 U/ml of *b*-kinase in 0.0025 M mercaptoethanol – 0.02 MTris – 0.02 M glycerophosphate buffer (pH 6.8); ATP-Na 0.003 M, Mg-acetate 0.01 M, glycogen in concentrations indicated

lase-*b*. The activating effect of glycogen was neutralized by the inhibitory effect of 5 mM glucose-6-P and surpassed by that of 10 mM glucose-6-P. Since the activating effect of 0.1 per cent glycogen was lower, it was neutralized by as little as 3 mM glucose-6-P. 5 – 10 mM glucose-6-P had a considerable inhibitory effect. Without the presence of glycogen the inhibition by glucose-6-P appeared already at low concentrations.

These results again point to the importance of the conformation of the substrate. The conversion rate greatly depends on the state of phosphorylase-*b* to be converted; the conversion by *b*-kinase of phosphorylase-*b*, as modified by glycogen, proceeds more rapidly than that of the native phosphorylase. Only higher concentrations of glucose-6-P will be able to convert phosphorylase-*b* modified by glycogen into a less readily convertible form.

This feed-back regulation may be highly important in the mobilization of glycogen. Glycogen accumulation promotes the rapid activation of phosphorylase, that is, its transformation into form *a*, while the depletion of glycogen, that is, a drop in its concentration below 0.1 per cent, permits automatically the operation of the inhibitory effect of glucose-6-P. This will slow down the further transformation of phosphorylase-*b* into phosphorylase-*a* thereby reducing the decomposition of the residual glycogen.

Discussion

Due to their physiological implications the conversion of phosphorylase-*b* into phosphorylase-*a* and the activation rate of *b*-kinase have been the object of detailed studies in recent years. Experiments so far have furnished conclusive results concerning the active and inactive states of *b*-kinase, the role of cyclic AMP and of Ca^{++} and glycogen mobilization brought about by adrenaline or electric stimulation. On the other hand, it has become clear that phosphorylase-*b* is an allosteric enzyme the activity of which is regulated by various effectors (ligands). The effectors display their action by changing the conformation of the enzyme.

In this work we have investigated the influence of the conformation of phosphorylase-*b* on the *b*-kinase reaction in which phosphorylase-*b* is not the enzyme, but fulfils the role of the substrate. Our results indicate that phosphorylase-*b* as a substrate may also be present in different conformations which are phosphorylated by *b*-kinase, i.e. converted into the *a*-form at different rates.

Some effectors despite of having a considerable influence on the activity of phosphorylase-*b* will not influence the rate of phosphorylation. Such effectors are AMP and glucose-1-P. Glucose-1-P affects neither native phosphorylase-*b* nor phosphorylase-*b* modified by glucose-6-P, i.e. it does not influence the rate of conversion of phosphorylase-*b*. AMP is ineffective by itself but is able to reverse the conversion of phosphorylase-*b* modified by glucose-6-P, i.e. to suppress the inhibitory effect of glucose-6-P. Glycogen converts phosphorylase-*b* as a substrate into a form more readily accessible to *b*-kinase. This is true for both the native and the glucose-6-P modified form of phosphorylase.

Consequently the influence of the effectors on phosphorylase-*b* is different depending on whether it concerns the regulation of enzyme activity or the conformation of the enzyme which serves as substrate. This is, however, not a contradiction, because activity means a binding of glycogen and glucose-1-P as well as the establishment of an interaction between the two, whereas the role of the substrate is to bind to *b*-kinase and to interact with ATP.

The probability of a modification of phosphorylase-*b* itself in the *b*-kinase reaction under the influence of certain effectors is in our opinion confirmed by the observation that the influence of the effectors decreases with increasing amounts of phosphorylase-*b*. This means that with increasing effector concentrations the influence increases sigmoidally. These observations may also indicate an allosteric modification of *b*-kinase, but the finding that in the presence of higher quantities of phosphorylase-*b* inhibition by glucose-6-P is suppressed only by

higher concentrations of AMP points to a binding of most of the AMP by phosphorylase-*b*. In this way most probably the latter will be modified. However, since an about ten fold increase in the quantity of phosphorylase can only be balanced by an about one hundred times higher AMP concentration, no conclusions can be drawn as to direct proportionality or an entirely unambiguous mechanism.

These observations and our experiments with phosphorylase phosphatase (Bot, Dósa, 1967) point to an entirely new approach to the allosteric effect. Hence, the conformations of phosphorylase-*b* and phosphorylase-*a* influence not only the activity of the enzymes, but also their mutual conversion, that is the rate of their phosphorylation and dephosphorylation. This effect of nucleotides and intermediates represents a further possibility of the regulation of glycogen mobilization. In this case the feed-back mechanism is realized not through the regulation of a single enzyme activity, but by means of the modification of an enzyme protein which serves as substrate.

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Electron Microscopic Demonstration of Differences between Succinic Dehydrogenase Activities of Brain Mitochondria in Homogenates and Mitochondrial Fractions

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(Received July 23, 1969)

Brain homogenates and pure mitochondrial fractions were stained for succinic dehydrogenase activity and studied under the electron microscope. In accordance with previous electron-histochemical investigations performed on intact nervous tissue a fairly high percentage of the mitochondria remained unstained. In pure mitochondrial fractions with normal respiratory control the number of non-reacting mitochondria was considerably higher than in rapidly prepared brain homogenates, indicating a certain loss of succinic dehydrogenase activity proportional to the duration of the preparatory procedure. The above findings demonstrate the genuine heterogeneity of brain mitochondria.

Introduction

The methods available for the biochemical analysis of mitochondria provide data concerning the whole population examined. By means of electron microscopic histochemistry differences have been revealed between mitochondria in the heart muscle and kidney regarding some enzymes of the electron transport chain (Ogawa, Barnett, 1964, 1965; Seligman et al., 1967; Ogawa et al., 1968; Kerpel-Fronius, Hajós, 1968). As for the nervous system recent electron histochemical studies (Hajós, Kerpel-Fronius, 1969) have shown that mitochondria in various parts of neurons exhibit different succinic dehydrogenase (SDH) activities. The most remarkable finding was the consequently negative reaction of mitochondrial situated in the presynaptic terminals of axons. This is in sharp contrast to the behaviour of strongly stained perikaryal ones. As a number of extramitochondrial factors may influence the reaction, the present work was aimed at ruling out these factors by studying brain mitochondria for SDH-activity in tissue homogenates and in isolated mitochondrial fractions.

Materials and Methods

Adult albino rats of both sexes were killed by decapitation. Brains including the cerebral hemispheres, the cerebellum and the cerebral trunk were rapidly excised and put into chilled homogenization medium containing 0.25 M sucrose, 10 mM tris-HCl buffer, 1 mM EDTA; the pH was 7.4. Homogenization was performed by using a Potter-Elvehjem-type homogenizer with a teflon pestle. All subsequent operations were made by using the MSE-17 refrigerated centrifuge

as described by Somogyi et al. (1962). Homogenates and pure mitochondrial fractions were incubated for the electron microscopic demonstration of SDH-activity according to the copper-ferrocyanide method of Kerpel-Fronius and Hajós (1968). In some cases mitochondria were preincubated in media containing 1 μg Rotenone or 5 μg Antimycin A and all components of the medium mentioned above except ferricyanide. Ferricyanide was added 5 minutes after the beginning of the incubation. Some reactions were carried out in the presence of 10 mg bovine serum albumin (BSA) added to the complete incubation medium. The total volume of the incubation media was in all cases 2.5 ml to which 0.1 – 0.2 ml of the sample was added by a micropipette. The incubation was carried out in centrifuge tubes at 37 °C for 30 minutes and terminated by a short centrifugation at 6000 *g* in a laboratory centrifuge. The pellet at the bottom of the tube was sufficiently coherent for the further manipulations. It could be removed from the tube either by washing it out with 0.1 M tris-HCl buffer or scratched out with a needle. Blocks of 1 – 2 mm^3 were cut under the buffer mentioned above and fixed subsequently in 1 per cent osmic acid, buffered at pH 7.4, dehydrated in graded alcohol series and embedded into Durcupan (Fluka). Ultra-thin sections were cut with the LKB Ultrotome, floated on distilled water and picked up on copper grids lacking film coating. Considering the dissolution of the membrane-bound copper-ferricyanide in highly alkaline lead stains (Kerpel-Fronius, Hajós, 1968) no contrast staining was employed. Electronmicrographs were made by the Tesla Bs 242 electron microscope.

Respiration was measured with a vibrating platinum electrode at 24 °C. The reaction mixture contained: 10 mM substrate (tris-salt); 24 mM tris-Cl buffer, pH 7.4; 5.3 mM tris-phosphate buffer, pH 7.4; 26 mM KCl; 14 mM sucrose in a total volume of 2.7 ml.

Results

According to parallel polarographic assays the mitochondria exhibited respiratory control (Fig. 1); their oxygen consumption increased markedly on adding ADP to the medium (State 3), and diminished again after its expenditure (State 4). It is interesting to note that in the case of succinate we could demonstrate respiratory control only in the presence of bovine serum albumin (BSA). This is in agreement with the results of Vogt and Basford (1968). In the absence of BSA the activated respiration did not return to the resting state. Even in the presence of BSA respiratory rate was higher after the exhaustion of ADP than before its addition. Dinitrophenol (DNP) accelerated respiration both with and without BSA, proving the coupled state of the isolated mitochondria under all experimental conditions.

As revealed by electron microscopy the brain mitochondria in homogenates, as well as in pure mitochondrial fractions, behave differently after incubation for SDH-activity (Figs 2,3). Although the reaction product is in all cases attached to mitochondrial membranes, three types of mitochondria can be distinguished according to the intensity of precipitation: (1) strongly reacting; (2) weakly react-

ing and (3) non-reacting. Neither ultrastructural characteristics nor the degree of preservation could be correlated with any of the above groups. Swollen or ruptured mitochondria that are not frequent in the preparates or those enclosed into intact axon terminals were not evaluated. It is noteworthy nevertheless, that the latter ones were always devoid of precipitate (Fig. 3). A marked difference was

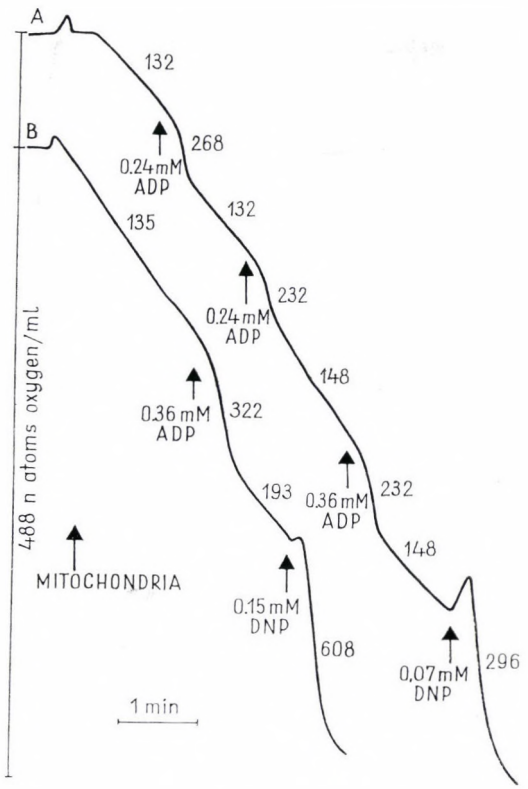

Fig. 1. Respiratory control with various substrates in rat brain mitochondria (2.7 mg mitochondrial protein).
A = 5 mM glutamate; 5 mM malate
B = 10 mM succinate; 1 mg Rotenone; 10 mg BSA
The numbers indicate n atoms of oxygen consumption/min.

ound between homogenates and mitochondrial fractions concerning the numerical distribution of the three types of mitochondria. This difference is indicated in Table 1.

This pattern of distribution remained unaltered in the presence of BSA. Rotetone caused an insignificant increase in the number of strongly stained mitochondria. Antimycin A did not influence the reaction, proving that the reduction of ferricyanide occurs at the level of SDH.

Discussion

It has been shown by the use of various electron histochemical techniques for the demonstration of SDH-activity (Ogawa, Barnett, 1964, 1965; Seligman et al., 1967; Ogawa et al., 1968; Kerpel-Fronius, Hajós, 1968) that in heart muscle and kidney strongly reacting mitochondria occur intermingled with weakly react-

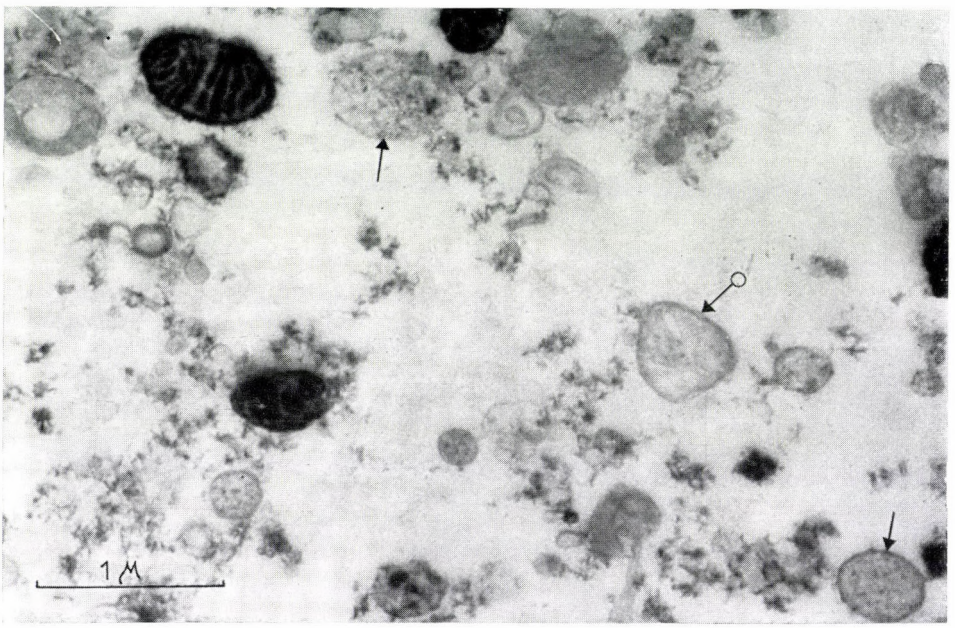

Fig. 2. SDH-reaction in rat brain homogenate. Besides mitochondria with a strong membrane reaction weakly precipitated (arrows) and non-precipitated (ringed arrows) ones can be seen. Slight precipitation in microsomal elements is probably due to fragments of ruptured mitochondria

Table 1

Numerical distribution of differently reacting mitochondria in brain homogenates and mitochondrial fractions (in per cent)

	Strongly reacting	Weakly reacting	Non-reacting
Homogenate	78.9	0.9	20.2
Mitochondrial fraction	26.0	25.3	48.7

ing and entirely unstained ones. In the nervous system the early observation of Szentágothai (1957) who found a strong SDH-reaction in the nerve cells of the ciliary ganglion but none at all in the oculomotor axon terminals, was the first to call attention to the possibility that mitochondria situated in different parts of

neurons may show different SDH-activities. This observation made by light microscopic histochemistry could be convincingly confirmed at the electron microscope level (Hirano et al., 1968; Hajós, Kerpel-Fronius, 1969). As shown by the systematic study of Hajós and Kerpel-Fronius (1969) in different regions of the brain the mitochondria of the presynaptic axon terminals remain consequently unstained, in sharp contrast to the perikaryal ones, which invariably exhibit a marked

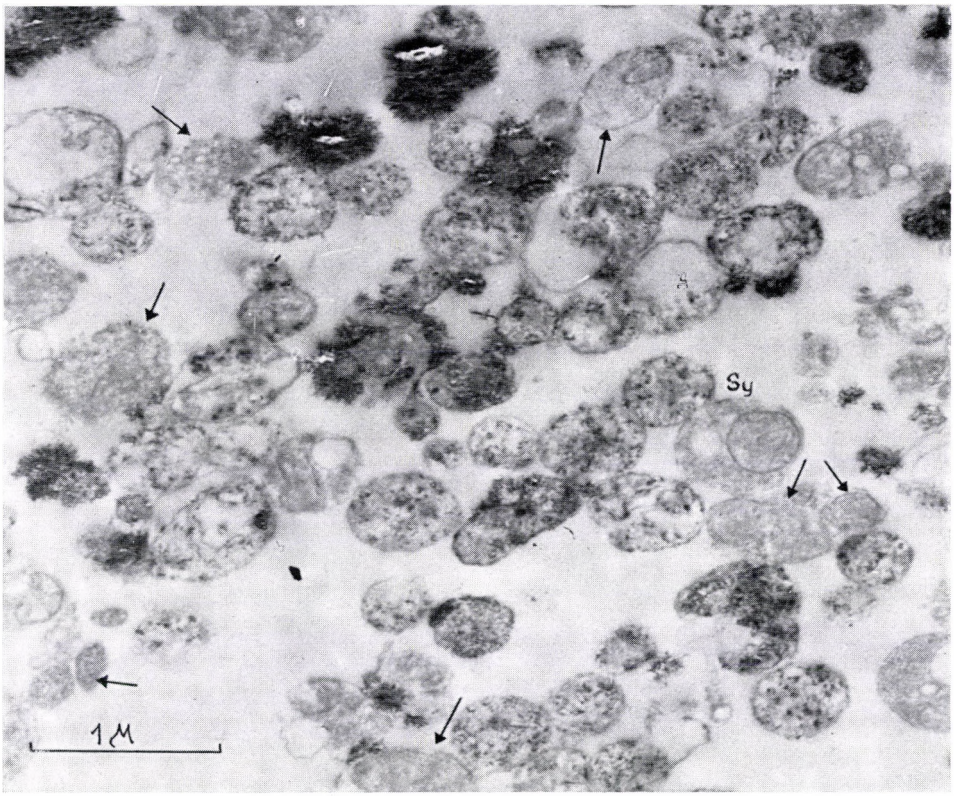

Fig. 3. SDH-reaction of isolated brain mitochondria. The three types of mitochondria as shown in Fig. 2, can be clearly distinguished. Arrows point to non-reacting mitochondria. Mitochondrion enclosed into a synaptosome (Sy) is also devoid of precipitate

SDH-activity. This finding, however, required further analysis in order to ascertain its genuine nature, as in the axon terminals a number of extramitochondrial factors could be responsible for the lack of reaction. The integrity of the presynaptic axolemma seemed to be the most relevant, inhibiting the free penetration of the incubating medium into the synaptic terminal the more so since synaptic function is closely connected with the permeability changes of synaptic membranes. If brain homogenates or mitochondrial fractions are incubated – disregarding the scarce intact nerve ending particles – all limiting plasma membranes, including

the synaptic ones, are disrupted and mitochondria are directly exposed to the incubating medium. In spite of this a marked percentage of the mitochondria did not exhibit any reaction. It seems unlikely indeed that apart from the synaptic disc the functional properties of the whole membrane bordering the presynaptic bag should differ fundamentally from that of the other plasma membranes of the neuron. The surprisingly high number of non-reacting mitochondria in mitochondrial fractions as compared to homogenates made us suspect that our preparations contained functionally damaged mitochondria. But this is ruled out by the observation that these fractions exhibit respiratory control. The number of non-reacted mitochondria could not be decreased either by adding BSA, known to be advantageous for mitochondrial respiration (Vogt, Basford, 1968) or Rotenone that prevents the formation of oxaloacetate, inhibiting succinate oxidation (Wojtczak, Wojtczak, 1964). The steady difference in the number of non-reacted mitochondria between homogenates and mitochondrial fractions indicates a considerable loss of SDH-activity during the isolation procedure.

Nevertheless, disregarding these quantitative differences the mere presence of differently reacting groups of mitochondria in homogenates and mitochondrial fractions corroborates recent electron-histochemical observations (Hirano et al., 1968; Hajós, Kerpel-Fronius, 1969) made in intact nervous tissue and seems to yield a satisfactory evidence for the genuine heterogeneity of brain mitochondria.

As to the possible causes of this heterogeneity the role of the axo-plasmic flow which transports mitochondria from perikarya towards axon terminals (Weiss, Pillai, 1965) should be considered. It is obvious that mitochondria situated far from the perikarya have travelled a longer distance. Hence, the decrease of SDH-activity in the distal portions of neuronal processes may be interpreted as a marker of mitochondrial ageing or some other alteration during axonal migration. Cytochrome oxidase seems to be less sensitive because it shows an equally strong activity both in the neuronal perikarya and axon terminals (Szentágothai, 1957; Kerpel-Fronius, Hajós, 1967). It can be hoped that further electron histochemical mapping of the occurrence of other mitochondrial enzymes will add to the understanding of the functional significance of such changes in the enzyme repertoire of brain mitochondria.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (1), pp. 25–31 (1970)

ATP-Dependent Enzymic Splitting of Mesitene Lactone and Triacetic Acid Lactone

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(Received September 11, 1969)

Mesitene lactone and triacetic acid lactone are decomposed by a pigeon liver extract in the presence of ATP and CoA into acetyl-CoA; in the same system they acetylate sulfanilamide.

1. Mesitene lactone is not hydrolyzed into β-methyl-δ-oxo-$\Delta^{\alpha\beta}$-hexenoic acid but directly into acetyl-CoA.

2. Neither is triacetic acid lactone hydrolyzed in the system investigated but decomposes in a way similar to mesitene lactone.

3. Also the keto acid corresponding to each lactone gives rise to acetyl-CoA in the system studied. However, the lactones do not act through acids but by some other mechanism.

It has been described in a previous paper (Alkonyi et al., 1969) that β-methyl-δ-oxo-$\Delta^{\alpha\beta}$-hexenoic acid (keto acid Fig. 1, I) is capable of acetylating sulfanilamide in a pigeon liver extract in the presence of ATP and CoA. The same was found with the lactone of the compound, mesitene lactone (Fig. 1, III), as well as with the decarboxylated product of the keto acid, mesityl oxide. It has also been described (Alkonyi et al., 1969) that acetyl-CoA is most probably formed directly from mesitene lactone and not from the keto acid which may originate from an occasional hydrolytic cleavage of the lactone ring. No enzymic activity which could have been responsible for opening the mesitene lactone ring in a hydrolytic way was found in pigeon liver. In the present paper an independent and direct evidence is presented to show that the acetyl donor role of mesitene lactone comes into play through the interaction of an ATP lyase, without the hydrolytic splitting of the lactone ring.

Since the time Lynen (1961) raised the possibility of TAL (Fig. 1, IV) being an intermediate in the biosynthesis of 6-methyl-salicylic acid and of various macrolide structures, Nixon et al. (1968) actually demonstrated the formation of TAL by means of a pigeon liver fatty acid synthetase complex. Most recently, Yalpani et al. (1969) carried out the synthesis of TAL with the aid of a fatty acid synthetase from baker's yeast. Much earlier Meister (1949) demonstrated the presence in rat liver and kidney of a lactonase capable of hydrolysing TAL into triacetic acid. According to these findings TAL is a substrate of several enzymes. However, its presence in living tissues has not been demonstrated so far.

Unusual abbreviations: TAL, triacetic acid lactone; keto acid, β-methyl-δ-oxo-$\Delta^{\alpha\beta}$-hexenoic acid.

In view of the above contradictory findings investigations on the possible occurrence in pigeon liver of an enzyme capable of splitting TAL in a non-hydrolytic manner seemed to be warranted. This question seemed us all the more interesting because we have often observed the splitting of mesitene lactone of a similar structure upon the addition of a pigeon liver extract. Thus, it can be assumed that an enzyme which splits TAL exists in pigeon liver. This enzyme will react with mesitene lactone only because of the structural similarity with TAL. No physiological role has been ascribed so far to this lactone.

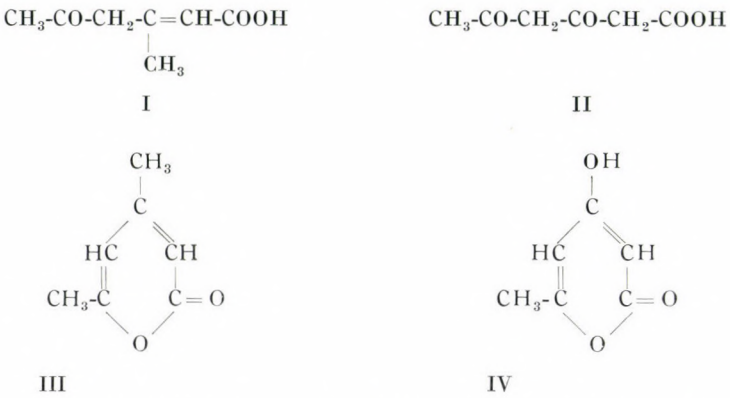

Fig. 1. Chemical structure of β-methyl-δ-oxo-$\Delta^{\alpha\beta}$-hexenoic acid (I), triacetic acid (II), mesitene lactone (III) and triacetic acid lactone (IV)

There is a hydrolase in rat liver, ox liver and rabbit liver as well as in rat kidney and rabbit kidney, which splits triacetic acid (Fig. 1, II) into acetic acid and acetoacetic acid. However, this enzyme in a purified form does not act on TAL (Witter, Stotz, 1948a). We have failed to demonstrate in pigeon liver any hydrolase capable of a hydrolytic splitting of TAL in the absence of ATP and CoA. In this respect there is an analogy between mesitene lactone and TAL. On the other hand, TAL does split in pigeon liver extracts when both ATP and CoA are present, just as does mesitene lactone. This decomposition can be measured either by a decrease of the amount of TAL or by the acetylation of sulfanilamide added to the reaction mixture. The capacity of TAL to acetylate sulfanilamide is much higher than that of mesitene lactone in an identical medium. Spectroscopic and paper chromatographic evidence will be presented to show that this decomposition is the result of the action of an ATP-dependent lyase.

Materials and Methods

CoA was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio; ATP, sulfanilamide Na-acetate and the organic solvents from Reanal, Budapest; glutathione from Serva, Heidelberg and mercaptoethanol from Merck, Darmstadt.

Triacetic acid was synthetized by the procedure of Witter and Stotz (1948b). After recrystallization from ether-petrolether the product had a m.p. of 30 °C, and an absorption maximum at 278 mμ with a molecular extinction coefficient of 2.740 $M^{-1}cm^{-1}$. TAL was prepared by the method of Collie (1891). After two recrystallizations of the crude product from water with the addition of charcoal the m. p. of TAL was 186 °C. The compound had an absorption maximum at 280 mμ with a molecular extinction coefficient of 7.880 $M^{-1}cm^{-1}$. Mesitene lactone was prepared according to Anschütz et al. (1890). After two recrystallizations from water and after one recrystallization from petrolether with the addition of charcoal the m.p. of the product was 47 °C. The compound had an absorption maximum at 294 mμ. The keto acid was obtained from mesitene lactone according to the procedure already described (Alkonyi et al., 1969). Paper chromatography was made on Schleicher-Schüll 2043 b paper, using 6 cm strips and the ascending technique at room temperature for 20 hours with the solvents as follows: 1 butanol – 96 per cent acetic acid – water (5 : 1 : 4, v/v/v).

The solvents were freshly distilled before use. 100 μl of the deproteinized incubation mixture were put on the start line; the compounds were located under ultraviolet light (filter UG-5). Extracts of pigeon liver were made according to the method previously described (Alkonyi et al., 1969); the fraction between 40 and 70 per cent saturation with ammonium sulfate was used.

Sulfanilamide in the incubation mixture was determined according to Bratton and Marshall (1939).

Results and Discussion

There are two different mechanisms which can explain the acetylation of sulfanilamide by mesitene lactone in the presence of ATP and CoA upon the addition of pigeon liver extract:

1. It is conceivable that mesitene lactone undergoes a hydrolytic cleavage to the corresponding keto acid (Fig. 1, I) which is then decomposed by a keto acid hydrolase into acetic acid and β-methyl-crotonic acid.

Several keto acid hydrolases are known. Recently Brock and Williamson (1968) have purified a fumaryl-acetoacetic hydrolase from rat liver. Acetic acid can then react with the acetate activating enzyme to form acetyl-CoA which, in turn, would acetylate sulfanilamide with the aid of pigeon liver acetyl transferase. Pigeon liver acetyl transferase was purified 48-fold by Jacobson (1961). Thus, if we assume that four enzymes are involved, the acetylating effect can be explained.

2. The second possible mechanism is the decomposition of the lactone ring into acetyl-CoA by a lyase without previous hydrolysis. Acetyl-CoA would, then, acetylate sulfanilamide by means of the transferase. According to this second mechanism only two enzymes are involved.

Fig. 2 and Table 1 clearly show that, in the present case, we are facing the second type mechanism. Fig. 2 shows the concentration dependence of the acetylating capacity of mesitene lactone (Fig. 1, III) and of the corresponding keto acid (Fig. 1, I). As it can be seen 20 μmoles of the lactone produce about the same

amount of N-acetyl-sulfanilamide as do 5 μmoles of the keto acid. This relation strictly holds in a range of 0 to 20 μmoles, i.e. the lactone acetylates to the same extent as does the keto acid which has only 25 per cent of the weight of the lactone. From this it follows that if lactone exerted its acetylating effect in form of the keto acid after cleavage of the ring, then, at least 25 per cent of the lactone should be opened hydrolytically. However, the data presented in Table 1 indicate that

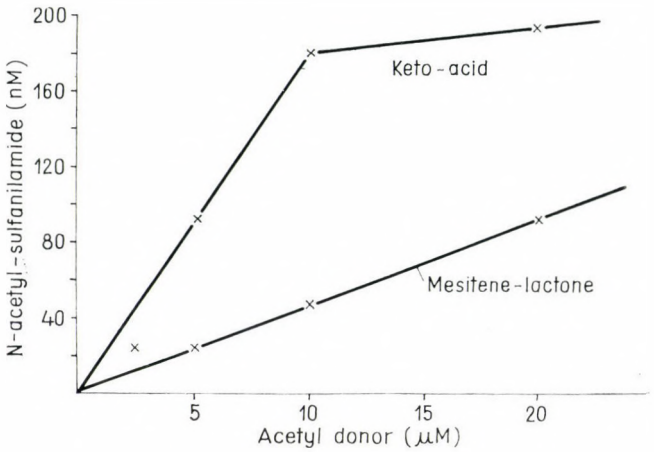

Fig. 2. Comparison of the acetylating ability of keto acid and mesitene lactone. The incubation mixture contained 0.4 μM sulfanilamide, 4.0 μM ATP, 100.0 μM $NaHCO_3$, 10.0 μM glutathione, 40.0 nM CoA, 0.3 ml of pigeon liver extract and increasing concentrations of acetyl donors. All the anions were neutralized with NaOH. Incubation time: 2 hours. Final volume: 1.1 ml. Temperature: 37 °C. Sulfanilamide was estimated as described earlier (Alkonyi et al., 1969)

Table 1

Study of the enzymic cleavage of mesitene lactone

All media contained 20.0 μM mesitene lactone, 0.4 μM sulfanilamide, 100.0 μM sodium bicarbonate and 10.0 μM glutathione. Tube 1 contained also 0.3 ml of a pigeon liver extract. This extract was added to Tube 2 at the end of the incubation and deproteinized immediately. No extract was given to Tube 3. Final volume: 1.1 ml. Time of incubation: 2 hours. Temperature: 37 °C. After incubation the volume of all mixtures was filled up with cold ethanol to 5 ml. After filtration two ml samples were taken and diluted with water to 100 ml. Extinctions of these solutions were measured at 294 mμ. For spectrophotometric measurement a reference solution containing the above mentioned constituents except mesitene lactone was used

Tube No.	Extinction at 294 mμ
1	0.576
2	0.572
3	0.563

no opening of the lactone ring occurs at all when no ATP and CoA are present in the incubation mixture. The keto acid exhibits an absorption maximum at 220 mμ; thus, a 25 per cent opening of the ring would be demonstrated by a marked decrease of the extinction measured at 294 mμ. This suggests that the cleavage of the mesitene lactone is due to the action of an ATP-dependent lyase.

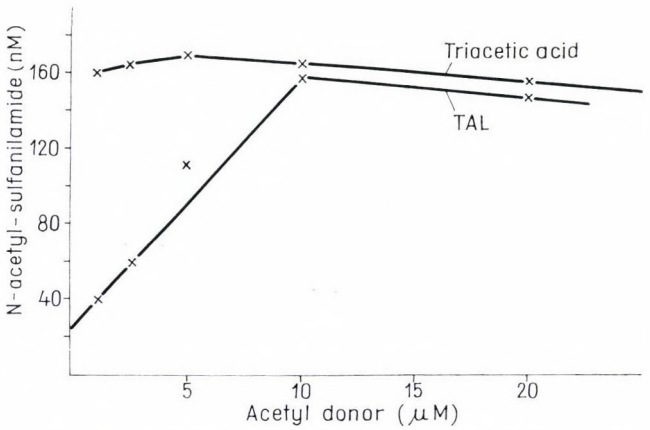

Fig. 3. Comparison of the acetylating ability of triacetic acid and TAL. Composition of the incubation mixture and the analytical procedures used were identical with those described in the legend to Fig. 2

Table 2

Study of the enzymic cleavage of TAL

All tubes contained 100.0 μM sodium bicarbonate, 10.0 μM mercaptoethanol, 10.0 μM $MgCl_2$ and 5 μM TAL. Extr. = incubation with 0.3 ml of fresh pigeon liver extract. Extr. prec. = 0.3 ml of pigeon liver extract added at the end of the incubation and deproteinized immediately. Cof. = addition of 10.0 μM ATP and 40 nM CoA. SA = addition of 1.6 μM sulfanilamide. Final volume: 1.1 ml. Time of incubation: 2 hours. Temperature: 37 °C. After incubation all mixtures were treated with 0.5 ml of 15 per cent TCA and centrifuged at 0 °C. 0.1 ml of the supernatant was subjected to paper chromatography as described in Methods. After chromatography the spots corresponding to TAL were cut out and eluted with 5 ml of distilled water. The extinction of the eluates was read at 280 mμ. Sulfanilamide in 0.1 ml of the supernatant after TCA precipitation was determined according to the method of Bratton and Marshall (1939). Each value corresponds to the mean of two measurements

Composition of the incubation mixture	Extinction of the eluate containing TAL	Decomposed TAL, per cent	N-acetyl-SA produced, nM
Cof.+SA+Extr.	0.288	12.0	202
Cof.+SA+Extr. prec.	0.328	—	—
Cof.+Extr.	0.297	10.0	—
Cof.+Extr. prec.	0.327	—	—
Extr. prec.	0.328	—	—

The ability of TAL to acetylate sulfanilamide under the conditions shown in Fig. 2 roughly corresponds to that of the keto acid. This means that, within the saturation limit, TAL acetylates some four times as much sulfanilamide as does an identical concentration of mesitene lactone. As to the mode of the splitting of TAL we have the same two possible hypotheses as in the case of mesitene lactone. Therefore, the first question to be answered is whether the acetylating effect of TAL comes into play through a hydrolysis into triacetic acid or by some other mechanism. In the system used by us triacetic acid acetylates very power-

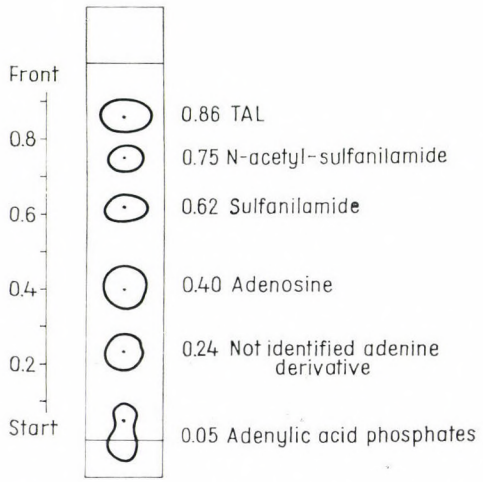

Fig. 4. Separation of TAL and N-acetyl-sulfanilamide from the incubation mixture. The incubation mixture contained 100.0 μM $NaHCO_3$, 10.0 μM mercaptoethanol, 10.0 μM $MgCl_2$, 5 μM TAL, 10.0 μM ATP, 40.0 nM CoA, 1.6 μM sulfanilamide and 0.3 ml of pigeon liver extract. Final volume: 1.1 ml. Incubation time: 2 hours. Temperature: 37 °C. At the end of the incubation the mixture was treated with 0.5 ml of 15 per cent TCA and centrifuged at 0 °C. 0.1 ml of the supernatant was subjected to paper chromatography

fully. This ability is probably based on a hydrolytic splitting of the molecule into acetic acid and acetoacetic acid. This hydrolytic process has already been demonstrated several times to occur in the liver (Witter, Stotz, 1948a; Brock, Williamson, 1968). In Fig. 3 the acetylating ability of TAL and that of triacetic acid are compared. As the ascending part of the curve which shows the acetylating ability of triacetic acid falls within a very low concentration range, the method used in Fig. 2 is not applicable any more. The amounts of TAL and triacetic acid resulting in an equal degree of acetylation are not commensurable. Thus, to measure the decomposition of TAL we used the quantitative determination of the compound after paper chromatographic separation from the incubation mixture. Such a paper chromatogram is shown in Fig. 4. The individual spots were identified by a comparison with authentic samples and by spectrophotometric methods. The second spot from the start line ($R_f = 0.24$) could not be identified. The most slowly moving spot ($R_f = 0.05$) may be ATP, ADP and AMP for these compounds are poorly separated in the system used.

The data in Table 2 were reproducible in three independent experiments. As it can be seen, incubation of TAL with pigeon liver extract in the presence of ATP, CoA and sulfanilamide as acetyl acceptor results in a 12 per cent decomposition of TAL. Decomposition decreases to 10 per cent when the sulfanilamide is omitted from the mixture; definitely no decomposition occurs when also the co-factors are omitted (last two rows in Table 2). The hydrolysis in the absence of co-factors was also investigated under the conditions shown in Table 1. In this case we used TAL instead of mesitene lactone and measured the extinction at 280 mμ. Also this experiment proves that TAL does not undergo any hydrolysis upon the addition of pigeon liver extract.

Thus, both the decomposition of TAL and that of mesitene lactone have the common characteristics that no hydrolytic splitting of the ring occurs and that probably the acetyl-CoA is split off from the ring of both substances. We have as yet no definite data on the mechanism of this ring opening or on the other substance produced in the reaction beside acetyl-CoA. Neither do the experimental conditions used in the present work allow any conclusion as to the exact manner of the splitting of ATP; when ATP was incubated with pigeon liver extract without any added substrate we similarly obtained the three slowly moving spots with R_f values of 0.05, 0.24 and 0.40, respectively, in the paper chromatogram of the incubation mixture. The enzyme source used contained some other ATP-splitting enzymes too which made the interpretation of the results difficult. This question can only be answered after additional purification of the lyase.

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Reversible Change in Iodine Sensitivity of Penicillinase by Alkali Treatment

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(Received July 29, 1969)

By means of incubation in alkali a penicillinase was prepared which at pH 6 was practically completely iodine sensitive. The effect of acidification to pH 6, after incubation in alkali, on the iodine sensitivity of the enzyme at pH 6 and pH 9 was studied. The iodine resistant activity of the native enzyme was found to be restored by different kinetics at the two different pH values. The iodine resistance measured at pH 6 of the alkali-treated enzyme was restored to that of the native enzyme within 0.1 minutes, while the iodine resistance measured at pH 9 approached that of the native enzyme only after incubation for 60–80 minutes at pH 6. The possibility that two different enzyme conformations with a different iodine sensitivity are responsible for the two inactivation reactions of penicillinase upon treatment with iodine has been excluded with high probability.

Introduction

Citri and co-workers have demonstrated that exopenicillinase produced by B. cereus undergoes under certain experimental conditions (alkali treatment, in the presence of urea and competitive inhibitors) reversible conformation changes. These conformation changes were followed by measuring the iodine sensitivity of the enzyme. The native enzyme is iodine resistant, but is rapidly inactivated in the presence of iodine after a conformation change due to the above treatments (Citri, 1958; Citri et al., 1960).

In our earlier experiments the effect of pH, temperature, iodine concentration, etc. on the iodine sensitivity of the enzyme was studied. It has been found that the inactivation reaction with iodine takes place in two steps (Csányi et al., 1970). These experiments have failed to show, however, whether the individual steps of inactivation are related to definite changes in enzyme conformation differing in iodine sensitivity or to the individual reactions of certain amino side-chains which contribute to enzyme activity. In an attempt to answer this question the fully iodine sensitive enzyme prepared by alkali treatment was studied.

Materials and Methods

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

Alkali treatment

The enzyme was subjected to alkali treatment in solutions containing 5000–6000 U/ml of penicillinase. The pH of the solution containing 0.05 M phosphate was adjusted to the desired value up to pH 12 with sodium hydroxide using a Radelkis "Blood pH-meter". For pH values above 12 standard NaOH solutions were used. Alkali incubation was performed at 0 °C in an ice bath. At the end of the incubation period a small aliquot (usually 0.1–0.5 ml) was pipetted into 3–5 ml of the 0.05 M phosphate solution which contained enough hydrochloric acid to give the desired pH value of 6 or 9 after mixing with the alkaline solution.

Treatment with iodine

After alkali treatment the sample was usually subjected to treatment with iodine by introducing 10^{-3} M iodine into the neutralizing solution immediately after the addition of the alkaline incubation mixture. Between neutralization and the addition of iodine usually about one second had elapsed. After one minute incubation in the presence of iodine the latter was removed by addition of sodium thiosulfate and the residual enzyme activity of the samples was measured by a method described earlier (Csányi, 1961).

Enzyme activity was expressed in percentage of the activity of samples which were not treated with iodine.

In the time curves the time between neutralization and the addition of iodine is taken into consideration.

Results

The two "steps" observed in the course of the inactivation of penicillinase with iodine are easy to distinguish when iodine treatment is applied in the presence of 0.001 M iodine at 0 °C for one minute at pH 6 and pH 9, respectively. When treatment is performed at pH 6 only a small percentage of the enzyme will be inactivated, while at pH 9 iodine treatment will lead to a 30–35 per cent decrease of activity. To facilitate the discussion of the results the following designations will be introduced: the residual enzyme activity after iodine treatment at pH 6 is expressed in percentage of the activity of the untreated enzyme and designated as A_{R6}, the residual activity after iodine treatment at pH 9 is also expressed in percentage of the activity of the untreated enzyme and designated as A_{R9}.

The effect of pH on iodine resistance

In our earlier publication we have reported on the effect of pH on inactivation during iodine treatment (Csányi et al., 1970). We have found that inactivation proceeds in two pH-dependent steps and that at pH values above 10 the enzyme is very rapidly inactivated. In the experiments to be described in this paper we have studied whether the incubation of the enzyme at a certain well defined pH will lead also in the absence of iodine to some lasting or temporary change in iodine

sensitivity, provided iodine treatment at pH 6 or pH 9 follows incubation in an alkaline medium.

The results are shown in Fig. 1. It appears from the values of enzyme activity measured after incubation in alkali for 20 minutes that penicillinase is stable at quite high pH values, that is incubation in strongly alkaline media, e.g. at pH 12, will cause an activity loss of not more than a few per cent. A more marked inactivation of the enzyme begins only at pH values above 12.5. Fig. 1 shows also the curves of the A_{R6} and A_{R9} values measured in the course of iodine treatment

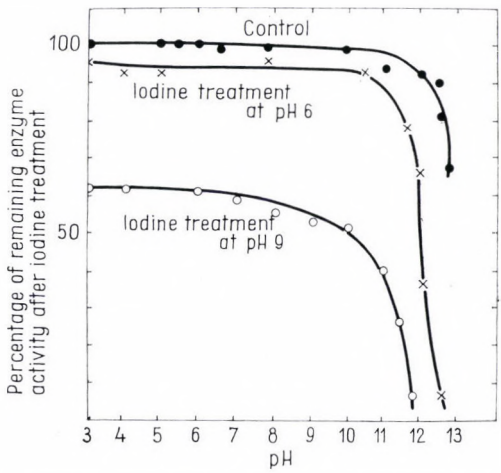

Fig. 1. 6000 U/ml of penicillinase was incubated at 0 °C for 20 minutes at a pH shown on the abscissa. 0.1 ml of this solution was pipetted into 0.05 M phosphate solution at 0 °C. The solution contained enough HCl to give a pH of 6 and 9, respectively, after mixing. Within one second iodine was added to the solution to make it 0.001 M with respect to iodine. After an incubation for one minute iodine was removed by the addition of thiosulfate and the residual enzyme activity was measured. The activity was expressed as percentage of the activity of the samples without iodine treatment

following pre-incubations at different pH values for one second. In the pH range from 3 to 10 pre-incubation has no marked effect on the iodine resistant activity measured immediately after alkali treatment. The value of A_{R6} is 95 per cent and that of A_{R9} 50–65 per cent.

There is a very marked reduction of the A_{R9} activity after pre-incubation at pH 11–12. At pH 12 the A_{R9} activity drops practically to zero. A_{R6} activity on the other hand changes but slightly in the same pH range (it is still 65 per cent at pH 12), but decreases rapidly in a pH range from 12 to 13 and at pH 13 it will also be close to zero. Hence, after pre-incubation at this pH value penicillinase will be fully iodine sensitive.

In the following experiments complete iodine sensitivity was produced by pre-incubation at pH 13 for two minutes. During this time the activity of the enzyme dropped by not more than 1–3 per cent, but its iodine resistance was 2–5 per cent both at pH 6 and pH 9.

We have examined the stability of the iodine sensitivity produced in the above manner. A given amount of the enzyme was treated in 0.1 N NaOH for 2 minutes at 0 °C and the pH of the solution was then adjusted to 6 and the phosphate concentration to 0.05 M. The A_{R6} and A_{9R} values were determined from aliquots at various intervals. The results are shown in Fig. 2. There is a significant difference in stability between the A_{R6} and A_{R9} values. The value of the A_{R6} activity returned within 0.1 minute from the few per cent measured immediately

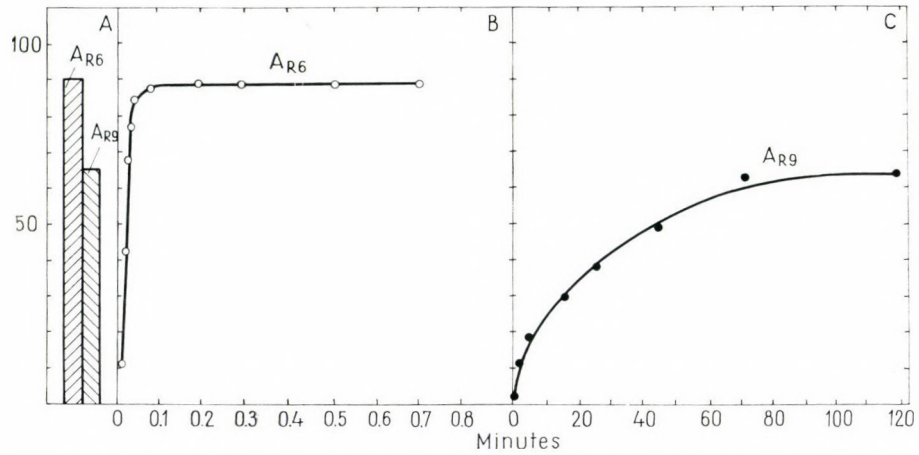

Fig. 2. 5000 U/ml of penicillinase was incubated in 0.1 N NaOH at 0 °C for two minutes, the solution acidified to pH 6 and made 0.05 M with respect to phosphate ions. The activities A_{R6} and A_{R9} were determined from aliquots at pH 6 and pH 9 at various times after acidification. *A* the activities A_{R6} and A_{R9} of the native enzyme without alkali treatment; *B* activity values A_{R6}; *C* activity values A_{R9}

after alkali treatment to 90–95 per cent, as measured prior to the treatment. A_{R9} activity which after alkali treatment was also nearly zero reached in about 60–80 minutes the 65–70 per cent value measured before alkali treatment.

It is important to know whether there is an equilibrium between the states characterized by the A_{R6} and A_{R9} values measured, or are these values independent of each other.

To answer this question the following experiment was performed. A given amount of penicillinase was treated with iodine at pH 9. This treatment resulted in a 36 per cent decrease of enzyme activity. The A_{R6} and A_{R9} values of the iodine-treated enzyme were measured after a repeated treatment with iodine. The values obtained were 98 and 97 per cent, respectively. The reason for the coincidence between the two iodine resistant activities is clear. At pH 6 a resistant, but at pH 9 a sensitive "enzyme activity" was inactivated by iodine. The enzyme treated in this manner was then subjected to the usual alkali treatment and the A_{R6} and A_{R9} values were again measured. The results are shown in Fig. 3. It can be seen that the A_{R6} and A_{R9} activities returned to their initial values according to the same kinetics as after the alkali treatment of the native enzyme. The difference

consists only in the return of the A_{R9} activity value to 86 per cent. This is almost the same as the A_{R6} value which in this experiment was about 93 per cent.

It follows from this experiment that the two kinds of iodine resistant activities are independent of each other. When the enzyme is "taken out" of the state which developed as a result of alkali treatment, the enzyme resumes the same A_{R6} and A_{R9} values it possessed prior to alkali treatment. Thus, there can be no transformation leading to some sort of equilibrium between the two states.

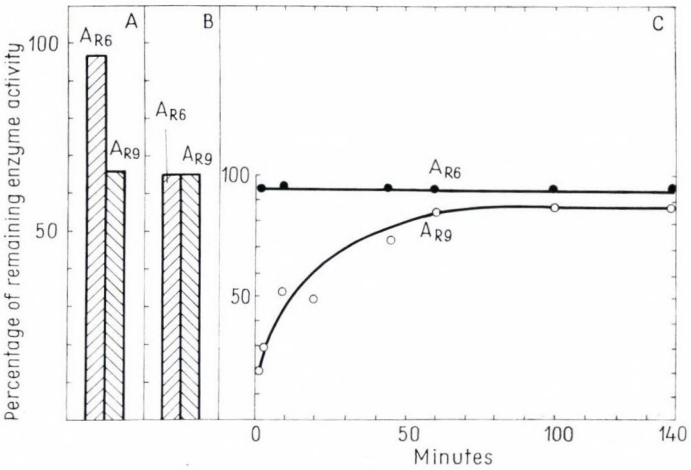

Fig. 3. 8000 U/ml of penicillinase was treated with 0.001 M iodine at pH 9 in 0.05 M phosphate at 0 °C for 5 minutes. The treated enzyme was incubated in 0.1 M NaOH at 0 °C for 2 minutes, the pH of the solution adjusted to 6 and the solution made 0.05 M with respect to phosphate ions. The activities A_{R6} and A_{R9} were measured at various intervals. The activities were expressed as percentage of the activity of the enzyme sample which received neither iodine nor alkali treatment. *A* the activities A_{R6} and A_{R9} of the native enzyme; *B* the activities A_{R6} and A_{R9} of the iodine-treated enzyme; *C* the activities A_{R6} and A_{R9} measured after alkali treatment

Discussion

Citri and co-workers (Citri, 1958; Citri et al., 1960; Zyk, Citri, 1967) have found that the inactivation reaction of penicillinase with iodine is suitable for following the conformation changes of the enzyme. We have shown in our earlier publication that the complete inactivation of the enzyme by iodine proceeds in two steps. In the first step 30–35 per cent of the enzyme is inactivated. The reaction is favoured by a temperature of 0 °C and pH value around 9. At higher temperatures (35–40 °C) the reaction takes place at lower pH values as well. In the second step the residual 65–70 per cent of the enzyme activity also disappears. The most favourable conditions for this second reaction are either 0 °C and a pH above 10, or a temperature above 50 °C and lower pH.

The use of the different experimental conditions of iodine treatment has clearly shown that the inactivation proceeds in the above two steps. As to the mechanism of this inactivation there are three alternatives:

1. For the two steps in the inactivation by iodine two penicillinases each with a different primary structure are responsible.

2. There are two enzyme conformations both with an identical primary structure but with a different iodine sensitivity.

3. In the penicillinase molecule there are two distinct groups each with a different iodine sensitivity (Csányi et al., 1970).

The aim of the present paper was to test the validity of the second alternative.

It is known from an earlier work of Citri (1958) that alkali treatment of penicillinase is accompanied by a considerable increase in iodine sensitivity. Our experiments have confirmed this finding. At the same time changes in iodine sensitivity upon alkali treatment have been analyzed in more details. After a short pre-incubation in 0.1 N NaOH the enzyme becomes fully iodine sensitive, inactivation takes place in a single step at 0 °C and pH 6 provided iodine treatment follows pre-incubation in alkali within less than two seconds.

Our experiments show that after alkali treatment the enzyme gradually regains its initial iodine resistance at pH 6. In accordance with the two inactivation steps encountered in the iodine treatment of the native enzyme the symbols A_{R6} and A_{R9} have been introduced to designate "iodine resistant" enzyme activities. The definition of these symbols may be found in Results.

We have found that the activities A_{R6} and A_{R9} resume their original value by entirely different kinetics. Activity A_{R6} regains its initial value in less than 0.1 minute while activity A_{R9} needs for this more than one hour. If we assume that for the two different kinds of resistance, that is, for the two steps of inactivation by iodine, two enzyme conformations of different iodine sensitivity are responsible, it should be possible to detect a certain transformation between the two conformations. 0.1 N NaOH is an extremely strong ionic medium in which the enzyme is supposedly present in a single conformation. This conformation is stable for a few seconds even after the removal of the alkali and is fully iodine sensitive.

It can be seen from the results shown in Fig. 2 that one hour after alkali treatment the activity values A_{R6} and A_{R9} fully agree with the corresponding values of the enzyme which has not been treated with alkali. To explain this result one might suppose that the conformations representing the activities A_{R6} and A_{R9} are restored from the form which exists in the alkaline medium in the initial proportion. If, however, part of the enzyme is first inactivated by iodine treatment at pH 9 (that part which is resistant to iodine treatment only at pH 6, but can be inactivated at pH 9) and this treated enzyme is then subjected to alkali treatment followed by reacidification, then – as shown in Fig. 3 – A_{R6} and A_{R9} values measured after the first iodine treatment rather than those characteristic of the native enzyme are obtained. In other words the existence of two conformations in equilibrium may be excluded from among the three alternatives.

Since some other experiments of ours have further excluded the possible existence of two different penicillinases differing in their iodine sensitivity (Mile et al., 1969), there is but one explanation of the two-step-inactivation of penicillinase under the influence of iodine, namely the presence in the same enzyme molecule of two distinct groups which differ in iodine sensitivity. The behaviour of

the two groups changes under the influence of alkali treatment, obviously as a result of the conformation change taking place in the extremely strong ionic medium. This change seems to be reversible; the native conformation is restored in the case of the group characterized by the activity A_{R6} within 0.1 minutes, and in the case of the group characterized by the activity A_{R9} within one hour.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (1), pp. 41–43 (1970)

The Influence of Concentrated Electrolytes on the Constitutive Penicillinase Synthesis of B. Cereus

(Short Communication)

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(Received July 29, 1969)

Penicillinase has earlier been described as inducible at high concentrations of electrolytes in B. cereus 569 cells (Csányi et al., 1967). The phenomenon was termed "aspecific induction" and the effect of electrolytes on the regulation of penicillinase synthesis was studied in detail. The mechanism of this induction is not yet known, but the conformational change of a protein which takes part in the regulation of the synthesis – presumably that of the penicillin binding factor described by Pollock (1950) – may well be suspected of being responsible for it. In addition, some other non-specific factors affecting the whole metabolism of the cell through a shift in the ion-equilibrium, must not be neglected in this respect, either. Therefore, it seemed to be reasonable to investigate the effects of electrolytes on B. cereus 569/H cells which produce penicillinase constitutively.

The medium used for the cultivation of B. cereus 569/H cells and the experimental conditions were the same as those earlier described for B. cereus 569 (Csányi, 1966). The cultures were shaken at 37 °C till the cells reached the logarithmic phase of growth. Then, at about 0.15–0.2 mg dry weight material/ml they were diluted fivefold with a fresh medium containing sufficient amounts of phosphate buffer, pH 7.0, to raise the phosphate concentration of the medium to 0.4 M. One of the cultures, also fivefold diluted with fresh medium containing only 0.01 M phosphate, was used as a control. In the course of subsequent shaking at 37 °C the growth of the cells was determined from time to time spectrophotometrically, and the amount of the yield of penicillinase was measured by the same method as previously reported (Csányi, 1961).

The growth of the cells in the medium containing 0.4 M phosphate was considerably slower than that of those cultured in "normal" medium with 0.01 M phosphate content (Fig. 1). The amount of penicillinase produced by the cells in the presence of 0.4 M phosphate was also smaller than that of the control culture. The kinetics of penicillinase synthesis is shown in Fig. 2. However, considering the differential values of the enzyme production (the ratio of penicillinase/ml to dry weight material/ml), there is no difference between the two cultures. Fig. 3 shows the production of penicillinase and that of the dry weight material in a "differential curve". It can be seen that cells growing in the presence of 0.4 M phosphate produce less penicillinase and less dry weight material than the control cells, but the slopes of the "differential curves" are the same.

The penicillinase constitutive mutant in which the regulation of the enzyme

synthesis is lost, produces penicillinase with a maximal synthesising capacity. In the case of inducible mutants this capacity is greatly repressed and becomes effective only on addition of penicillin, the natural inducer, or in the presence of electrolytes in high concentrations.

Our present results show that the inhibitory action of concentrated phosphate has no direct influence on the rate of penicillinase synthesis, although it definitely inhibits the rate of growth. The same results were obtained by using different electrolytes such as sodium chloride, sodium sulfate, etc.

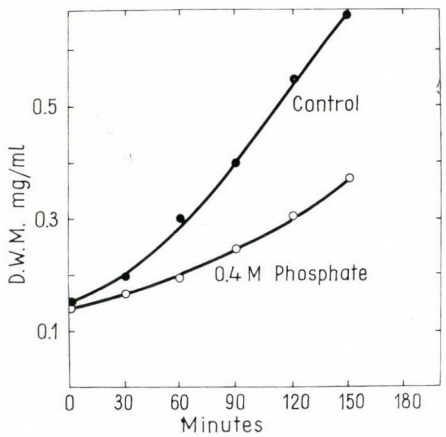

Fig. 1. The effect of 0.4 M phosphate on the growth of the cells. The cultures of B. cereus 569/H at the logarithmic phase of growth diluted fivefold with fresh medium containing phosphate and shaken at 37 °C. ●—● 0.01 M phosphate (control); o—o 0.4 M phosphate

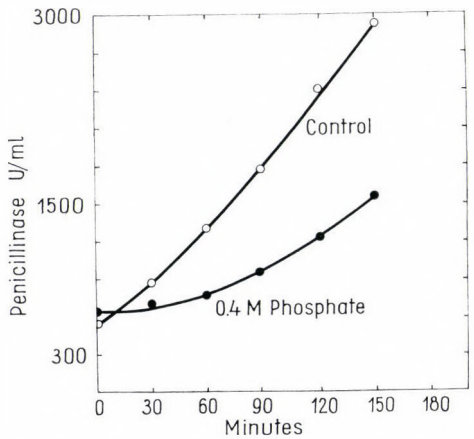

Fig. 2. The effect of 0.4 M phosphate on the penicillinase production of B. cereus 569/H cells. The same experiment as shown in Fig. 1. o—o 0.01 M phosphate (control), ●—● 0.4 M phosphate

In the case of inducible wild type B. cereus we have found a similar inhibition of growth, but, in contrast, a very high increase in the rate of penicillinase formation. We termed this phenomenon "aspecific induction" contrasted with induction elicited by penicillin. Yet our present results show that the effect is not due to a general influence on protein or penicillinase synthesis, but it is specifically concerned with the regulation of the penicillinase system.

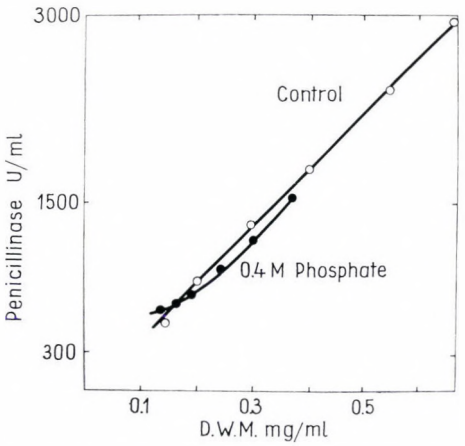

Fig. 3. The effect of 0.4 M phosphate on penicillinase and dry weight material production o; B. cereus 569/H. The same experiment as shown in Fig. 1. o—o 0.01 M phosphate (control) ●—● 0.4 M phosphate

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (1), pp. 45–48 (1970)

The Subunit Structure of Light-Meromyosin

(Short Communication)

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(Received July 28, 1969)

It was observed first by Szent-Györgyi and Borbiró (1956) that light-meromyosin (LMM), the tryptic fragment arising from the filamentous part of the myosin molecule depolymerizes upon treatment with urea to relatively low molecular weight (5000–12 000) peptides termed "protomyosins". Later, based on the kinetic analysis of tryptic digestion of myosins Mihályi and Harrington (1959) suggested that protomyosins were formed by the jagging of the polypeptide backbone of myosin during enzymatic splitting of the myosin molecule to meromyosins. Thus the idea of an eventual functional significance of the protomyosins was abandoned. If the polypeptide chains of LMM were in fact randomly jagged, some important further structural work (e.g. N- and C-terminal determinations) would be meaningless. There is, however, the possibility that protomyosins arise only during the urea treatment. Some proteolytic contamination might be carried along with LMM during purification. Urea dissociates the trypsin-trypsin inhibitor complex (Jacobson, 1955) and denaturation by urea can enhance proteolysis rates enormously (c.f. Okunuki, 1961). Szent-Györgyi and Borbiró (1956) argumented against this possibility by showing that protomyosins were easily and extensively digested by trypsin. They tried to exclude the presence of proteolytic activity in urea-treated LMM by alcohol titration method. Several observations of Szent-Györgyi and Borbiró (1956) may be explained, however, equally well by the activation upon urea treatment of some proteolytic contamination present in LMM. Such are e.g. the existence of a "urea optimum" (5 M urea being more effective than 8 M urea), the time dependence of "depolymerization" and the specificity of urea in contrast to guanidinium-HCl. These facts are hardly reconcilable with the commonly known features of the effect of these structure disrupting solvents (c. f. Tanford, 1968).

In more recent works the failure of guanidinium-HCl to induce the formation of protomyosins was confirmed (Weeds, 1967; Woods, 1968), the observations on the effect of urea, however, are conflicting. Woods (1968) corroborated practically all the aspects of the effect of urea as described by Szent-Györgyi and Borbiró (1956), while McCubbin and Kay (1968) observed a reversible dissociation of LMM to two subunits of equal size (observed by using osmometric methods) in 8 M urea.

Our experiments briefly reported here seem to give full support to the view that protomyosins are formed upon urea treatment by proteolytic contaminations, provided they are present.

LMM-fraction-1 (LMM purified by an alcohol precipitation procedure) was prepared essentially as described by Szent-Györgyi et al. (1960), but with inhibition of tryptic activity by diisopropylfluorophosphat (DFP; see Bálint et al., 1968). LMM-fraction-1 was then treated with different denaturing solvents and its molecular weight was determined by the intrinsic viscosity method of Tanford et al. (1967).

The protein redissolved after alcohol treatment in 0.5 M KCl was reprecipitated by dilution and to the centrifuged precipitate an ethanol-water mixture (3 : 1) was added. The resulting suspension could be kept in the deep-freeze for months as evidenced by the unchanged disc electrophoresis pictures.

For viscosity experiments an aliquot of the alcoholic precipitate was freeze-dried and the dry powder was distributed gravimetrically to make stock-solutions in the different denaturing solvents. Protein concentration of the dry powder was estimated by the biuret method (Gornall et al., 1949).

Fig. 1 shows the reduced viscosity of LMM-fr-1 in different structure-disrupting media. From the intrinsic viscosity observed in 6 M guanidinium-HCl we calculated the number of monomers (n) by the formula of Tanford et al. (1967):

$$100 \times [\eta] = 0.716\ n^{0.66}\ ([\eta] \text{ expressed as dl/g})$$

The calculation gives a value of 642 for n, or, by calculating with an average residue weight of 115, a molecular weight of 74 000. By assuming the two subunits to be identical, this leads to a molecular weight of 148 000 for LMM in excellent agreement with accepted literary data (Mueller, 1964: 151 000; Young et al., 1964: 146 000).

The experiments with urea cannot be evaluated exactly with respect to molecular weight, since the formulas of Tanford do not apply to this solvent. Intrinsic viscosity in 5 M and 8 M urea (0.39 and 0.41 dl/g, respectively) does not differ much from the value obtained in guanidinium-HCl (0.51 dl/g). Therefore no serious degradation is expected in these solvents either.

The results of the reassociation experiments shown in the disc electrophoresis pictures of Fig. 2 indicate that after the removal of the denaturing agent by dialysis, the bulk of the proteins reassociates to LMM.

It is clear that the most complete reassociation occurs in guanidinium-HCl and the least complete reassociation in 5 M urea. McCubbin and Kay (1968) obtained complete reassociation after treatment with 8 M urea as revealed by the sedimentation pattern of the reassociated protein. In our experiments carried out with the extremely sensitive disc-electrophoresis method some unidentifiable diffuse protein components arose forming "clouds" ahead of and especially after the LMM disc. These formations, however, cannot be protomyosins. When protomyosins are actually formed reassociation experiments fail completely (see below). The bulk of the new components formed during the reassociation experiments seem to be, on the basis of their electrophoretic behaviour, comparable in size with LF-1, the longest subfragment of LMM.

We attribute the absence of "protomyosins" in our urea treated LMM preparations to a more complete blocking of proteolytic activity by DFP as compared to the effect of the soybean inhibitor, and/or to the complete irreversibility of DFP

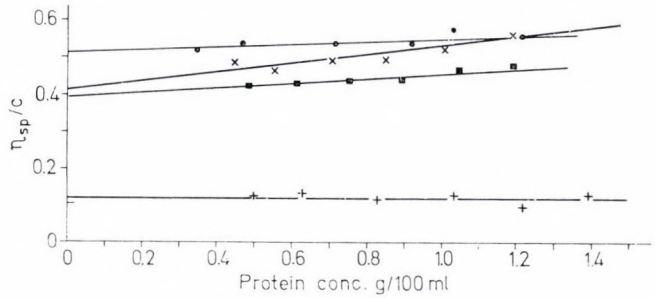

Fig. 1. Reduced viscosity of LMM-fraction-1 preparations treated by denaturing solvents. ●: 6 M guanidinium-HCl; x: 8 M urea; ■: 5 M urea; + : 5 M urea, after addition of a trypsin-trypsin inhibitor mixture (1 : 2) and washing with alcohol-water (3 : 1). The measurements in guanidinium-HCl were carried out at 25 °C some two hours after dissolving (see Tanford et al., 1967), whereas those in the other agents at 20 °C and on the third day after dissolving, as in the experiments of Woods (1968). An Ubbelohde-type viscosimeter was used. All solutions contained in addition to the denaturing agent 0.02 M Tris-HCl, pH 7.5, 0.02 M EDTA and 0.1 M mercaptoethanol. For standardization of protein content see the text

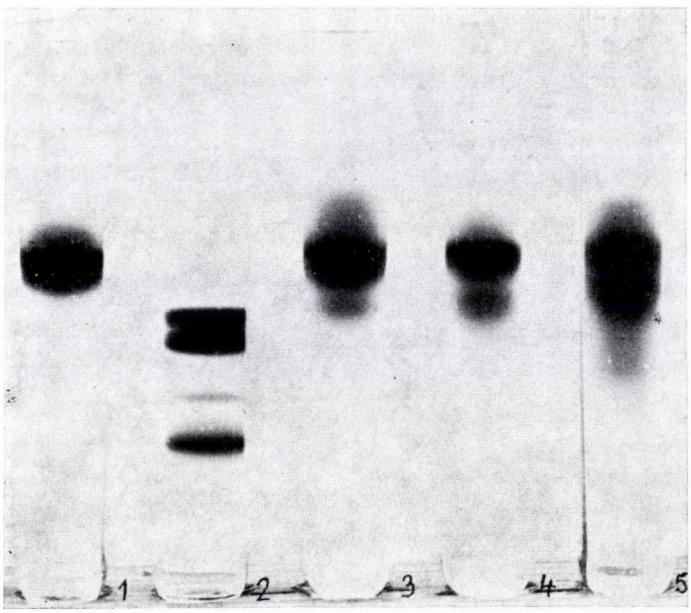

Fig. 2. Disc electrophoresis pictures of LMM preparations treated with denaturing solvents followed by dialysis. Reassociation was affected by dialysing the solutions against 500 vols of 0.5 M KCl, 0.02 M EDTA, 0.02 M Tris-HCl, pH 7.5 and 0.02 M mercaptoethanol. 1. Untreated LMM-fraction-1 preparation. 2. A mixture of LF-1, LF-2, LF-3 (see Bálint et al., 1968), and of H-meromyosin-subfragment-2 (see Bálint et al., 1970). Column 2 is included as a marker of the positions corresponding to the helical fragments of myosin shorter than LMM. 3—5. Reassociated LMM-fraction-1 preparations. 3. 6 M guanidinium-HCl treated, 4. 5 M urea-treated, 5. 8 M urea-treated. Electrophoresis was carried out as described by Bálint et al. (1968)

inhibition. Attempts were made to obtain a direct proof of this assumption by adding trypsin-trypsin inhibitor complex to LMM and observing its effect. To the LMM-fraction-1 precipitated by dilution a trypsin-trypsin inhibitor complex (1 : 2) was added in the ratio of 1 part trypsin to 300 parts LMM. After some minutes of standing, the suspension was centrifuged, the precipitate was washed with the alcohol-water (3 : 1) mixture used in the procedure for the preparation of fraction-1 from LMM. Further procedures for the measurement of viscosity in 5 M urea were carried out as in the experiments described above. As it can be seen in Fig. 1 (crosses), viscosity was reduced by urea treatment to 0.12 dl/g. The intrinsic viscosity in 5 M urea of the protomyosins prepared by Szent-Györgyi and Borbiró was less, i.e. 0.06 dl/g, but there is no doubt that our preparation was also heavily degraded. Using Tanford's formula, an average molecular weight of about 8000 is obtained. This is of course a very rough estimate only, since Tanford's formula refers to guanidinium-HCl and not to urea.

In some other similar experiments alcohol treatment was affected only after two washings with 0.02 M KCl – 0.01 M phosphate, pH 6.5, approaching thus closely the experimental conditions used by Szent-Györgyi and Borbiró (1956). After standing for 3 days in 5 M urea, an intrinsic viscosity of 0.12 dl/g was obtained as compared to a value of 0.55 dl/g for the control which was treated in the same way but without the addition of trypsin-trypsin inhibitor complex. These experiments seem to support the assumption that some trypsin-trypsin inhibitor complex becomes rather tightly absorbed to the precipitated LMM and is activated upon urea treatment.

When "protomyosin", i.e. preparations with low viscosity in urea were dialyzed free of urea no material stained by amido-black was obtained in our disc electrophoresis experiments, even if the anion front was not allowed to leave the column. In all probability, the peptides present were not fixed during staining and were removed during the destaining procedure.

The experiments described here clearly show that protomyosins are proteolytic artefacts. LMM is built up of two continuous polypeptide chains of approximately equal size, and this is a fact of considerable importance for further structural studies on LMM and its subfragments.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (1), pp. 49—52 (1970)

Selective Reaction of Tyrosyl Side Chains with Iodine in Glyceraldehyde-3-phosphate Dehydrogenase

III. Specificity of the Reaction in Urea. Determination of Reacting Histidine and Tryptophan

(Short Communication)

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(Received October 17, 1969)

The substitution of iodine in the tyrosyl side chains of proteins in aqueous solution at slightly alkaline pH is considered a specific reaction. Our investigations reported in previous papers of this series (Libor, Elődi, 1970, 1970a) have supported this assumption disregarding the oxidation of free sulfhydryl groups, which was observed by Móra et al. (1969). In the presence of urea, however, the reaction of additional side chains was observed, both a substitution reaction with histidine resulting in the formation of iodohistidine and an oxidation of tryptophan.

Glyceraldehyde-3-phosphate dehydrogenase contains 11 histidines and 3 tryptophans per subunit (Harris, Perham, 1968). In the present paper the determination of unreacted histidine and tryptophan in the iodine treated protein is described. The amount of histidine can be conveniently measured by a spectrophotometric method described earlier (Ovádi et al., 1967). This is based on the reaction of histidine with diethylpyrocarbonate. The amount of residual tryptophan can also be determined spectrophotometrically by measuring the decrease in absorption at 280 mμ upon N-bromosuccinimide oxidation (Witkop, 1961).

Determination of the unreacted histidine. The procedure of iodination has been described in detail in an earlier paper (Libor, Elődi, 1970).

When the experiments were carried out in the presence of 7 M urea with radioactive $K^{131}I_3$, the quantity of bound iodine was higher than that expected from the amount of diiodotyrosine formed. Moreover, in the fingerprint of the iodine-treated protein labelled peptides were found also in the region of basic peptides which contained histidine but no tyrosine. It was found with other proteins (Wolff, Covelli, 1966) that histidine can also form substituted derivative(s) with iodine. Thus, we have assumed that in the protein studied iodohistidine can also be a product of iodination in the presence of urea.

The number of histidines in the various protein samples was determined by carbethoxylation with diethylpyrocarbonate (Ovádi et al., 1967). The iodine treated samples were first passed through a Sephadex G-50 column to remove iodide from the solution, since it interferes with the spectrophotometric determination of histidine at 240 mμ.

Carbethoxylation was carried out in 7 M urea, pH 7.0 at 0 °C by addition of 100 moles of diethylpyrocarbonate per subunit. After 30 min incubation the

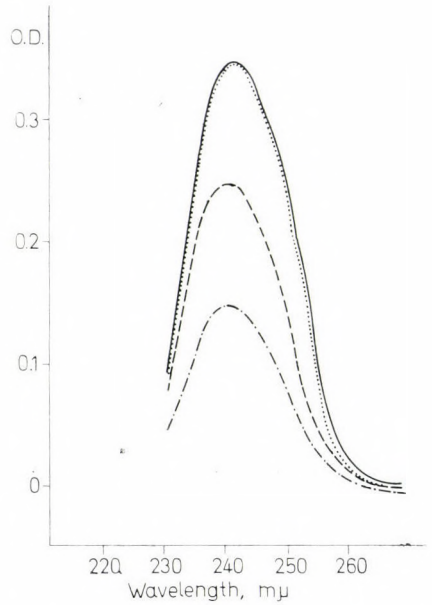

Fig. 1. Difference spectra of carbethoxylated histidyl residues in glyceraldehyde-3-phosphate dehydrogenase 5×10^{-5} M protein solution was reacted with iodine at 0 °C for 30 min in 0.2 M borate buffer, pH 9.2, or in 7 M urea, pH 9.2. Excess iodine was removed by addition of arsenite and the protein was gel-filtered on a Sephadex G-50 column equilibrated with 7 M urea, pH 7.0. 1×10^{-5} M enzyme was then carbethoxylated with 4×10^{-3} M diethylpyrocarbonate. After a fourfold dilution with 7 M urea, pH 7.0, difference spectra were recorded against untreated (non-carbethoxylated) protein. *a*,—— control (without iodine treatment); *b*, . . . and *c*, — — — iodinated with 33 moles KI_3 per subunit in 0.2 M borate buffer, pH 9.2, and in 7 M urea, pH 9.2, respectively; *d*, — · — iodinated with 66 moles KI_3 in 7 M urea

Table 1

Oxidation of tryptophyl side chains in glyceraldehyde-3-phosphate dehydrogenase

1×10^{-5} M protein or 1.2×10^{-4} M N-acetyl-tryptophan ethylester was treated at room temperature with 5×10^{-4} M N-bromosuccinimide in 0.1 M acetate buffer, pH 4.5, containing 7 M urea. Decrease in absorption was measured at 280 mμ and the number of tryptophan residues oxidized per subunit was calculated

Experimental conditions	AE_{280}	Tryptophan oxidized per subunit
Untreated control	0.500	3.0
Iodinated in 0.2 M borate buffer, pH 9.2, with 33 moles KI_3 per subunit	0.515	3.0
Iodinated in 7 M urea, pH 9.2, with 33 moles KI_3 per subunit	0.360	2.15
Iodinated in 7 M urea, pH 9.2, with 66 moles KI_3 per subunit	0.160	0.95
N-acetyl tryptophan ethylester iodinated in borate buffer, pH 9.2	0.510	3.0

absorption was measured at 240 mμ and the amount of carbethoxyhistidine formed was calculated by using a molar extinction difference of $\Delta\varepsilon_{240} = 3260$. As shown in Fig. 1 in the protein iodinated in aqueous solution 11 ± 0.5 histidines can be found (Fig. 1, curves *a* and *b*). In the protein which was iodinated in urea, however, only 8 ± 0.5 histidyl residues could be detected. The histidine content of these samples was also determined by amino acid analysis (Hirs, 1956) which revealed similar amounts of histidine to those found by spectrophotometry.

The reaction of 3,5-diiodohistidine with diethylpyrocarbonate was excluded by incubating 4×10^{-4} M 3,5-diiodohistidine prepared according to Brunings (1946) with 4×10^{-3} M diethylpyrocarbonate in phosphate buffer, pH 6.0. Absorption difference at 240 mμ characteristic of carbethoxyhistidine was not found with the diiodo derivative of histidine.

Determination of the tryptophan content of the iodine treated protein. Hartdegen and Rupley (1964) described that tryptophyl side chains of lysozyme can be oxidized by iodine. We have also observed in experiments with glyceraldehyde-3-phosphate dehydrogenase that upon iodination in 7 M urea a decrease in absorption occurs at 280 mμ (Libor, Elődi, 1969). In addition, a new flat maximum appeared around 325 mμ, probably due to the formation of an oxidized indole derivative (Covelli, Wolff, 1966).

The tryptophan content of the protein iodinated in urea and in aqueous solution, respectively, was determined in 0.1 M acetate buffer, pH 5.0, by the addition of 25 moles N-bromosuccinimide per subunit. The amount of oxidized tryptophan was calculated from the decrease in absorption at 280 mμ according to Witkop (1961; Green, Witkop, 1964). Table 1 shows that both in the untreated protein and in the protein iodinated in aqueous solution three tryptophans can be oxidized. This corresponds to the total tryptophan content of the protein (Harris, Perham, 1968.).

An increase in iodine concentration above 33 moles per subunit raises both the extent of histidine substitution (Fig. 1, curve *d*) and of tryptophan oxidation.

From the above data we may conclude that urea decreases the specificity of the reaction of iodine with various side chains in glyceraldehyde-3-phosphate dehydrogenase. This may be the case with other proteins, too. In addition to tyrosine substitution and sulfhydryl oxidation, formation of iodohistidine and oxidation of tryptophan can also be detected in the presence of urea. The reactions of these two side chains upon iodine treatment can easily and conveniently be determined by spectrophotometric methods, by means of carbethoxylation of unreacted histidine with diethylpyrocarbonate and by the oxidation of tryptophan with N-bromosuccinimide.

The skilful technical assistance of Miss A. Bertók is gratefully acknowledged.

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Acta Biochim. et Biophys. Acad. Sci Hung. Vol. 5 (1), pp. 53–55 (1970)

Transformation of a Serine Protease of Aspergillus oryzae into a Thiol-Enzyme

(Preliminary Communication)

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(Received October 31, 1969)

It has been previously shown that the reactive serine residue at the active site of two different subtilisins can be replaced by a cysteine residue (Polgár, Bender, 1966; Neet, Koshland, 1966; Polgár 1968). By studying the activity of these thiol-enzymes new information was obtained on some important features of the active site, and the mechanism of action of the parent serine proteases (Neet Koshland, 1966; Polgár, Bender, 1967; Polgár, Bender, 1969). The general validity of these conclusions has to be tested with some other serine proteases. For this purpose we have isolated a serine protease from Aspergillus oryzae and prepared its thiol derivative.

Fungal protease concentrate (85 000 HU), a product of the Miles Chemical Co., Inc., was used as starting material which contained at least three different proteases. From these we isolated a component which was highly active towards p-nitrophenyl N-benzyloxycarbonyl-glycinate, and which could be readily inactivated by phenylmethylsulfonyl fluoride, a characteristic reagent for serine proteases.

The protease was isolated by ion-exchange chromatography. The commercial preparation was extracted with water. From this extract the protease bound very specifically to the CM-cellulose column equilibrated with 0.01 M phosphate solution, pH 5.5. Most of the dark colour and of the protease activity did not bind under those conditions. The protease bound to the column was eluted with 0.05 M phosphate buffer, pH 6.5, and further purified on a DEAE-cellulose column equilibrated with 0.01 M phosphate buffer, pH 7.0. In this run the protease appeared in the first fractions, while most contaminations bound stronger. The final purification was performed by equilibrium chromatography on a Whatman CM11 column using 0.01 M phosphate buffer, pH 6.5. The protease obtained as the main homogeneous peak from the column is different from aspergillopeptidase B isolated previously from the same commercial product (Subramanian, Kalnitzky, 1964a). Namely, on the basis of gel filtration on a Sephadex G75 column, we have found a molecular weight of 22 – 27 000 for this protease while 17 920 was described for aspergillopeptidase B (Subramanian, Kalnitzky, 1964b). The molecular weights of the proteases isolated from Aspergillus oryzae by Bergkvist (1963) are not known.

In principle, the thiol derivative of the protease was synthetized similarly to the thiol-subtilisins. The protease reacted rapidly and stoichiometrically with ^{14}C-l'-phenylmethylsulfonyl fluoride at pH 7.0 – 7.5. The resulting inactive phenyl-

methylsulfonyl-enzyme was treated with thiolacetate ion. The acetyl-thiol-enzyme formed in this way was hydrolyzed by the very catalytic entity of the enzyme. The displacement of the phenylmethylsulfonyl group by thiolacetate ion had to be performed at high temperature (40 °C, 20 hours) similarly to the preparation of the Carlsberg thiol-subtilisin (Polgár, 1968).

The specific substrates of the original serine protease, like haemoglobin and casein, are not hydrolyzed by the thiol-enzyme. On the other hand, p-nitrophenyl acetate is hydrolyzed by the thiol-enzyme and 95 per cent of the hydrolytic activity

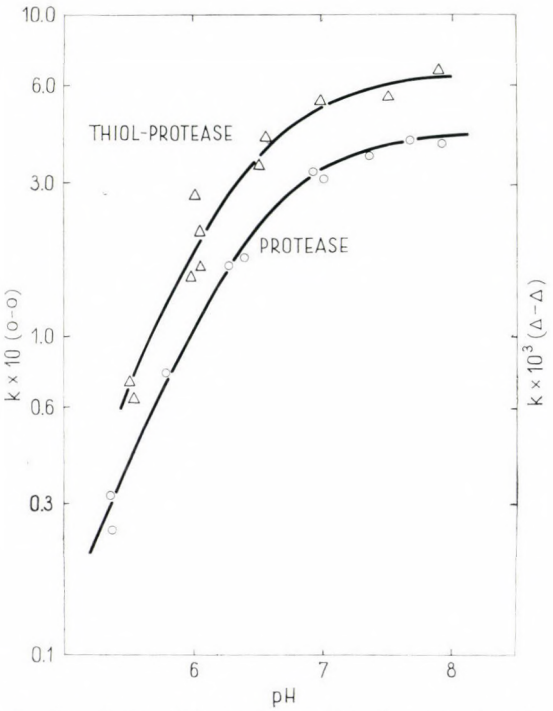

Fig. 1. Semilogarithmic plot of the pH-dependence of the k_{cat} of p-nitrophenyl acetate hydrolysis catalyzed by a protease of Aspergillus oryzae and by its thiol derivative at 25 °C

can be inhibited by p-chloromercuribenzoate, a reagent for SH-groups. The hydrolysis of p-nitrophenyl acetate catalyzed by the serine protease is not inhibited by p-chloromercuribenzoate.

Fig. 1 shows the pH-dependence of the overall rate constants of the hydrolysis of p-nitrophenyl acetate catalyzed by the protease, and by its thiol derivative, respectively. It is seen that the decrease in the value of k_{cat} for the thiol-enzyme is about 65-fold, while about 40-fold decrease was found with the subtilisins. A histidine residue seems to participate in the hydrolysis of both the acetyl-serine and the acetyl-thiol-enzyme, since both reactions depend on a single ionizable group with a pK of 6.5.

Thanks are due to Professor M. L. Bender for some of the fungal proteases, and to Miss E. Mátrai for determining the molecular weight of the protease.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (1), pp. 57—69 (1970)

Bound Water in Physics and Biology*

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(Received November 1, 1969)

1. Contrasting with van't Hoff's conception of osmosis the formula of Raoult emphasizes the role of water. 2. The structure of pure water indicates that the water molecules are bound to a certain degree. 3. Water contained in a swelling system is bound to a certain degree. 4. Water contained in a solid system (porosity, capillarity) is also bound. Due to *capillary condensation* the "polymer water" in capillaries is also bound. 5. The water content of muscle can be considered as bound to a certain degree. 6. Heavy water in biological systems is also bound. 7. The amount of bound water and the sizes of tissue spaces possibly could be applied to the problem of senescence.

1. The physical properties of water and its role in biology have again and again set problems throughout my scientific career.

1.1. It is known that on the basis of Nollet, Dutrochet, Traube and Pfeffer's results, according to van't Hoff — in agreement with the formula of the law of gases

$$PV = RT$$

— the osmotic pressure of a solution is

$$P = \frac{1}{V} RT = c\ RT,$$

where c stands for molar concentration. Thus, according to this theory, solutes exert gas-like pressure in an aqueous solution where water is only a passive medium, similarly to vacuum in a system of gases. All objections from outstanding experts have been in vain; this view can, even today, be found in books of high standard on physics (Budó, Pócza, 1962) and other subjects; in fact in Müller-Pouillet's "Handbuch der Physik" Eucken says (1926): "In principle, osmotic pressure would be measurable simply manometrically in a completely closed space." On the whole this is how also Einstein saw the question (Einstein, 1905).

On the other hand, the importance of the role of water is indicated, simultaneously with van't Hoff, by Raoult's formula, according to which the activity

* Part of a lecture delivered on the invitation of the Loránd Eötvös Physical Society on November 18, 1969.

of water, or its vapour pressure, decreases by its acting as a solvent; according to the relation

$$\frac{p_0 - p_1}{p_0} = \frac{n_1}{n_0 + n_1}$$

the relative lowering of vapour pressure numerically agrees with the mole fraction of the solute (p_0 = vapour pressure of pure water, p_1 = that of the water in the solution, n_1 = the number of g moles of the solute, n_0 = that of the water). Accordingly, osmotic pressure should be interpreted by saying that pure water of higher vapour pressure will pass into the solution of lower vapour pressure. In fact, in "Thermodynamics" by Lewis and Randall (1923) vapour pressure is regarded as the characteristic data of the solution, and according to it the osmotic pressure

$$P = \frac{1}{v} \; RT \; \ln \; \frac{p_0}{p_1},$$

where v = volume of 1 g mole of water.

1.2. van't Hoff's calculation, or rather the concept of gas-like osmosis sharply clashes with the facts in certain cases. It was in this way that I arrived at the biologically important thesis of thermoosmosis; sc., in my consideration (Fig. 1), if two solutions of identical concentration are of different temperatures, then a) according to van't Hoff $P_1 = c\,RT_1$ and $P_2 = c\,RT_2$, and since $T_1 < T_2$, therefore $P_1 < P_2$; thus solvent would pass from P_1 into P_2, from a colder place to one of higher temperature. On the other hand, b) on the basis of the vapour pressure view, if $T_1 < T_2$, the vapour pressure $p_1 < p_2$, and thus more water would be distilled from p_2 of greater vapour pressure into the solution with the lower vapour pressure of p_1, i.e. from a higher temperature to a place of lower temperature, a problem raised by Kohnstamm as early as 1911. In certain cases our experiments on thermoosmosis showed the latter situation (Ernst, Homola, 1952; Katchalsky, Curran, 1965; Vető, 1969), which is a very important result from a biological point of view; viz., it clarifies the so far unexplained fact that the active and, due to its metabolism, warmer cell forces fluid out of itself, e.g. during production of saliva.*

1.3. This conception can be supported by mathematical formalism; viz., I have re-written Clausius-Clapeyron's equation

$$\frac{dp}{p} = \frac{HdT^{**}}{RT^2}$$

* A conclusion *contradictory* to this fact was drawn e.g. by the botanist Münch (1930), who tried to account for the water uptake by saying that the cell being warmed as a result of metabolism is — according to van't Hoff — of higher osmotic pressure, therefore it sucks in water from its surroundings.

** H = molar heat of vaporization of water

with an approximative accuracy into

$$\Delta T = -\frac{RT^2 \ln \frac{p_0}{p_1}}{H},$$

which formula establishes a simple relation between temperature change and vapour pressure change and which – without newer theoretical considerations – directly gives the numerical values of molar lowering of freezing point and molar elevation of boiling point with an accuracy of 0.01 in accordance with the familiar data: – 1.86 and 0.51, respectively, in an aqueous solution.

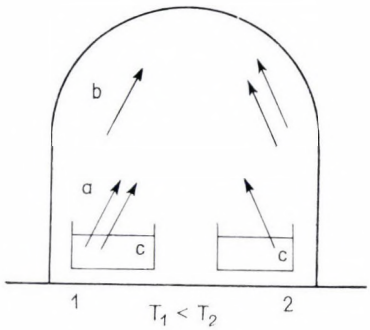

Fig. 1. Thought-experiment for "heat osmosis"

But what does elevation of the boiling point mean? Pure water has to be heated to 100 °C, if its vapour pressure is to be 760 mm Hg, and a 1 molar solution to a temperature of 0.51 °C above 100 °C. I.e., the decreased activity of the water in the molar solution has to be raised by a higher temperature, if vapour pressure is to reach the value of 760 mm Hg. This condition can be described by saying that, by acting as a solvent, water has passed to *a more strongly bound state.* And lowering of the freezing point means that in a molar solution the vapour pressure of water has decreased by exactly as much as is the difference in vapour pressure between solid ice and undercooled water (see § 2.1.), i.e. *water is more strongly bound in solid* than in liquid form.

2. What does a "bound" state mean? For this purpose let us first consider the properties *of pure water itself.* From this analysis it becomes evident that even in pure water there exists a certain degree of binding of water molecules.

2.1. The phase diagram of water (Fig. 2) shows that at the same temperature the vapour pressure of overcooled water is greater than that of ice*: i.e. in solid state pure water is bound to an increased degree.*

2.2. This becomes clearer if the *macromolecular* structure of water is examined. It is known that in water, when in liquid form, the water molecules with a diameter of 2.76 Å form associates of various sizes. In this the dipole character

of the water molecules is implicated, but the fact is more important that when a molecule of pure water dissociates electrically:

$$H_2O \rightarrow H^+ + OH^-,$$

then one water molecule joins the proton, and – as is known – they form an OH_3^+ hydroxonium ion. In pure water this formation is very rare, one in every 10^9 molecules, but not as a stable structure, the proton being bound to different water molecules (Fig. 3). From the figure it is evident that the *H-bonds indicate a certain degree of binding* of water in the macromolecules of water.

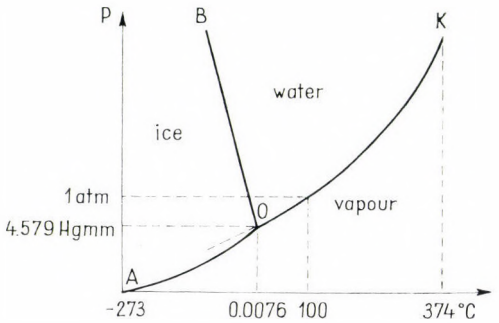

Fig. 2. Phase diagram of water

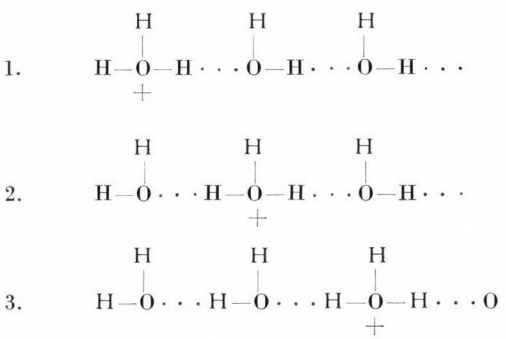

Fig. 3. The water "macromolecule" with H-bridges

The molecular movements due to temperature changes, however, keep on destroying the "macromolecules" of liquid water, in contrast to solid ice the stable structure of which is shown in Fig. 4. Summarizing: *in solid form the water molecules are even more strongly bound, which finds expression in the decreased vapour pressure of ice as compared with the fluid state.*

3. Thus a certain degree of binding of water can be established already in pure water, but more clearly in a solution, in which binding is expressed also

numerically by the lowering of vapour pressure. In general, other *water-containing systems with several components* can only be discussed at a proper scientific level on the basis of the vapour pressure concept.

3.1. Accordingly, on the basis of the vapour pressure view of *swelling*, exactly the same formula could be deduced for the pressure of swelling (Katz, 1918) as for osmotic pressure:

$$P = \frac{1}{v} RT \ln \frac{p_0}{p_1} .$$

Thus solutions and swollen systems both with aqueous media can be dealt with also mathematically, in the same way; this agrees with Ostwald's view, according to which "the relationship between solosmosis and gel-swelling is close" (Ostwald, 1932).

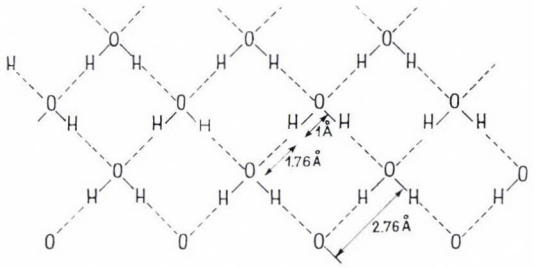

Fig. 4. Crystalline structure of ice in two dimensions

3.2. In spite of the applicability of the same formula to them, solutions and swollen systems behave quantitatively very differently. Let us just remember that, e.g. the osmotic pressure of a NaCl-solution of the highest concentration cannot exceed 200–300 atmospheres, it is able to bind water down to −22–23 °C, and at lower temperature the total water content will freeze. In contrast, an aqueous swollen system can retain its decreasing water content against a pressure of several thousand atmospheres and a much lower (freezing) temperature. This difference is shown in Fig. 5 with the quantitative data of relative lowering of vapour pressure in solutions and a swollen system.

This difference in water binding makes the biological question understandable, whether e.g. muscle should be regarded as a system of solutions or as a swollen system. In 1925 I carried out the following experiment (Ernst, 1926): A living frog muscle kept in a so-called physiological saline solution, takes up a certain amount of water (Fig. 6), but the stretched parallel does much less; when stretching is discontinued, then this muscle makes up for the lag. Later, experiments conducted together with my co-workers Tigyi and Nagy (Ernst et al., 1954) confirmed this finding and established also quantitatively that while a normal muscle is in a state of water-equilibrium with a 0.72 per cent NaCl-solution, an about 25 per cent lower concentration of the NaCl suffices to maintain the state of water-equilibrium with the stretched muscle. Since according to other estimations

by us (Ernst et al., 1951) stretching of the muscle is not associated with any significant change of volume and yet the muscle's ability to suck in water has decreased considerably, this experiment shows that *the muscle cannot be treated as a simple solution, as a so-called osmotic sack but as a structured system, which possibly binds water by means of swelling.*

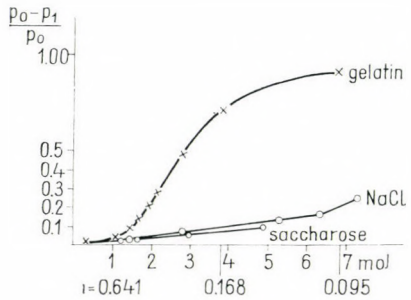

Fig. 5. Water binding as indicated by a relative lowering of vapour pressure in swelling gelatin and in solutions

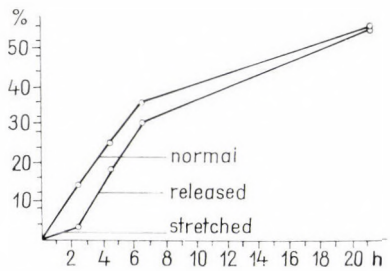

Fig. 6. Water binding by the muscle in states of rest and stretch

3.3. The question of swelling of the muscle and that of binding its water content have long been the subject of an extensive debate in biology. My co-workers and I have also seriously taken part in it (Ernst, Czimber, 1931; Ernst, Fricker, 1934; Ernst et al., 1950) and have thought – together with authors abroad (e.g. Hechter, 1965) – that we had succeeded in proving the binding of water by muscle. Together with Tigyi and Zahorcsek I demonstrated that the relative lowering of vapour pressure of the muscle, as a function of water content, shows a curve similar to the swelling of gelatin, as seen in Fig. 7 (cf. Fig. 5). Yet the denial of bound water has cropped up again and again in the literature of biology (e.g. Mountcastle, 1968).

3.4. This has made it timely to resume, with the collaboration of colleague Pócsik, our investigations of bound water, to see how the density of water would change in the gradually drying muscle. Namely, since according to our mentioned

experiments muscle water has proved to be bound to a certain degree, therefore the question of the hydrosphere of muscle substance comes to the fore. And in connection with the hydrosphere, electrosriction (Ernst, 1963), according to which the volume of (bound) water of hydratation decreases, i.e. its density increases, irrespective of whether water is linked to ions or to protein molecules. Along this

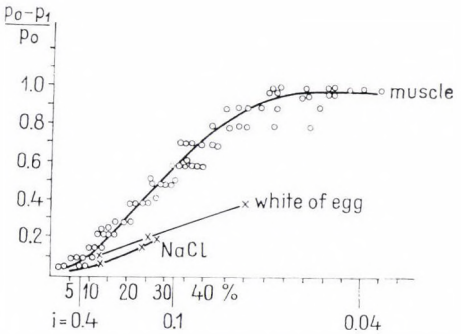

Fig. 7. Relative lowering of vapour pressure of the muscle water in cases of various water contents

line it becomes clear that if muscle water, or part of it, is bound, then the binding, i.e. the density of the water remained in the muscle in the course of drying, must increase.

Fig. 8 shows the experimental data of Pócsik (1967); in the gradually drying muscle the density of residual water increases gradually from the starting value of 1.01 to 1.28 g/cm^3. If it is assumed that the proportion of the compressibility coefficient ($k = 5 \times 10^{-5}$ atm^{-1}) to pressure is maintained even at very high pressures, this would mean that $0.28 = 5 \cdot 10^{-5}\, x$ and

$$x = \frac{0.28}{5 \cdot 10^{-5}} \sim 6 \cdot 10^3,$$

	i	d_i (g/cm^3)	D_i (g/cm^3)
1	4.01	1.012	1.059
2	2.99	1.014	1.073
3	1.69	1.025	1.113
4	1.40	1.032	1.129
5	0.58	1.076	1.209
6	0.30	1.141	1.261
7	0.19	1.192	1.282
8	0.14	1.234	1.292
9	0.12	1.26	1.296
10	0.10	1.28	1.299

Fig. 8. Increase of the density of muscle water after gradual drying

i.e. that, at this stage of drying, the residual water is bound by the muscle with a force corresponding to about 6000 atmospheres.

It must be admitted that this is a result which sets one thinking, although, in the course of our earlier work we also (Ernst et al., 1950. Ernst, 1963) had obtained similar results. Viz. we continuously dried the muscle (frog sartorius), and determined water content and vapour pressure at a certain stage of drying. When the weight of a sartorius muscle had dropped from 180 mg to 34.8 mg, the vapour pressure of the muscle (water) was found to be 1.5 mm Hg ($p_0 = 17.5$); on the basis of the above equation (*p.* 61) the pressure of swelling could be computed to be about 3000 atm.

3.5. At any rate these results again confirm our standpoint that water in muscle, or some part of it, is bound, mainly by the protein substance of the muscle. At the same time this is in agreement with the increasingly uniform view expressed in the literature of recent years that *water attaching itself to proteins* is bound (Hechter, 1965).

This agreement, however, also harbours newer contradictions. Namely, according to several data in the literature (Szent-Györgyi, 1957; Klotz, 1958) this bound water forms a *so-called iceberg*. On the other hand, it is common knowledge that the volume of ice is larger, by about 8 per cent, than that of water. In this way, according to our experimental data showing increased density, we came to be at variance with the view that bound water forms an ice-like structure.

Therefore we made further experiments in which we (Pócsik, 1969) established that if a muscle was frozen at -4 -7 °C, i.e. a veritable ice structure was produced in the muscle, then the volume really increased, and density decreased. Therefore we cannot accept the iceberg hypothesis, because it clashes with the results of our measurements, according to which *muscle water is bound, its density* > 1.

4. However, we obtained our old and new results regarding the binding of water by muscle as if it were a simple process of swelling and used mathematics appropriate to this: we judged the degree of binding of water on the basis of Katz's formula (Katz, 1918)

$$RT \ln \frac{p_0}{p_1} = \frac{ab}{(b+i)^2},$$

which shows the vapour pressure as a function of the relative water content (i). But Fig. 6 indicated the importance of the structured nature of the system; furthermore, even in simple hydrogel *the structure of the system* plays an important role. From the point of view of water-binding it is the porosity of the system, the size of the pores, the clefts and passages, that come to the fore.

4.1. Even porosity in itself acquires importance, since in porous solid substances the heat of vaporization of water may be as much as twice the familiar 10 kcal/mol. This fact shows that vapour pressure of water in the pores can greatly diminish, i.e. *porosity alone can also contribute to the degree of binding water*.

4.2. Thus, the structure of an aqueous system having several components is important from the point of view of binding water, therefore, it is necessary to

take into account a few data of *capillarity*. According to the simplified form of Thomson's (or Kelvin's) equation

$$\Delta p = -N\frac{1}{r},$$

i.e. vapour pressure changes inversely to the radius of curvature of the water surface. This is positive in the case of a concave surface and negative in that of a convex one; i.e. as a result of formation of a convex surface (e.g. drop) Δp is posi-

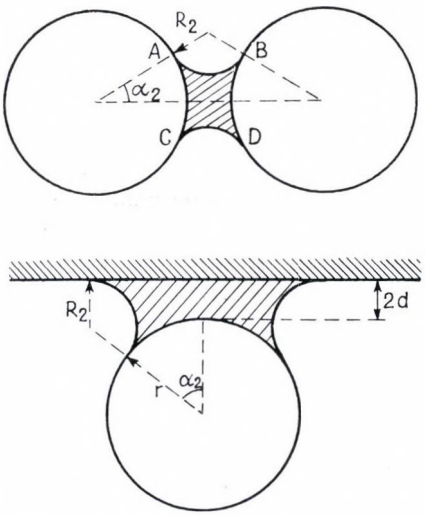

Fig. 9. "Quasicapillary" behaviour of water in spaces between "parallel cylinders"

tive, vapour pressure increases; *if the surface is concave, then Δp is negative, that is, vapour pressure decreases*. Water wets the wall of the capillary; thus, as a result of its concave surface its vapour pressure decreases; i.e. *the binding of water contained in the capillary increases.*

4.3. What has so far been said of capillarity does, however, not make up the whole of the complex of phenomena involved in water binding, which complex is of outstanding importance in physics and biology. Namely, the physical data concerning capillarity mainly deal with phenomena occurring in capillary *tubes*, and it is only in quite recent years that the phenomena have been studied which take place in the spaces between threads, between fibrils or in those between them and membranes (Fig. 9) (Princen, 1969). The phenomena of capillarity occurring in the spaces between these "*parallel cylinders*" correspond, in principle, to the classical descriptions of capillarity.

4.4. In the next step we shall briefly discuss the process called *capillary condensation*, which can be directly related to Thomson's (or Kelvin's) equation mentioned earlier. According to this, as a result of decreased vapour pressure due to

the concave surface the vapour of water is condensed in the capillary space, or viewed from another aspect: the vapour pressure of the water in the capillary space is smaller than that of "macroscopic" water with a free surface. Generalizing our discussion so far, this means that *the binding of water in capillary or other narrow spaces increases.*

4.5. This, however, is only an introduction to the developments in the past decade. After several earlier communications, Fedjakin (1962) reported in his article entitled "On the changes of the structure of water in the case of condensation in capillaries" in Kolloidnii Zhurnal that the vapour pressure of water condensed in the capillaries was even lower than expected and that the water molecules were in a more orderly arrangement. In continuation of these investigations this year there appeared in the Science Lippincott and his co-workers' (1969) "Polywater", i.e. an article on *polymer water*. This paper, summarizing and confirming the data of earlier workers, reports of this kind of water which can be termed "*capillary-condensed*", that (1) its vapour pressure is small, (2) its behaviour differs from that of common water at low and high temperatures, (3) its density may reach 1.4 gcm^{-3}. This will be understandable if it is accepted that, as compared with the 2.76 Å diameter of the normal water molecule, the diameter of this sort of water molecule is 2.3 Å. In short, these data indicate *the high degree of binding of polymer water in the capillary.* At the same time I should like to remark that these newer physical data lend good support to our data of a density of 1.28 gcm^{-3} concerning muscle water.

5.1. In connection with this statement it is in order to quote the microstructure of muscle. In our earlier works we ourselves have also expounded (Ernst et al., 1950; Ernst, 1963) that muscle is a heterogeneous system, and at least three phases can be distinguished in the fresh muscle. It consists a) of about 15–20 per cent *interstitial fluid* which can be regarded as a more dilute solution than blood plasma, and of muscle fibres making up 80–85 per cent of the muscle. The fibres contain b) the ground substance, the *sarcoplasm* and c) the *fibrils*. The *sarcoplasm* is a thick enough colloidal solution containing about 20 per cent dry matter; it makes up about 40 per cent of the muscle.* In this sarcoplasm is embedded the chief representative of mechanical function of the muscle: the bundle of fibrils;** at a first approximation the single fibril may be regarded as swollen gel. The approximative transverse dimensions of these structural elements figuring here: the muscle fibre is about 50–100 μ in thickness, the tissue spaces between the fibres are of a size of several μ; the thickness of the fibril is about 1 μ, and, by and large, the sarcoplasm layer between the fibrils may be considered to be of the same width.

5.2. If, as a first step, we consider the substance of the muscle – being in three different phases and having various consistencies – as we did also in our earlier work quoted above, then Fig. 7 itself will show sharp differences from the point of view of water binding. It can be seen that in the case of gradual drying the value of the relative lowering of vapour pressure indicating water binding is different in a crystalloid solution, in the native egg-white solution containing 18 per

* Frog muscle.

** The further finer structure is left out of consideration in our present discussion.

cent protein and in the muscle. On this basis we assumed that in the course of gradual drying it was the interstitial part of the muscle that lost water most easily and most quickly, then the sarcoplasm and in the last place the fibril. Accordingly we assumed that, after strong drying (relative water content $i \sim 0.1$), the swollen substance of the fibril contained the small amount of water still left in the muscle.*

5.3. It is conceivable that the results of the microphysical investigations detailed above can be transferred to the muscle, considering that the above-described structure of the fibre – with the fibrils embedded in sarcoplasm – corresponds to the "ensemble of parallel cylinders". On the basis of all these data it is possible that *the microclefts in the muscle take part in binding the fluid contained in them.*

6. As a further aspect let be added to what has been said above also the data on *heavy water* according to which in living systems the D_2O/H_2O ratio is, even if in a small degree, greater than in free water; furthermore, that the vapour pressure of D_2O is generally about 20 per cent smaller than that of H_2O.

6.1. The idea of the binding of water molecules was proved correct by experiments in which normal water was replaced by heavy water, or more precisely, in which deuterium was substituted for the hydrogen of water. It is known that the energy of the *H-bridge* amounts to 2–6 kcal/mol, or simplified, to about half the heat of vaporization; this energy is required for the disruption of the H-bridges. On the other hand, the molar heat of vaporization of D_2O is 1.4 kcal/mol larger than that of H_2O. If this is attributed to *a stronger binding of the D-bridges*, it can be worked out that** a D-bridge amounts to 1.69 Å as compared with the 1.77 Å of the H-bridge, which would point to *an even more increased binding of heavy water.*

6.2. The literature shows that the biological experiments with D_2O have led to greatly differing results; of the experiments conducted in our institute I mention that when barley germs growing in a nutritive solution prepared with 1 per cent D_2O had reached a height of 10–20 cm they exerted, on a statistical average, greater force than they did in a solution prepared with aqua bidest. Furthermore, according to the experiments of my co-workers Niedetzky, Dalnoki and Járai, frog sperm and saccharomyces, respectively, live longer and multiply more readily in D_2O than in a solution prepared with tap water or distilled water. From another experiment conducted in collaboration with Metzger I demonstrate three pictures (Fig. 10) of a vineflower (Vitis vinifera) in a flower pot; the first is the normal flower; then, for about one month, it was given 20 ml of bidistilled water every other day; the second picture shows the plant in a poor state; then it was given 1 per cent heavy water in a dosage equal to the former for one month; the third picture shows conspicuous recovery. These results may be attributed (at least partly) to an *increased binding of the D_2O molecules.****

The above-mentioned phenomena definitely argue in favour of a high degree of binding of its water content by the biological system of muscle; this can pos-

* A basis for this view is provided by the fact that the fibril portion of the muscle fibre – in contrast to the two other phases – is a mesomorphic, quasicrystalline substance.

** If the force is in inverse proportion to the third power of the distance.

*** The 99.8 per cent D_2O was obtaind from the Soviet Union and West Germany.

sibly be enhanced if there is heavy water in the muscle. But, to continue this trend of thought, the facts roughly outlined so far might make general *the concept of bound water in biology*.

7. In conclusion let us examine the further perspectives of water binding in biology, at least in a single sector.

7.1. I shall briefly mention the conditions of vapour pressure of silica gel as a function of time (Zsigmondy, 1920): the vapour pressure of fresh silica gel

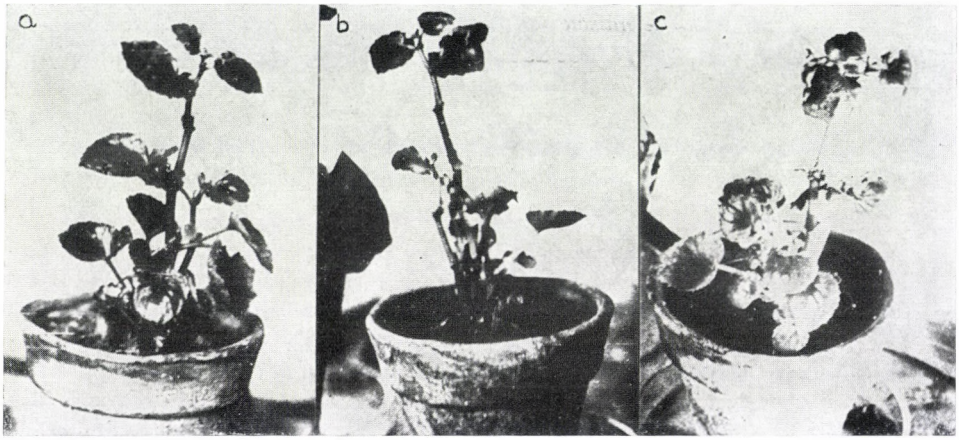

Fig. 10. Vine-flower: (a) normal, (b) after being watered for a month with bidistilled water, (c) after being watered with the same amount of 1 per cent D_2O

composed of 1 gmol of SiO_2 and 1 gmol of H_2O is 4 mm Hg, after 8 months about 6, after 2 and 1/2 years 9 mm Hg, and in its "final" condition it approches to the value of $p_0 = 13$ mm Hg corresponding to pure water (~ 15 °C).* That is to say the high degree of water binding in fresh silica gel is indicated by the observation that, at first, the vapour pressure of the water greatly decreases and only after a longer period of standing approches to the vapour pressure of pure water; at the same time its water binding decreases continuously. This process has long been regarded as the "aging" of silica gel which is associated with the phenomenon that the diameters of the clefts developing in the gel are at first of the order of magnitude of 1 μ and increase to even tenfold in the course of aging.

As to possible investigations in the future, what has been said might indicate that through examining the amount of bound water and the sizes of tissue spaces we shall perhaps be able to approach the problem of aging in biological systems.**

* In the case of a higher starting water content the capacity to bind water (e.g. 1 gmol of SiO_2 + 1 1/2 gmol of water; $p_0 = 13$ mm Hg) decreases, vapour pressure being 6 mm Hg in the fresh state, 8 after 8 months and 10 after 2 and 1/2 years.

** Advancing in years (biologically inevitable) and growing old (more or less pathological, not common to all living systems) are to be distinguished.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (1), pp. 71–79 (1970)

Computer Simulation of the Pattern Transfer of Large Cerebellar Neuronal Fields

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(Received August 11, 1969)

A computer simulation method is applied to the cerebellar neuron circuit, giving an opportunity to study the activity of many neurons simultaneously. As a morphological basis a schematic connection chart is deduced from the original neural net. Each neuron is supposed to fire if the number of their excited input channels reaches a threshold value. The patterns of excited neurons at a particular instant of time are computed and displayed. Four types of cerebellar neurons are taken into consideration in the model. The simulated structure contains altogether 64,260 neurons. Through the patterns displayed one can get an insight into the possible activities in neuronal fields composed of 31,510 neurons, where the complete set of connections of each element has been considered.

I. Introduction

This work aims at simulating the holistic behaviour of neural fields of realistic structure. Earlier attempts at modelling hypothetical networks built up of "formal neurons" show that their study requires simulation methods that permit to consider the networks as a whole.

The earliest discrete representation of a network was made by Rochester et al. (1956) to test the postulates of Hebb and Milner on a quasi-random connected net of 512 formal neurons. Farley and Clark (1961) simulated the propagation of activity-spots in a planar net composed of 1296 elements with interconnections specified by two dimensional probability distributions.

The attempt, to be reported in the present paper, at simulating the neuron network of the cerebellar cortex differs from preceding studies in two important aspects:

(1) The structural characteristics of the network are deduced from the known histological structure of the cerebellar cortex.

(2) The smallest part of the cerebellar cortex that might be considered as a functional unit of higher order and hence worth while to be simulated is composed of far more (in the order of 10^4) neurons even if the model were drastically simplified.

Owing to limitations of the computer available the network, although conforming in principle to the real neuron network of the cerebellum, has been reduced in neuron numbers. It has additionally been simplified by placing all kinds of the neurons considered into two-dimensional fields and by considering their state of

activity for a particular moment only. In this respect the elements of the model are typical McCulloch–Pitts neurons. This implies that no other functional parameter of the neurons can be taken into consideration in this model, than a threshold value for each one: i.e. the units are necessarily considered threshold elements for the time being.

II. Simulation of the Cerebellar Neuronal Network

The neuronal network of the cerebellar cortex offers considerable advantages for the construction of simplified wireing models:

(1) It consists of 5 types of neurons only. Their connections

(2) form a three-dimensional rectangular lattice (if the curvature of the surface is neglected).

(3) The distances bridged by various types of connections, and

(4) the numbers of other elements with which any given type of connection is established, and finally

(5) the physiological properties of all known elements are fairly well understood (Eccles et al., 1967).

(6) It has two input channels.

The connectivity model and its functional interpretation for one of the two input channels – the mossy afferents – has been proposed by Szentágothai (1963). The present attempt of computer simulation is based on his model, shown in its essential features in Fig. 1. In this model the second input channel, the climbing fibres, are neglected for the time being. This is thought permissible as the climbing fibres contact the Purkinje cells directly, so that whatever occurs between the mossy afferent input and the Purkinje cell is not influenced by the climbing fibres.[1]

The present simulation considers only (part of) what may happen if an input reaches the cerebellar cortex through the mossy fibres: the pattern of excitation and inhibition that is set up by this input in the matrix of Purkinje cells.

As seen in Fig. 1 (A and D) mossy afferents establish contacts with the claw-shape dendrites of granule cells. Each granule cell has four dendrites, and each of them becomes involved with a terminal thickening of a mossy afferent. The granule cell gives origin to an ascending axon that upon reaching the superficial (molecular) layer of the cortex divides in T-shape fashion into parallel fibres (Fig. 1 D). The parallel fibres run for about 2–3 mm in the longitudinal direction of the folia and make excitatory contacts with the flat dendritic trees of all Purkinje cells, which they cross. As between two neighbouring Purkinje cells inhibitory interneurons (so-called basket cells) are positioned, the parallel fibres excite also the basket cells. The basket cells, in turn, establish inhibitory connections with Purkinje cells, which are situated in lateral position from the group of parallel fibres that have been excited simultaneously through an incoming mossy fibre volley. The concept of Szentágothai (1963), which has been substantiated by the studies of Eccles and co-workers (summarized by Eccles et al., 1967) is based on the assump-

[1] This is not quite true, but for this first approach this simplification may be justified.

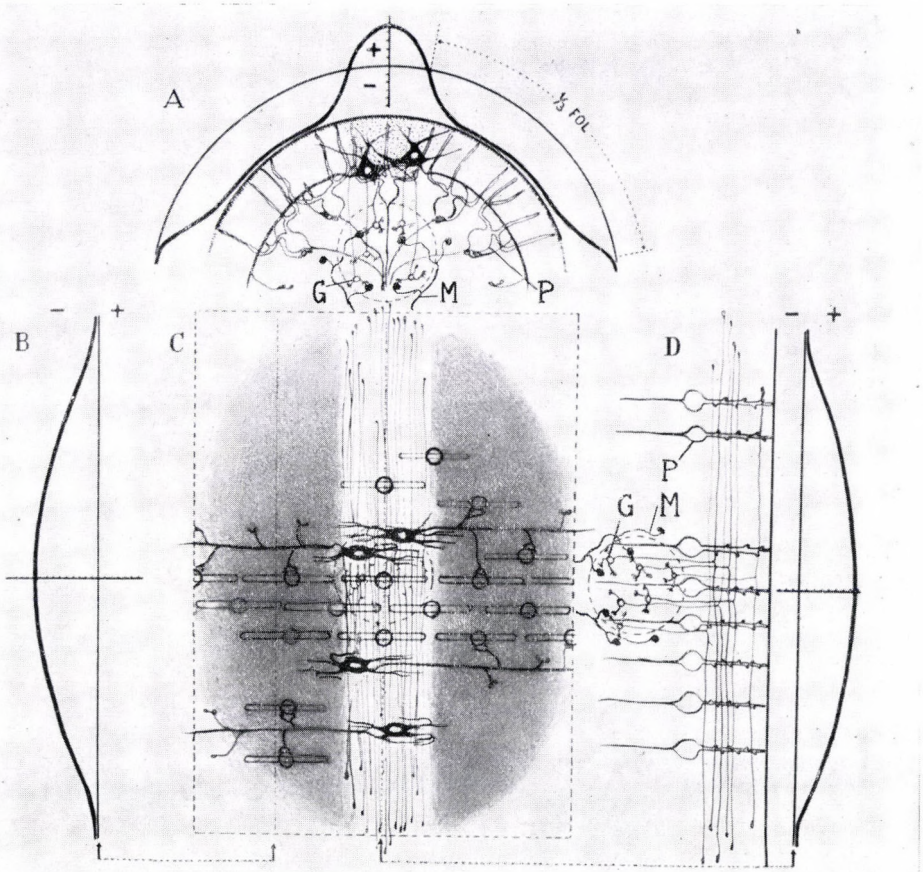

Fig. 1. Diagram illustrating the main neuronal circuit of the cerebellar cortex, detailed explanation in the text. After Szentágothai (1963, 1965)

Fig. 1 shows a composite diagram of a small piece (top of a so-called folium) of the cerebellar cortex. Part *A* of the diagram shows a transverse section of the folium with mossy afferents (*M*) entering from below. They establish synaptic contacts with the claw-shape dendrites of the small granule neurons (*G*). Axons of these neurons ascend into the superficial layer of the cortex where they divide in T-shape fashion to form the parallel fibres. This is seen in part *D* of the diagram representing a longitudinal section of the cortical folium. A comparison between the shapes of the Purkinje cells (*P*; drawn in outlines) as they present themselves in the transverse (*A*) and the longitudinal (*D*) sections, explains that the dendritic trees of these cells are forming flat sheets that are pierced at right angles by the parallel fibers. Part *C* of the diagram shows the cerebellar cortex as it would appear if looked at from above. The Purkinje cells are shown here in highly schematic way only as a circle (for the cell body) and a transverse bar (for the dendritic sheet). Only some representative cells are indicated in the diagram, in reality the space available is fully filled by similar cells in the density and arrangement as can be deduced from the drawing. Inhibitory basket cells are shown in full black in parts *A* and *C* in the Figure. Their axons extend in transversal direction and establish inhibitory synapses with the Purkinje cell bodies. The functional operation of the model is explained as follows: let us assume that a number of mossy afferents terminating in the regions of the

dashed circles become excited simultaneously and set up an excitatory volley in a beam of parallel fibres that arise from this excited focus. Consequently all Purkinje cells the dendritic trees of which are crossed by this excited beam of parallel fibres (white in part *C*) would also be excited. This is indicated by a curved arbitrary scale drawn above part *A*, in which the thick line represents the state of excitation (or inhibition) of the Purkinje cells situated below. A similar arbitrary scale and curve (at the extreme right) indicates the state of excitation of the Purkinje cells along the axis of the excited parallel fibre beam. As some of the parallel fibres terminate towards both ends of the beam the state of excitation of the Purkinje cells falls off at both ends. As can be deduced from parts *A* and *C* also the basket cells situated in the beam of excited parallel fibres would become excited. As their axons are directed laterally in either direction, and as their function is known to be an inhibitory one, they can be assumed to set up inhibition of Purkinje cells on both sides of the excited parallel fibre beam. This is indicated by gray shadowing in part *C* and by the thick line in part *A*. Part *B* shows the supposed state of inhibition of Purkinje cells in a longitudinal section along the dotted line in the left part of *C*. This is logically a mirror image of the curve of excitation as assumed to prevail along the axis of the excited beam of parallel fibres as indicated at extreme right of part *D*. Inhibition could be supposed to fall off in correspondence to both ends of the beam.

tion that if a beam of parallel fibres would become excited by a near simultaneous volley of mossy fibres, the Purkinje cells crossed by this beam would be excited (Fig. 1 C) and the longitudinal row of excited Purkinje cells would be flanked from both sides by a fringe of inhibited rows of Purkinje cells.

The model simplifies this structure by placing each kind of elements into a two-dimensional field. The interconnections of the fields are determined in accordance with the actual relative distances, directions, and numbers of connections as they are found in the real cerebellar neuronal net.

As an input to the system a random binary pattern is assumed to enter through the mossy afferents and to create at each mossy terminal either 1 for being in excited state or 0 for not being excited. Arbitrary assumptions are made for the conditions under which any further element is excited above threshold. These assumptions concern the fraction of synaptic contacts that have to be excited simultaneously from the total number of the contacts of each respective neuron type.

III. The Geometrical Structure of the Model

Consider a set of matrices having r rows and c columns each. Every matrix represents a different neuronal field consisting of one kind of neuron only. The neurons are placed at particular matrix points, so that the arrangement should be similar to the original neuronal network as much as possible. The value of the matrix elements is 1 if the neuron at that place is in an excited state, else 0. Every matrix is denoted by a capital referring to the name of the neuron type concerned. The neurons are denoted by the same capital having an r, c pair of subscripts which indicates the row and column where the neuron is located in the matrix.

The four types of neurons considered in the model are the following:

M: Mossy fibre terminal
G: Granule cell
P: Purkinje cell
B: Basket cell

The structure of the model is characterized by the following list, which indicates the four sets of matrix points containing neurons:

$M_{i,j}$ $[1 : 153,\ 1 : 175]$
$G_{i,j}$ $[1 : 153,\ 1 : 175]$
$P_{i,j}$ $[1 : 153,\ 3 + 5k]$ $(k = 1 : 34)$
$B_{i,j}$ $[1 : 153,\ 3 + 5k]$ $(k = 1 : 34)$

(The $X_{i,j}$ $[a : b,\ c : d]$ formula – also used in the following – means that the i, j subscripts take all the values between the lower a and upper b limits (for i) or between c and d limits (for j) respectively.)

This means that mossy fibre terminals can be found at every matrix point and so can the granule cells. The number of Purkinje cells and that of basket cells is supposed to be equal, but it is five times less than the number of granule cells. Every fifth column only contains Purkinje (and basket) cells. (The first column which contains Purkinje and basket cells has the column subscript 3.)

IV. Interconnections among the Neurons in the Model

A granule cell is connected with 4 mossy fibre terminals and every mossy fibre terminal receives also 4 granule cell dendrites, so both the divergence from the mossy fibre terminals and convergence to the granule cells are 4. The system of connections is:

$$M_{i,j} \rightarrow G_{[(i-1) : i,\ (j-1) : j]}$$

$$G_{i,j} \leftarrow M_{[i : (i+1),\ j : (j+1)]}$$

(The directions of the interconnections are indicated by arrows.) Every granule cell is connected by its longitudinal (columnal), bifurcated parallel fibre with 51 Purkinje cells situated in a column. The Purkinje cell dendritic trees are supposed to span 5 matrix columns in width and not to overlap one another. In such a way $51 \times 5 = 255$ granule cell parallel fibres get through each Purkinje dendritic tree. An important assumption is that all the parallel fibres getting through a given Purkinje cell have functional connection with that cell. The system of the connections among granule and Purkinje cells is the following:

$$G_{i,j} \rightarrow P_{[(i-25) : (i+25),\ k]}$$

$$P_{i,j} \leftarrow G_{[(i-25) : (i+25),\ (j-2) : (j+2)]}$$

And similarly the system of interconnections among granule and basket cells is:

$$G_{i,j} \rightarrow B_{[(i-25) : (i+25),\ k]}$$

$$B_{i,j} \leftarrow G_{[(i-25) : (i+25),\ (j-2) : (j+2)]}$$

(Where k is the subscript of the nearest column containing Purkinje and basket cells to the column j.)

Basket cells are connected with 9—9 Purkinje cells placed symmetrically in both lateral sides:

$$B_{i,j} \rightarrow P_{[(i-1):(i+1),\ (j-4):(j-2)\ \text{and}\ (j+2):(j+4)]}$$

$$P_{i,j} \leftarrow B_{[(i-1):(i+1),\ (j-4):(j-2)\ \text{and}\ (j+2):(j+4)]}$$

V. The Logic of the Model

The conditions (thresholds) which determine whether a granule-, Purkinje-, or basket cell in the model is excited or not, are summarized in the following equations:

$$G_{i,j} = \begin{cases} 0 \\ 1 \end{cases} \quad \text{if} \quad \sum_{k=i}^{i+1} \sum_{l=j}^{j+1} M_{k,l} \begin{cases} < 3 \\ \geq 3 \end{cases} \quad *$$

$$P_{i,j} = \begin{cases} 0 \\ 1 \end{cases} \quad \text{if} \quad \sum_{k=i-25}^{i+25} \sum_{l=j-2}^{j+2} G_{k,l} \begin{cases} < 79 \\ > 79 \end{cases} \quad **$$

$$B_{i,j} = \begin{cases} 0 \\ 1 \end{cases} \quad \text{if} \quad \sum_{k=i-25}^{i+25} \sum_{l=j-2}^{j+2} G_{k,l} \begin{cases} < 79 \\ \geq 79 \end{cases}$$

The influence of the basket cell's inhibition upon a Purkinje cell is expressed by an $I_{i,j}$ factor, namely:

$$I_{i,j} = \begin{cases} 0 \\ 1 \end{cases} \quad \text{if} \quad \sum_{k=i-1}^{i+1} \sum_{l=j-4}^{j-1} B_{k,1} + \sum_{k=i-1}^{i+1} \sum_{l=j+1}^{j+4} B_{k,1} \begin{cases} < 9 \\ \geq 9 \end{cases} \quad ***$$

Therefore an element of the P matrix after being modified by the basket inhibition system (denoted by $P^B_{i,j}$) can be calculated by

$$P^B_{i,j} = P_{i,j} \wedge \overline{I_{i,j}}$$

VI. Results

The patterns of the excited mossy terminals, granule cells, Purkinje cells having been computed (without and with considering the basket cell inhibitory system) the G, P, and P^B matrices can be displayed as follows:

* The nearest majority of the 4 granule cell dendrites.

** This threshold is chosen in order to have an output Purkinje pattern with 50 per cent rate of activity.

*** A simple majority of the 18 basket cell axons arriving at a Purkinje axon hillock.

In the matrices having r rows and c columns an x represents the elements of the matrix with the value 1, and a space the element of the matrix with the value 0. (According to the excited and not excited points respectively.)

It can be easily understood that the neurons situated near to the margins of the fields cannot have all their inputs because some of their input elements lie outside of the matrix. That is why in the following all the patterns presented display

Fig. 2. An input pattern of the system, supposed to be existing in the field of the mossy fibre terminals at a particular moment. Every point of the 101 row-, 130 column matrix represents a mossy fibre terminal marked with x when excited. The states of the mossy fibre endings in this random binary pattern are considered independent of each other. The pattern of granule cells is transformed from this field: the granule cell, for example, connected with 4 glomeruli lying in the frame will be excited if 3 of its inputs are excited

Fig. 3. The pattern of the excited granule cells, transferred from the previous M matrit. The emergence of concentrated excitatory spots as a result of the local averaging effected by the granule cells is worthy of mention. The granule cells being connected with particular Purkinje cell can be seen in the frame. The Purkinje cell itself is in the middle of the frame (in black) and its dendritic tree is also indicated, spanning five granule cells in width

the central part of the matrices only, where the neuronal interconnections are complete from this point of view. This means that the patterns (which have 101 rows and 130 columns) display the array of the matrices from the row subscript 27 till 127 and from co lumn subscript21 till 150. The general formula for the matrices is the following:

$$N[27 : 127, \quad 21 : 150]$$

The vertical directions in the pictures correspond to the longitudinal axis of the cerebellar folium.

The input pattern of the system is shown in Fig. 2 and the G, P, P^B matrices are presented in Figs 3–5.

Although it is not proposed to discuss here the special functional meaning of these patterns, some characteristic features of the transfers are remarkable at the first sight. For example, I wish to draw attention to the emergence of concentrated excitatory spots as a result of the local averaging effected by the granule

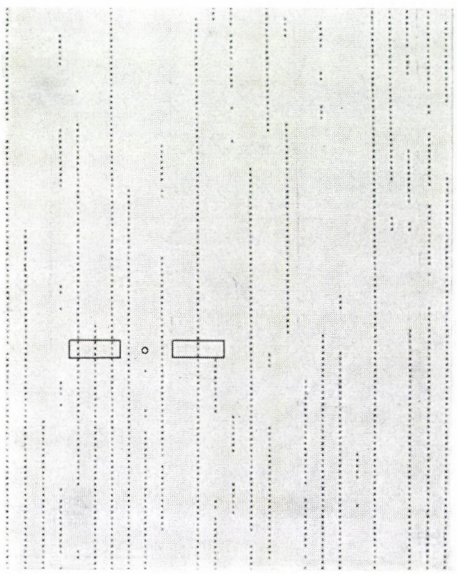

Fig. 4. The pattern of the excited Purkinje cells, transformed from the G granule cell matrix. The Purkinje cells to be inhibited by a single basket cell (marked with a circle) are framed by two rectangles

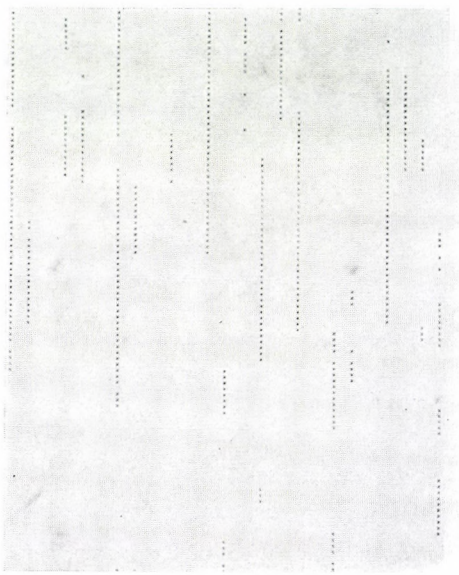

Fig. 5. The pattern of the excited Purkinje cells after being modified by the basket cell inhibitory system. Besides the remarkable columnal activity of the Purkinje cells, the single or very short columns of excited Purkinje cells are also worth while noting

cells (Fig. 2). The arrangement of the active Purkinje columns separated by inactive or inhibited surrounding is also worth while studying (Fig. 4). It is of particular interest that no such pattern could be observed if the histologically established fact were not taken into consideration that the basket cell has little if any inhibitory effect on Purkinje cells situated in immediately neighbouring rows, whereas the inhibitory effect becomes pronounced in the second neighbouring row. It is also interesting to note that in contrast to the speculations of Szentágothai (1963) single Purkinje cells or very short rows of Purkinje cells could be set into action by such a mechanism. This result could not have been expected by conventional reasoning.

The simulation has been carried out with a computer of ODRA 1013 type. The time required has been granted by the Chair of Numerical and Computer Mathematics of the Roland Eötvös University. It is gratefully acknowledged to the Head of the Chair: Dr. J. Mogyoródi. The author is indebted to Dr. A. Meskó as well, for his valuable assistance in the programming.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (1), pp. 81—90 (1970)

Quantitative Researches into the Volume Decrease of the Muscle, I

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(Received July 17, 1969)

For registering and measuring quantitatively the volume decrease following muscle activity a piezoelectric pressure transducer was constructed (Ernst et al. 1951) the transmission of which was examined with square-wave and graded functions produced by electro-mechanical apparatuses. According to our experiments the lower limiting frequency of the pressure transducer is 0.3 c/s, its upper limiting frequency is 300 c/s, its phase delay in this frequency band is within the accuracy of the measurement, and its amplitude-sensitivity is $(3.38 \pm 0.03) \times 10^{-7}$ cc./mV.

Introduction

Several direct and indirect methods of examination have been developed for recording the volume decrease occurring during the activity and the passive tenf sion of the muscle; a greater experimental problem was caused by the analysis o- the quick effect going together with muscle activity.

Formerly the capillary dilatometer has been used with the direct method of examination (Ernst, 1962, 1963; Meyerhof, Möhle, 1933; Meyerhof, Hartmann, 1934; Dittler, Hannappel, 1940). Essentially the same method has been used by later authors (Abbott, Baskin, 1962; Mórocz-Juhász, Örkényi, 1966); the only difference from previous measurements being that the nonlinear change of the capacity of the condenser formed by the liquid-surface and the wire introduced into the capillary have been registered ("proximity transducer") instead of the direct photographical recording of the liquid-meniscus movement.

The authors mentioned above dealt with the examination of the transmission of the capillary volumeter only in part; Ernst and Uj (1934) treated it as a "maximum method" considering the time-course, thus referring to the occurring difficulties. Lately the capillary dilatometer was examined theoretically by Pasechnik (1968).[1]

Ernst et al. (1951) transformed the volume decrease of the muscle into a pressure change: the muscle chamber was closed by a piezoelectric quartz crystal, and the potential-difference developing on the crystal was registered. Baskin and Paolini (1966) used a capacitive pressure transducer; Pasechnik (1968, 1969) also

[1] The procedure of Kühne and J. R. Ewald, perfected by Ernst, can be considered to be the first indirect method: he measured the relative density change occurring parallel with the volume decrease of the muscle (Ernst, 1925).

performed experiments with a piezoelectric pressure transducer. During the application of pressure transducers experiments spread out also to the determination of the frequency characteristics of the apparatuses.

The exact analysis of the volume change of muscle needs a reliable experimental method; therefore, in the present work, we tried to calibrate the pressure transducer used by us and to determine the limits of its applicability.

Experimental Methods

The experimental method has been developed by taking the following requirements into consideration:

1. The pressure transducer used for measuring the volume change should be a practically inertia-free instrument of possibly linear characteristics.

2. The electronic system should amplify in a wide frequency range, without distortion.

3. The signal introduced into the muscle chamber (reference signal) by the apparatus used for calibrating the frequency and amplitude of the pressure transducer should be well definable.

The first requirement was fulfilled by a piezoelectric quartz crystal (Ernst et al., 1951) and the second one by a special dual-ray oscilloscope. In order to fulfil the third requirement an electro-mechanical apparatus was built, and a capacitive transducer appropriate for registering its quick movement was constructed to it.

The experimental arrangement together with the auxiliary apparatuses serving for calibration can be seen in Figs 1a and 1b. The muscle chamber is a well-closed glass cylinder with a firmly fixed round piezoelectric quartz crystal at the one end. The volume change of the muscle placed into a closed vessel filled with Ringer's solution of room temperature results in a pressure change in the liquid, which causes a slight deformation of the crystal. The deformation is followed by a potential-difference developing on the crystal capacity without delay. The amplification and registration of the electric signal proportional to the volume change were ensured by the first channel of the electric apparatus. The pre-amplifier (2) contained an electrometer tube of small grid current placed at some cm distance from the crystal and used as a cathode follower, and it also contained an alternating current amplifier stage. The output of the pre-amplifier was fed into a three-stage amplifier (3), the signal to be examined getting on the first pair of deflector plates of the cathode ray tube (6). The second channel either passed on a signal proportional to the input volume change or the time-mark of 50 c/s frequency to the second pair of deflector plates of the cathode ray tube.

The critical first channel of the electric apparatus has been rated by considering the low frequency substituting image of the piezoelectric quartz crystal (Fig. 2a). The crystal can be considered to be a capacitive generator with a charge production proportional to the pressure change. Fig. 2b indicates the elements measurable experimentally: C is the resultant of the crystal capacity and the spurious capacity, which is closed by the resultant (R) of the leakage resistance (R_l) and the grid leak resistance (R_g). In the case of small changes in pressure the pressure-converting

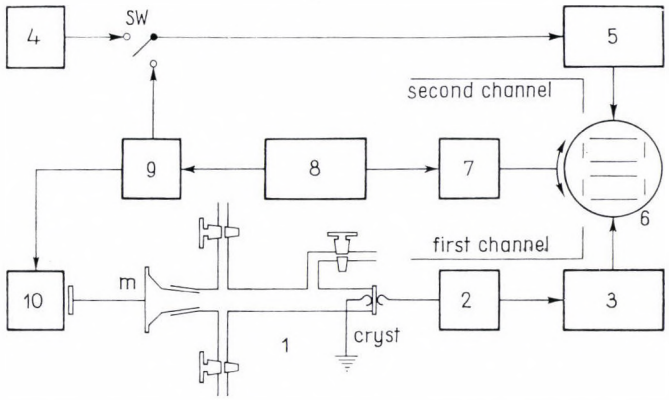

Fig. 1a. The scheme of the pressure transducer measuring the volume change and of the instrument creating the rectangular volume decrease. (1) muscle chamber, (2) pre-amplifier, (3) and (5) amplifiers, (4) time-mark generator, (6) dual-ray cathode ray tube, (7) Kipp generator, (8) square-wave generator, (9) additional resistance, (10) electromagnet, cryst = quartz crystal, sw = switch, m = membrane

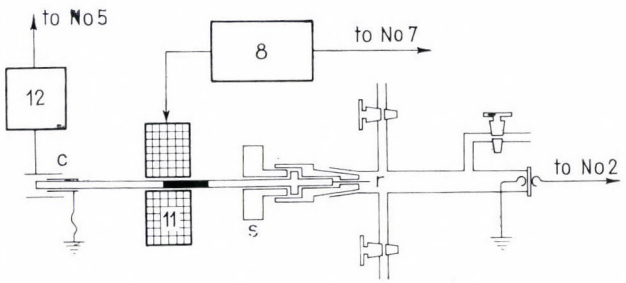

Fig. 1b. The scheme of the instrument creating the graded volume change. (8) square-wave generator, (11) electromagnet, (12) capacitive transducer, s = micrometer screw, r = ram, c = cylinder condenser (cp. Fig. 1a)

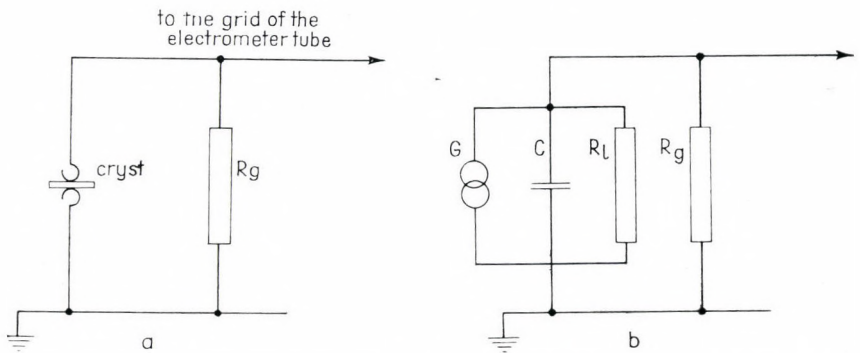

Fig. 2a, b. The crystal-circuit (a) and its equivalent circuit (b). cryst = quartz crystal, R_g = grid leak resistance, R_l = leakage resistance, C = input capacity, G = charge generator

characteristic of the quartz crystal is linear (Gohlke, 1959), so the transmission of the low-frequency components is primarily determined by the time constant ($\tau = RC$) constituted by the above elements. Since the effect is small, the appropriate time constant can only be reached by the increase of R and R_g, respectively. (The increase of C would necessitate further amplification, which would increase the noise-signal relation.) It is useful, therefore, to build an electrometer tube of high input resistance into the first stage of the amplifier, and to forward the signal through an impedance transformer (Nastuk, 1964; Grave, 1965). As the expectable duration of the muscle signal to be registered is of ms order of magnitude, it is appropriate to use an alternating current amplifier of small lower limiting frequency and of greater stability instead of a direct current amplifier.

The frequency transmission of the amplifiers (Fig. 1a) was examined with the aid of a sine-generator; according to this the frequency band of the three stage amplifier of the first channel (3) is 0.15 c/s to 5 kc/s, which is modified by the preamplifier (2) as follows: the lower limiting frequency varies between 0.3 and 15 c/s depending on the grid leak resistance (R_g) and the upper limiting frequency is 1 kc/s. The frequency band of the amplifier constituting the second channel (5) is 0.1 c/s to 5 kc/s.

As the direct determination of the frequency characteristic met technical difficulties, the transmission of the piezoelectric pressure transducer was examined with the aid of square-wave and graded functions produced by electro-mechanical instruments.

The rectangular volume decrease was created by the impulse-like moving of a round glass-membrane fixed to the wall of the muscle chamber filled with liquid (Fig. 1a). The membrane (m) was joined to the tongue of an electromagnet (10) through a thread;[2] the current of the electromagnet coil was yielded by a square-wave pulse generator (8). During the outward tension of the membrane there comes about a liquid expansion corresponding to the volume decrease, and during its release there comes about a liquid compression corresponding to the volume increase. The decreasing voltage on the additional resistance (9), series-connected with the electromagnetic coil served as a reference signal.

The graded volume change was produced by moving the liquid-proof ram introduced into the muscle chamber (Fig. 1b). Moving back the ram (r) corresponds to a volume decrease and its moving forth corresponds to a volume increase. The axis to which the ram of a cross-section of $q = r^2\pi = 0.015^2 \times \pi\ \mathrm{cm}^2 \sim 7 \times 10^{-4}$ cm^2 was fixed was moved by an electromagnet (11); as a permanent magnet was used as an iron core, the direction of the displacement of the ram could be regulated by changing the direction of current in the coil. The length of the displacement of the ram was adjusted with an accuracy of 2×10^{-4} cm with the aid of a micrometer screw (s). In these experiments the displacement of the ram was used as a reference signal (input signal). The displacement was measured with a capacitive transducer (12) in the oscillating circuit of which a cylinder condenser (c) changing its capacity parallel with the displacement of the ram was interposed (Mórocz-Juhász, Örkényi, 1966).

[2] The possible elasticity of the thread had to be taken into account.

Our experiments performed with the apparatus outlined here, the parameters of which were chosen by considering the results of our preliminary muscle experiments, were recorded photographically.

Results

Figs 3a–e show "crystal signals" coming about under the effect of rectangular volume decreases of 200 ms duration in the case of grid leak resistances of ∞, 300, 30, 10 and 1 Gohm (Figs 3a–e, lower curves). Of course, the volume

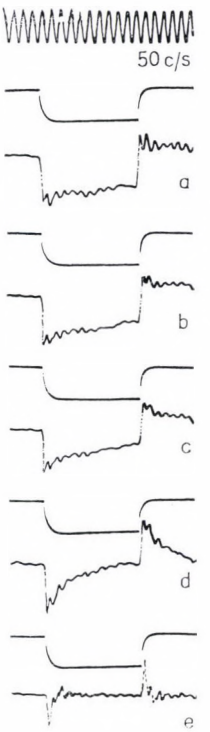

Fig. 3a–e. Transmission of a rectangular volume decrease of a duration of 200 ms in the case of a grid leak resistance of ∞, 300, 30, 10 and 1 Gohm (a–e). Upper curve: change, proportional to the input signal; lower curve: output signal

decrease signals are late as compared to the current of the coil (upper curves), their runing up is not momentary; notwithstanding, the time constants belonging to the enumerated grid leak resistances can be determined graphically, with good accuracy, on the basis of these signals. The time constants are: 440 ± 10, 375 ± 10, 195 ± 10, 70 ± 10 and 9.0 ± 1.5 ms. The oscillation of a frequency of 55 c/s appearing on the volume decrease curves is due to the vibration of the stretching thread.

The oscillograms of Figs 4a–h show graded signals of volume decrease (4a–d) and volume increase (4e–h) appearing under the effect of the displacement of the ram 2.5×10^{-3}, 5×10^{-3}, 10×10^{-3} and 20×10^{-3} cm outward and inward, respectively (Δl); the absolute values of the volume changes (ΔV) are $1.75\times$ $\times10^{-6}$, 3.5×10^{-6}, 7×10^{-6} and 14×10^{-6} cc. ($\Delta V = q\Delta l = 7\times10^{-4}\times2.5\times10^{-3}$

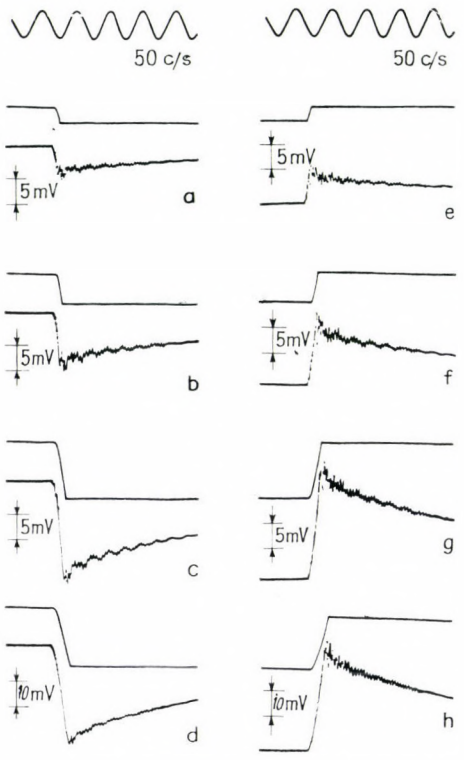

Fig. 4a–h. Transmission of a graded volume decrease (a–d) and volume increase (e–h). Upper curve: input signal, lower curve: output signal. (The size of the volume change vertically: 1.75×10^{-6}, 3.5×10^{-6}, 7×10^{-6} and 14×10^{-6} cc.)

cc. $= 1.75\times10^{-6}$ cc.). The oscillograms indicate that the duration and magnitude of the running up of the displacement (upper curve) and of the volume change (lower curve) vary parallelly.[3]

The curves of Figs 5, 6 and 7 have been obtained by adaptation to the data of the experiments shown in Fig. 4.

[3] The volume change signals are disturbed by a self-oscillation of a frequency of 120 c/s and by one of 585 c/s. The amplitude of the former can be considerably influenced by changing the coupling of the electro-mechanical system causing the volume change and of the muscle chamber.

The downflow of the displacement (Δl) and the volume change (ΔV) in time in the case of normalized ordinate units have been shown on a common curve in Fig. 5. The common curve is accounted for by the fact that no difference beyond the accuracy of the measurement (0.3 ms) was found between the time data of the displacement and the volume change.[4]

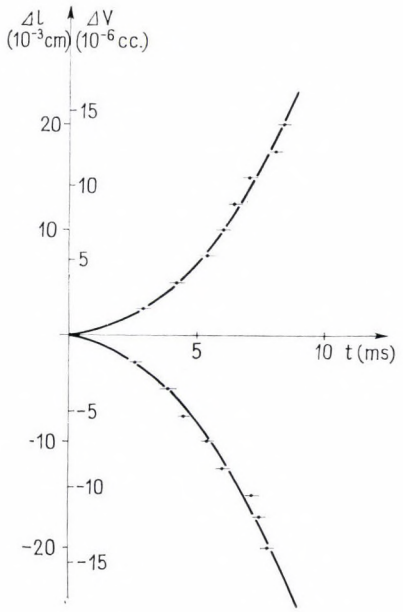

Fig. 5. Running up in time of the graded volume change

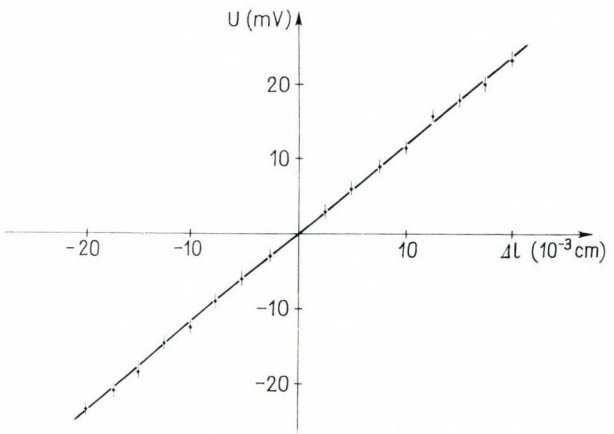

Fig. 6. Calibration curve of the capacitive transducer recording the displacement of the ram

Fig. 6 proves the linearity of the capacitive transducer registering the displacement of the ram: the output signal of the transducer changes proportionally to the change in the magnitude of the displacement; the slope of the line is (1.18 $\pm$ $\pm$ 0.01) mV/10^{-3} cm.

Fig. 7 presents the actual *calibration curve* of the volumeter and the pressure transducer, respectively. The slope of the straight fitted to the points of measurements is (2.97 $\pm$ 0.03) mV/10^{-6} cc. So the sensitivity of the apparatus is (3.38 $\pm$ 0.03) $\times 10^{-7}$ cc./mV.

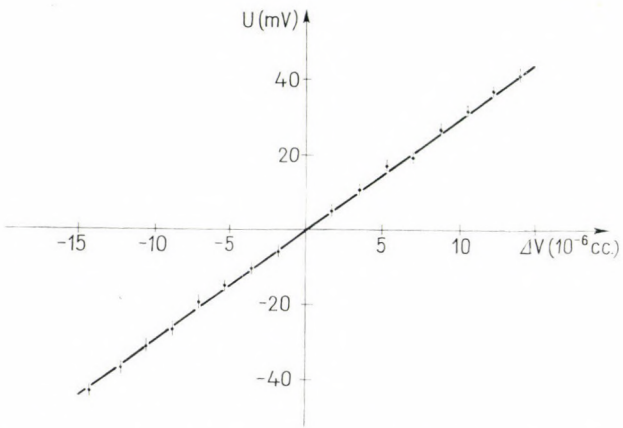

Fig. 7. Calibration curve of the pressure transducer registering the volume change

Discussion

The limits of the serviceableness of the volumeters can be determined on the basis of their frequency characteristics. The basic difficulty lies in choosing a source which is able to create a volume change of optional frequency and of constant amplitude. To bridge over this problem it seems to be useful to examine the transmission of rectangular and graded volume changes. On the basis of the decay of these signals we can namely conclude on the lower limiting frequency of the volumeter and, on the basis of their running up or of the natural frequency vibration of the apparatus, we can conclude on its upper limiting frequency.

[4] Supposing the electromagnet to ensure a constant accelerating power and the power hindering the movement to be independent of the velocity, the functions $\Delta l = \frac{a_l}{2} t^2$ and $\Delta V = \frac{a_V}{2} t^2$, respectively, were matched to the points of measurements, where a_l is the acceleration of the displacement, a_V is the acceleration of the volume change and t is the time. According to our calculations the acceleration of the outward and inward displacement of the ram is: (-0.64 ± 0.03) $\times 10^{-3}$ cm/ms^2 and (0.58 ± 0.03) $\times 10^{-3}$ cm/ms^2, resp.; the acceleration of the volume decrease is (-0.44 ± 0.02) $\times 10^{-6}$ cc./ms^2, the acceleration of the volume increase is (0.40 $\pm$ 0.02) $\times$ $\times 10^{-6}$ cc./ms^2.

Our results show that the lower limiting frequency of the pressure transducer used by us is determined by the electronic apparatus. This is proved by the fact that the change in the input data of the amplifier is followed by the running off of the rectangular volume decrease of a duration of 200 ms. The value of the smallest lower limiting frequency attainable is 0.3 c/s. In fact, this is the data meaning the main difference from the piezoelectric pressure transducer used formerly in our institute (Ernst et al., 1951): the input resistance of 20 Mohm has been increased to a Gohm order of magnitude, so the transmission frequency band has been significantly widened towards the low frequencies.

The lowest natural frequency of the muscle chamber calculated from its geometrical data (> 10 kc/s), the natural frequency of the quartz crystal (> 100 kc/s) and the upper limiting frequency of the amplifier (1 kc/s) – respecting the expectable time data of the biological effect to be measured – do not influence the transmission of the signal to be registered. Inasmuch as the oscillation of a frequency of 585 c/s already mentioned is considered to be the natural frequency equivalent to the pressure transducer, of a univariant system able to vibrate, we can determine, on the basis of the damping factor, that the upper limiting frequency of the pressure transducer is 300 c/s. It can be easily demonstrated that the phase delay in the frequency band of 0.3 to 300 c/s is negligibly small.

The "rod method" used by us for calibration has been first applied by Baskin and Paolini (1966), but the frequency-characteristic of the pressure transducer has not been determined.

Pasechnik (1968) does not find the "rod method" appropriate for determining the right frequency characteristic; instead, he recommends the examination of the volume change caused by gas formation and he publishes his results concerning the frequency characteristic of the volumeter on the basis of this experiment. It is, however, just the gas formation because of which Pasechnik's method is unsuitable for calibrating the amplitude of the pressure transducer, as the effective compressibility of the liquid is significantly influenced by the presence of the gas bubble.

In our oppinion the condition of the undistorted transmission of the volume change is that the surface velocity of the object producing the effect should be lower than the wave velocity of the voice in liquid. Under this condition the change in pressure can be considered to be the same in the whole volume of the muscle chamber filled with liquid, so the registration follows truly the volume change in the muscle chamber. This condition is fulfilled in this experiment, because the maximum velocity under the calibration does not reach the value of 5 cm/s.

The above data concerning the frequency characteristic and the velocity as well as the linearity of the pressure transducer support the experimental result that the pressure transducer is able to register, without distortion, the volume change of an acceleration of 0.44×10^{-6} cc./ms^2 and of a maximum amplitude of 14×10^{-6} cc.

The chief merit of the pressure transducers used for measuring the volume change as compared to the capillary dilatometers is that the former ones – due to their frequency characteristics – are more appropriate for measuring the dynamic volume changes. Pasechnik thinks the capillary dilatometer to be able to

register the volume change of the muscle. Meyerhof et al. held the same opinion though Meyerhof and Möhle (1933) pointed out a phase delay equivalent to 7 ms with a volume change of a frequency of 50 c/s, and Meyerhof and Hartmann (1934) measured natural frequencies of about 100 c/s. These data led us to conclude on a cut off at higher frequencies and so to a destorted transmission of the examined signals. The uncertainties occurring during the motion of the liquid in the capillary cannot be eliminated by electrical observation either (Abbott, Baskin, 1962; Mórocz-Juhász, Örkényi, 1966).

A pressure transducer described in the present paper enables us to register the signals of volume change of an amplitude of 10^{-7} to 10^{-5} cc. containing components of a frequency of 0.3 to 300 c/s with a good accuracy, and to measure them quantitatively.

The authors express their thanks to Prof. Ernst for his advices during this work and to J. Örkényi for his help in constructing certain parts of the experimental equipment.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (1), pp. 91–97 (1970)

Quantitative Researches into the Volume Decrease of the Muscle, II

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(Received September 25, 1969)

The volume change of the striated muscle has been investigated with a piezoelectric pressure transducer. In a tensionless condition – when the frog gastrocnemius muscle was stimulated indirectly by single and series electric impulses – it became possible to register the time course, amplitude and maximum frequency of the initial volume decrease (not of the one going together with tension). According to our experiments performed at room temperature the initial volume decrease is reversible; it reaches its maximum, with a value of: $(1.5-3.5)\times 10^{-6}$ cc./g muscle, in 10 ms after the nerve stimulus; the effect follows stimulus series of 200 c/s, sometimes even of higher frequency.

Introduction

The volume change, one of the phenomena going together with the activity of the striated muscle, is a much debated theme of muscle-biophysics and -physiology (Ernst, 1962, 1963). Though nowadays different mechano-electric transducers are used for registering the effect instead of the formerly used capillary dilatometers, there is no agreement in the literature even concerning the character of the initial volume change. Ernst et al. measured volume decrease with a piezoelectric pressure transducer, thus corroborating their qualitative results obtained formerly with a capillary dilatometer. Abbott and Baskin (1960, 1962) as well as Baskin and Paolini (1965, 1966) have registered also volume increases with two kinds of capacitive transducers ("proximity transducer" and "pressure transducer"). On the other hand, Mórocz-Juhász and Örkényi (1966) using the "proximity transducer" demonstrated volume decreases. Recently, Baskin and Paolini (1967a, 1967b) as well as Baskin (1967) have given accounts of volume decreases. Pasechnik (1968, 1969) – on the basis of his experiments performed with a piezoelectric pressure transducer – considers the positive phase of the volume change, i. e. the volume increase of the muscle, to be verified.

It is the aim of the present work to characterize, with quantitative data, the initial volume decrease of the striated muscle.

Experimental Methods

The experiments have been performed in a tensionless condition of the muscle. As muscle preparations frog ischiadicus gastrocnemius preparations, isolated from Rana esculenta were used. A modified version of the piezoelectric pressure

transducer developed formerly in this institute (Aradi, Futó, 1970) has been used in the experiments.

Fig. 1 shows the schematic arrangement of the experiments performed on muscle. The preparation was placed into a liquid-proof glass cylinder – into a so-called muscle chamber (1) – in such a way that the nerve lay on the pair of stimulating electrodes introduced through the stopper; *the muscle and the stopper were not stiffly connected to each other.* The muscle chamber was filled with a non-oxygenized Ringer's solution of room temperature in such a way that it did

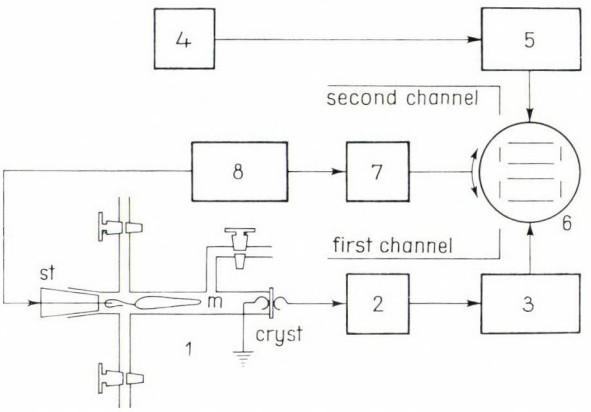


Fig. 1. Scheme of the apparatus measuring the volume change of the muscle. (1) muscle chamber (cryst = quartz crystal, m = muscle, st = stopper*), (2) pre-amplifier, (3) and (5) amplifiers, (4) time-mark generator, (6) dual-ray cathode ray tube, (7) Kipp generator, (8) stimulator.
* The muscle and the stopper are not stiffly connected

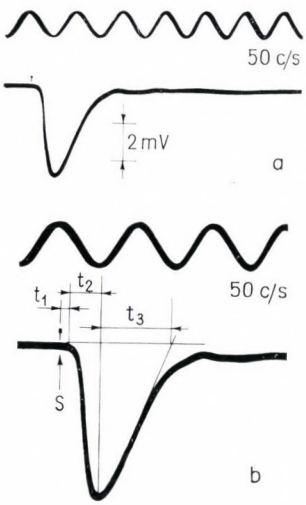
not contain bubbles. In the closed muscle chamber, i.e. under isochor circumstances, the pressure change caused by the volume change of muscle results in a potential difference on the piezoelectric quartz crystal. This signal, proportional to the volume change of the muscle, was amplified by the first channel of the electric apparatus (2 and 3). The stimulus signal was also introduced into this channel through a capacitive connecting, so the stimulus break in and the signal to be examined got on the first pair of deflector plates of the cathode ray tube (6). The second channel (5) passed on the signal of 50 c/s frequency of the time-mark generator (4) to the second pair deflector plates of the cathode-ray tube.

The indirect stimulation of the muscle-nerve preparation was done with a square-wave generator (8) which at the same time also started the Kipp-generator (7). The amplitude of the electric stimulus was 1 Volt and its duration 0.1 ms.

Results

Fig. 2 shows a photograph of the volume decrease of the muscle coming about under the effect of a single indirect stimulus (Oscillogram 2b is the magnified picture of 2a); the downward deviation of the lower graph indicates the vol-

ume decrease, the stimulus break in can be seen before the signal. On the basis of the sensitiveness of the pressure transducer ((3.38 $\pm$ 0.07) $\cdot$ 10^{-7} cc./mV) the magnitude of the initial volume decrease varied between 1.5×10^{-6} and 3.5×10^{-6} cc./gmuscle. According to the evaluation indicated in Fig. 2b the average time data of the initial volume decrease are the following: time of latency $t_1 = (2.5 \pm 0.5)$ ms, the duration of the running up and decay of the signal $t_2 = (7.5 \pm 0.5)$ ms and $t_3 = (20 \pm 5)$ ms respectively; thus the whole duration of this reversible change lasts 30 ms.



Figs 2a–b. Oscillograms of a single initial volume decrease of frog gastrocnemius muscle. S = stimulus break in, t_1 = latency time, t_2 = duration of the running up of the signal, t_3 = duration of the decay of the signal. The mass of the muscle is: 0.875 g (Oscillogram 2b is a magnification of 2a)

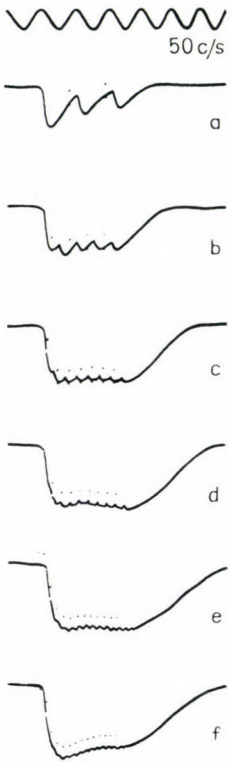
Fig. 3 shows the curves of volume decrease produced by series stimulus of different frequency. The duration of the excitation is 50 ms, the frequency of the stimulus is 50, 100, 150, 200, 250 and 300 c/s, resp. (Fig. 3a–f).

Fig. 4 proves the fact that a stimulus break in does not cause an effect similar to the volume decrease; these photographs were made by exciting the died muscle; the stimulus-frequencies were 100 and 300 c/s (Fig. 4a–b).

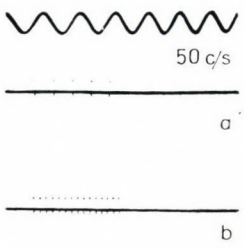
In the course of our previous work "artificial" volume changes comparable in magnitude and duration to the volume decrease of the muscle were created (Aradi, Futó, 1970); the reliability of our quantitative data concerning the initial volume decrease is supported by the findings as follows: 1. It can be demonstrated on the basis of the frequency spectrum of the volume decrease signal that the 0.4 to 300 c/s frequency band of the experimental apparatus is suitable for transmitting components of both low and high frequencies. 2. The duration corresponding to the phase delay occurring in the above frequency band is smaller than 0.3

ms, thus the latency time is not a consequence of the inertia of the pressure transducer. 3. The pressure transducer – through its linearity – ensures the right determination of the magnitude of volume decrease.

Volume increase never occurred in several hundred experiments.



Figs 3a–f. Oscillograms of a volume decrease following a series-stimulation of frog gastrocnemius. Frequency 50, 100, 150, 200, 250 and 300 c/s (a–f)



Figs 4a–b. Examination of the artefacts brought about by "stimulating" a died muscle. Frequency of stimuli: 100 and 300 c/s (a–b)

Discussion

According to our experiments, in a tensionless condition of the muscle each volume change following a stimulus is unambiguously a volume decrease. Contrasting with Pasechnik's opinion (1968) the cause of the contradictory literary data should be looked for not only in the complex nature of the phenomenon under examination; the contradictions may be primarily solved by clarifying the experimental circumstances. Abbott and Baskin (1960, 1962), Baskin and Paolini (1965, 1966) as well as Pasechnik (1968, 1969) gave preference to the existence of the initial volume increase on the basis of their experiments performed with stretched muscles, though in this case the active and automatic sections of the muscle activity (Ernst, 1963) overlap each other. The tensionless condition of the muscle makes it possible to register only the volume change prior to the mechanic activity and taking place in the active period, and to eliminate the effect going together with tension.

Even though in Baskin and Paolini's opinion the duration and size of the physical process serving as a basis for the initial volume increase do not depend on the initial length of the muscle (Baskin, Paolini, 1966, 1967a), in their recent communications – supporting our above statement referring to the experimental circumstances – *they already give account of an initial volume decrease in the tensionless state of the muscle as well* (Baskin, Paolini, 1967a, 1967b), though formerly they had measured initial volume increase in the same state of the muscle (see: Baskin, Paolini, 1966, Fig. 9). Moreover Baskin (1967) demonstrated an initial volume decrease also "at lengths below in situ length".

The initial volume change registered by us corresponds in its character to the results of several works of Ernst et al. appearing after 1925 and those of Meyerhof et al. after 1932 (Ernst, 1963), as well as to those of the paper of Mórocz-Juhász and Örkényi (1966), even though there are differences as to the data of time.

The measurements performed with capillary dilatometer – independent of the way of registering – gave data considerably different from each other: the whole duration of the volume decrease in the experiments of Meyerhof and Möhle (1935) was 65 to 85 ms, but only 15 ms according to Mórocz-Juhász and Örkényi (1966). This difference is due to the fact that the running down of the signals was determined by the geometrical data of the capillary.

Ernst et al. – using piezoelectric pressure transducer – were led to the conclusion that the initial volume decrease takes place within the first 10 ms following the stimulus. A possible explanation of the quick running down of the signal is that the electronic apparatus, through its relatively high lower limiting frequency cut off the components of low frequency; though in the present work this deficiency has been eliminated by widening the transmission frequency band, the methodological question still remains open.

In a non-analyzed oscillogram of Baskin and Paolini the volume decrease measured in tensionless state of the muscle reaches its maximum in about 20 to 22 ms after the stimulus (see: Baskin, Paolini, 1967a, Fig. 8).

The high frequency of the initial volume decrease in the case of tetanic stimulation is a well-known fact (Ernst, Koczkás, 1935; Meyerhof, Möhle, 1935; Dittler, Hannappel, 1940). According to our results the signals of volume decrease due to successive stimuli are already superponed at stimulus frequencies over some 50 c/s but, in most cases, up to a frequency of 200 to 300 c/s they appear as peaks distinguishable from each other which indicates that the "dead-time" of the initial volume decrease is smaller than 3 to 5 ms; the saturation character of the resultant signal indicates the reversibility of the effect. The running down of the tetanic volume decrease curve is determined by the dependence of the amplitude and of the time data on the frequency and duration of the stimulus and on the state of the muscle in general. A more detailed analysis of the curve may bring to light further data.

Comparing the time data of a single volume decrease with the time data of the contraction and action potential it is evident that the appearance of the maximum of the initial volume decrease precedes the maximum of the contraction considerably; its latency time (2.5 ms) corresponds well to the latency time of the action potential of the indirectly stimulated frog gastrocnemius; the time (10 ms) necessary to reach the maximum from the stimulus break in is equal with the duration of a whole period of the action potential (including also latency time).

Under the effect of a series of stimuli the tetanic contraction shows a smooth running down already at a frequency of 50 c/s while the initial volume decrease – similarly to the action potential – follows even stimuli of 200 c/s, moreover, sometimes those of higher frequency.

On the basis of our experimental results we can conclude on the parallel course of the running up period of the volume decrease and of the action potential.

Ernst et al. consider the initial volume decrease – just like the action potential – to be one of the data of the excitatory state. In the opinion of Meyerhof et al. the reversible period of the volume decrease of the muscle is in connection with the basic chemical process of the contraction. Abbott and Baskin (1960, 1962) as well as Baskin and Paolini (1965, 1966) refer to the connection of the volume decrease to the active state of the muscle. Baskin and Paolini (1967b) as well as Baskin (1967) refer to the connection between the contractile component of the muscle and the volume decrease. The results of Mórocz-Juhász and Örkényi (1966) support the concept of Ernst et al.

The initial volume decrease of the striated muscle is undoubtedly a characteristic of the quick phase transformation which follows the stimulation, but the above data are not sufficient for our dealing with the molecular or submolecular explanation of the phenomenon. Similarly, further examinations are needed to decide the question which phase of the muscle activity is this effect strongly connected to.

The author expresses his thanks to Professor E. Ernst for his counsels.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (1), pp. 99–104 (1970)

Frequent Excitation of the Nerve and Muscle

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(Received July 17, 1969)

Experiments were performed on frog sciatic-gastrocnemius preparations to investigate the frequent excitation of the nerve treated with glycerine and decalcifying agents. It was found that (a) the motor nerve can transform a short electric stimulus into frequent excitatory impulses, (b) to a certain degree the muscle can show independent behaviour. The results are interpreted from the point of view of electron processes and of information transmission across the excitatory channels.

Introduction

Great importance has been attributed to the frequency modulation in excitatory processes of the nerve with respect to communication of information within the organism (Ernst, 1968). It is generally known that the stimulus produces a receptor potential continuing in sensory nerves as action potential of different frequency depending upon the stimulus intensity (Katz, 1950; Hensel, Zotterman, 1951; Gray, Sato, 1953). The generator potential due to electric stimulation also elicits frequent action potentials in the Carcinus axon (Hodgkin, 1948).

The hypothesis about the ability of the motor nerve to generate frequent excitatory impulses under the effect of a d. c. stimulus has been put forward by Frey (1883) and investigated by several authors (Jasper, Monnier, 1933; Erlanger, Blair, 1935–36; 1938; Fessard, 1936; Arvanitaki, 1939; Biró, 1968). However, it is a further question whether a short stimulus can, or cannot, give rise to frequent excitation of a motor nerve. The reduction of the water and Ca content of the excitable tissues is a condition favourable for investigation of frequent excitation (Kühne, 1888; Biedermann, 1896; Katz, 1936; Brink et al., 1946; Monnier, 1947; Ernst et al., 1967).

In connection with the examinations for generation of frequent action potentials in the motor nerve, the present paper deals with the frequent excitation of the frog's sciatic-gastrocnemius preparations after treating the nerve with glycerine and decalcifying agents.

Methods

The experiments were performed on sciatic-gastrocnemius preparations of autumn, winter and spring frogs (Rana esculenta).

Responses were elicited by stimulation of the nerve through platinum or non-polarizable electrodes. The action potentials of the nerve and the muscle were

recorded by pairs of platinum electrode. The distal end of the muscle stretched to its resting length was attached to a mechanoelectric transducer (Mórocz-Juhász, Örkényi, 1967) recording the muscle contraction. The experimental arrangement is shown in Fig. 1.

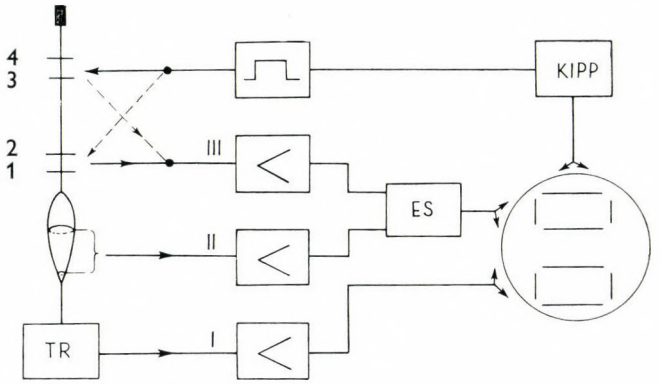

Fig. 1. Scheme of the experimental arrangement. TR: transducer, ES: electron switch. The distances were as follows: between the muscle and electrode 1 1.5 cm, 1 and 2, 2 and 3, 3 and 4 0.5 cm, respectively. One of the stimulating electrodes (namely the nearer one to the recording electrodes) always was grounded

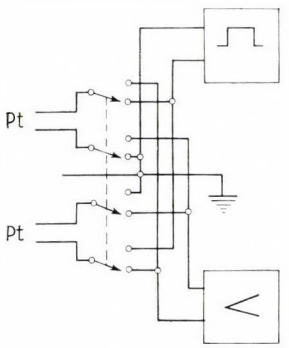

Fig. 2. Circuit diagram of the selector switch

A three-channel oscilloscope was used. The lower beam (channel I) recorded the muscle contraction together with the time marker of 10 msec. The action potentials of the muscle and nerve were recorded by the middle beam (channel II) and the upper beam (channel III), respectively. The middle and the upper beams of the oscilloscope were produced by an electron switch. The stimuli were delivered by a stimulator which produced square-wave impulses of different amplitude (0.01 to 100 V) and duration (0.04 msec to 10 sec).

In a part of the experiments the function of the electrodes at the nerve (i.e. stimulation and recording) was interchangeable by means of a special selector

switch. The moving plate of the selector switch is a circular flexible plate of copper on which two pairs of contact are mounted. The stator plate is an insulator sheet generally used in printed circuits. The copper layer on the stator plate is removed within the close range of the contacts. The copper plates were grounded for shielding, nevertheless, the appearance of a. c. noise in the oscillograms could not be completely prevented because of the high amplification and of the relatively large size of the selector switch. Turning off the moving plate causes the pairs of contact to interchange (Fig. 2).

During the experiments the proximal part of the nerve was soaked in the following solutions:

1. Ringer's solution containing 35 volume per cent of glycerine. (Soaking time: 15 to 20 minutes.)

2a) Mixture of Ca-free Ringer's solution[1] and of a decalcifying solution:[2]

Ca-free Ringer : isotonic citrate	= 1 : 4
Ca-free Ringer : twice isotonic oxalate	= 2 : 1
Ca-free Ringer : isotonic dibasic phosphate	= 1 : 1
Ca-free Ringer : isotonic EDTA	= 2 : 1

(Soaking time: 5 to 50 minutes)

b) Ca-free Ringer's solution containing citrate (6 mM) or oxalate (4 to 12 mM) or dibasic phosphate (16 to 24 mM) or EDTA (2 to 5 mM). (Soaking time: 1 to 5 hours in a refrigerator at 8 °C.)

The nerves were kept in wet atmosphere during experiments in order to avoid drying of the preparation. The records were made at room temperature (19 to 26 ° C).

Results

The oscillograms obtained by stimulating the nerve *at the soaked* part with a square-wave stimulus of 0.1 msec show different responses of the preparations. One of the often recorded responses is shown in Fig. 3 where, in the case of treating the nerve with Ringer's solution containing glycerine,[3] a stimulus of 2V and of 0.1 msec elicited a frequent nerve activity accompanied by a train of action potentials of the muscle and by tetanic contraction.

However, after soaking the nerve in Ringer's solution containing glycerine the frequent excitations coming into existence at the same time both in the nerve and the muscle display differences in their appearances. On the one hand, a significant increase in the nerve activity is accompanied by a short tetanus only and, on the other, a train of muscle action potential together with tetanic contraction appears without significant increase of the nerve activity (Fig. 4). Turning off the selector switch, i.e. when the nerve is stimulated at its distal part and the action potentials are led from the proximal part, the responses are usually similar to that shown in Fig. 4.

[1] $CaCl_2$ was substituted by NaCl.

[2] Sodium salts were used.

[3] In the experiments performed with glycerine, medical student P. Vadon has taken part.

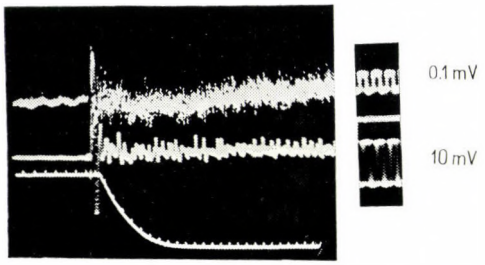

Fig. 3. Frequent excitation of the nerve and the muscle. Stimulus: 2 V to 0.1 msec. Upper beam = nerve action potentials, middle beam = muscle action potentials, lower beam = muscle contraction

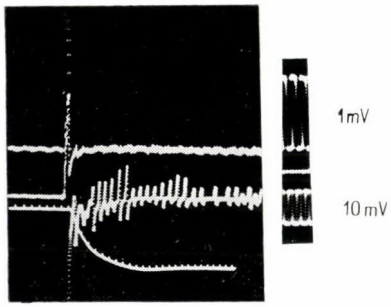

Fig. 4. Frequent responses of the preparations in the case of treating the nerve with glycerine. Stimulus: 2 V to 0.1 msec

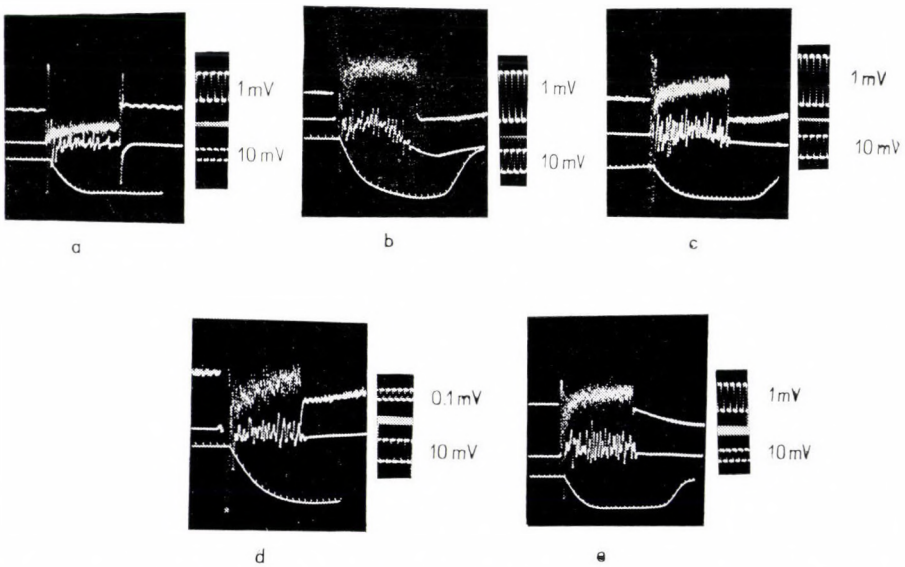

Fig. 5. Frequent excitations during a stimulus of 0.5 V and 150 msec in the case of treating the nerve with glycerine (a), citrate (b), oxalate (c), dibasic phosphate (d), or EDTA (e)

In the case of soaking the nerve in Ca-free Ringer's solutions containing decalcifying agent, increase in the nerve activity due to a short stimulus did not come into existence, although the muscle tetanus sometimes appeared.

On stimulating the nerve with a long square-wave pulse (150 ms, 0.5 V) the oscillograms show increased nerve activity and tetanic contraction under the effect of all the solutions used (Fig. 5).

Discussion

The observation presented in Fig. 3 shows that a short electric stimulus can produce frequent action potentials in the motor nerve which give rise to a tetanic contraction of the muscle generating a train of action potentials. Though the nerve trunk contains the motor fibres together with other fibres, the frequent excitation of the muscle demonstrates the active participation of the motor fibres in the frequent excitation recorded from the nerve. This result shows an analogy with the finding reported by Ernst et al. (1967) on drying nerve.

The other registrations in Figs 4 and 5 support the idea about the independent behaviour of the muscle (Klinghardt, 1951; Partridge, 1966). This independence may be connected, to a certain degree to the natural frequency of the muscle (Ernst, Koczkás, 1938; Bethe, 1951; Ernst, 1963). Concerning the development of the rhythmic muscle response, important role is ascribed to the motor end plate (Neuroth, 1951). Such frequent excitation generated in the neuromuscular synapse can be explained by a coding process taking place in the motor end plate (Ernst, 1961; Biró, Királyfalvi, 1966; Biró, 1969).

The experimental results presented above demonstrate that, on the one hand, the myelinated motor nerve fibres are capable of responding to a short stimulus with frequent excitatory impulses, and on the other, they support Frey's hypothesis (1883) that the motor nerves produce frequent excitation under the effect of a long-lasting square-wave stimulus.

In the mechanism of transforming the stimulus into frequent excitation, electron processes taking place in the nerves can play an important part (Ernst, 1963; 1968; Lakatos, Kollár-Mórocz, 1966; 1967; Lakatos, 1969; Királyfalvi, 1968a; 1968b; Biró, Gábor, 1969). The mentioned properties of the motor nerve seem to be significant with respect to communication of information along the nerve channels.

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

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (1), pp. 105–107 (1970)

Employment of Collagen for Constructing a Mechano-chemical Machine

(Short Communication)

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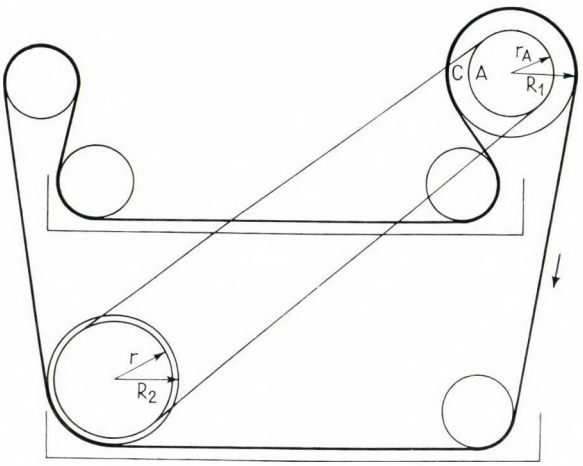
(Received July 19, 1969)

The experiments of Katchalsky and his collaborators, founding the construction of a machine performing rotary movement on the reversible contraction and relaxation of the collagen thread, belong to the experiments carried out for studying the mechano-chemical transformation on a simple model. Such an apparatus was presented by Oplatka during the Symposium on Muscle organized in Budapest, but no description appeared about it in the publication of the Symposium (1968).* K. Laki (Bethesda, Md. USA) was kind enough to inform us about the method by letter: the thread has to be soaked in formol of 0.25 per cent adjusted to pH 7 with NaOH then, after rinsing with water, the ability to contract and relaxe has to be determined in order to be able to find the optimum period of time necessary for the "tanning". This procedure creates cross-links.

The Ethicon Co. (Somerville, New Jersey, USA) was so kind as to place at our disposal a collagen band 12 m long, 1 mm wide and about 0.06 mm thick. Wet threads of a length of 10 cm, lengthened with yarn at both ends, were used for the investigations. One end was fastened to the lower, hooky end of a vertically fixed thin glass-rod, and the other piece of yarn was led through a plexit disc in such a way that a weight of 10 g fastened to its end kept it spanned. We could obtain data about the degree and the time course of the contraction by observing the movement of the weight while lifting 7 M KSCN solution upward on the thread in a big test-tube. The relaxation was brought about by using a test-tube filled with water. First the processes were slow but, by repeating the administration of the salt solution and water 15 to 20 times alternatively, well reproducible values were obtained. The proportion of the length of the relaxed and of the contracted thread was called quotient K, but here the length of the relaxed thread is no more the original 10 cm but 4.9 to 6.6 cm depending on the time-period of tanning (1 and 18 hours respectively); this is the length to which the contracted thread can stretch in water. The length of the contracted thread does not depend to such a degree on the time period of the treatment with formol (3.4–4.0 cm). A quotient K of 1.65 to 1.67 can be obtained by a 12 to 18-hour treatment. The

* So for the reproduction of this striking model, valuable also didactically, we were only referred to the communication of Steinberg et al. (1966) not describing the method of the pre-treatment of the collagen thread; the other deficiencies of the communication will be pointed out later.

most striking thing is the effect of the time of tanning on the speed of relaxation which decreases from 14 seconds (treated for 1 hour) to 1.5 seconds (treated for at least 12 hours). This quick relaxation is one of the conditions of a good performance of the thread in the apparatus. After a treatment of 12 hours both the relaxation and the contraction are quick (the latter is such quick already after a 1-hour treatment). When the thread was made contracted by 7 M KSCN at a



temperature of 60 °C for 10 minutes after formol treating, and then rinsed with water, the processes were at once as quick as if they had been treated 20 times with salt solution and water alternatively. Naturally, the long thread to be put in the apparatus was treated in the same way.

As to the principle of operation of the model itself, the reader is referred to the communication of Steinberg et al. (1966), first of all to their simplified Fig. 2 according to which the apparatus rotates anti-clockwise if the lower bath contains concentrated salt solution and the upper one contains water. Our figure shows that, on the right side, the position of the upper guide roll and the pair of discs driven by the yarn has been reversed: this makes the functioning more steady. A similar transversal yarn-drive could be seen on the apparatus presented by Oplatka on the Symposium.

Basing our deduction on Fig. 2 of Steinberg et al. together with its signs we obtained the following relation between quotient K and the size of the discs of a working model. The C–A pair of discs fixed together rotates n times while the D–B pair m times. The C disc continually receives relaxed thread and if it is, for instance, 100 cm long, a 60 cm long piece emerges from D during the same period of time. So $100/60 = 1.67$ is obtained for quotient K. Thus the two revolution numbers are:

$$n = \frac{100}{2R_1\ \pi} \quad \text{and} \quad m = \frac{60}{2\ R_2\ \pi}.$$

The revolution number of the A disc is n, and that of the B disc is m; the length of the yarn passing through the two discs is the same: $n2R_a\pi = m2r\,\pi$. When the values of n and m are substituted and then the formula is reduced, we obtain the following relation:

$$\frac{R_1\, r}{R_2\, R_A} = 1.67 = K$$

The soundness of our formula was proved as follows: according to our collagen of quotient K 1.67 a model with the following sizes was constructed: $R_1 = R_2 = r = 3.4$ cm and $R_A = 2.0$ cm. The signals correspond to our figure; in the sketch r is drawn to be somewhat smaller, in order to avoid the impression as if the collagen and the yarn were passing on the same disc. In such a way we obtained a machine which really rotated, and did it in the expected direction, i.e.clockwise. The speed of the motion of the thread was 7.2 cm/sec. Using the sizes given in the text by Steinberg et al. we could not produce a model in going condition: by substituting these data into our formula we obtain $K = 1$.

We can find a short allusion also in the communication of Steinberg et al. concerning the connection desirable between the size of the discs and the characteristics of the collagen. In their energetical calculations they set forth the finding that the contracted collagen thread emerging from the salt solution must be of the same length as the relaxed one emerging from the water in a continually working apparatus. The authors apply the expression "specific length" by which they mean the length of the thread of the same weight in relaxed and contracted conditions, respectively. Their quotient means the same as the one explained by us as quotient K. For the sake of simplicity they suppose that the discs driven by yarn are equal (as in their Fig. 2), i.e. $r = R_A$. In their opinion in this case R_1/R_2 must give the value expressed by us as K. Thus the result corresponds to one of the cases of the application of our formula of general validity.

We also examined whether the collagen thread can be used for rotating the model after preserving it dry in a refrigerator for 2 years. We found that also this material can be made suitable for the given purpose by treating it with formol, but the contraction itself is somewhat slower, and the relaxation is even more slower; the approximately equal speed of the two processes cannot be reached. Thus we can understand the fact that such a thread (its quotient K being 1.47) reaches only a speed of 5.7 cm/sec in a model calibrated to fit it. A thread which had been used several times and has been preserved at + 4° C in water ($K = 1.25$) reached a speed of 2 cm/sec even after 2 years.

We express our thanks to Joseph Lőrinczi, mechanic of our Chair, for his valuable and untiring collaboration.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (1), pp. 109–111 (1970)

Investigation on a Method Measuring Low Energy X-Ray Doses with Energy-dependent Film Dosemeter

(Short Communication)

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(Received July 26, 1969)

Dosimetry in the low energy X-ray range has been a less cultivated territory both as regards the measuring method and its accuracy.

Although the maximum permissible dose levels for the critical organs are not ordered to energy, as regards radiation effect, the doses due to the 0.01 to 3 MeV energy range are not of the same importance. Undoubtedly, doses of medium and high energy radiations are more important; still, it is advisable to pay some attention to the low-energy range, too. If e.g. the effect of low doses (Loványi, Predmerszky, 1962; Geszti et al., 1968; Jorke, Wedekind, 1968) is assessed on the basis of the haematological examination of the peripheral circulatory system the problem arises to which energy threshold should the observed lesion be ordered. Another problem to be elucidated is the role hard γ-, medium and low energy X-ray exposures play in the damages due to occupational radiation exposure. Therefore, it is reasonable to deal with the proper measuring problems of X-ray doses of energies below 30 keV.

Practically, mainly film and glass dosimetry should be reckoned with for measuring soft X-ray doses. The measuring problems connected with film dosimetry will be discussed in the following.

As established, the sensitivity of the film depends on radiation energy. The various measuring methods surmount this difficulty in two different ways. The film dosemeters based on filter analysis are suitable to measure doses above 30 keV energy. The energy dependence of the films sensitivity should be corrected for by the energy correction factors obtained for the differences in absorption of the filters of different atomic weights and thicknesses (Dresel, 1956). With the usual copper filters this evaluation method is unsuitable for the measurement of doses below 30 keV. Other methods are based on the principle of energy compensation. The A. E. R. E. film dosemeter (Jones et al., 1965) as well as the film dosemeter developed by Rothe (1967) are based on this principle. The latter enables to reduce the dose into several components and the dose may be taken for the sum of these components. This renders dose measurements possible even in the range from 12 keV to 2 MeV. In the interval between 12 and 90 keV the limits of error are ranging from -40 to $+50$ per cent. The energy-independent measuring method performed by multi-element filters is unsuitable for measurements below 40 keV; the system published by Storm and Shlaer (1965) is suitable for measurements in the range from 40 keV to 1.2 MeV within an accuracy of 30 per cent.

However, adopting this method for the low-energy region encounters difficulties. First of all, filters consisting of several elements of high atomic numbers, conventionally used in the range of medium energy X-rays, represent an absorption too high for low energies. Secondly, the compensation of film sensitivity is more complicated in the 12 to 90 keV than in the 40 to 90 keV range.

Accordingly, the problem of measuring low energy X-ray doses in the 10 to 30 keV range should be summarized as follows. The energy-independent film dosemeter is not suitable for measuring low energy X-ray doses in such a way as to ensure adequate energy compensation in the whole energy range mentioned. As for the dosemeters based on the principle of energy compensation, they usually measure low energy X-ray doses with a higher error than those above 40 keV. The evaluation method, based on the use of the energy correction factors (Dresel's system) may be adopted for the range of low X-ray doses. However, when selecting the filters one has to consider the requirement of *low* absorption.

Dresel's method was investigated from this point of view. The exposures that may result from the use of picture amplifiers and electron microscopes justify the necessity of measuring low X-ray doses. As there was no demand for measuring mixed radiation, it seemed to be advisable to develop a film dosemeter for the proper measurement of soft X-ray doses. Investigations have not been completed as yet. The results thus obtained are reviewed as follows.

ORWO RD 4 films were used for the measurements. For the selection of the adequate filters, 18 Al filters of different thicknesses were tested. From the point of view of Dresel's extrapolation 3 filters were taken into consideration. The film response measurements were made with open window and with 0.53 mm Al; 1.06 mm Al; 1.5 mm Al filters. The films were irradiated with a Siemens Dermopan X-ray apparatus within the dose interval of 20 mR to 6 R. The standard doses were measured with Siemens Dosemeter. Homogeneous radiation was used; the effective energy was determined by H.V.L. measurement (Table 1). The energy correcting factors f_1, f_2, f_3 obtained with the three different filters are shown in Fig. 1, as a function of the apparent dose-ratio.

Table 1

X-ray qualities used for irradiation

The E_{eff} values were calculated by H.V.L. measurement

Exciting potential (kV_p)	X-ray current (mA)	Filter (mm)	HVL (mm Al)	$\sim E_{eff}$ (keV)
10	25	1.0 Be+ 0.66 Al	0.12	9
29	25	1.0 Be+ 0.568 Al	0.28	12
29	25	1.0 Be+ 1.02 Al	0.502	15
29	25	1.0 Be+ 3.58 Al	0.9	20
29	25	1.0 Be+ 10.63 Al	1.5	23
43	25	1.0 Be+ 5.01 Al	(3.0)	(31)

According to the control measurements, the films evaluated by the above method have a maximum limit of error of ± 30 per cent in the interval of 12 to 25 keV. The inaccuracy mostly arises from graphic extrapolation. Namely, the selection of filters of optimum thickness is very critical because, here, the method is more sensitive to the alteration of absorption conditions than is at higher energy levels. By modifying the thickness of the filters used up to now we hope to obtain more accurate results in the range from 12 to 25 keV. Further investigations of this problem are in progress.

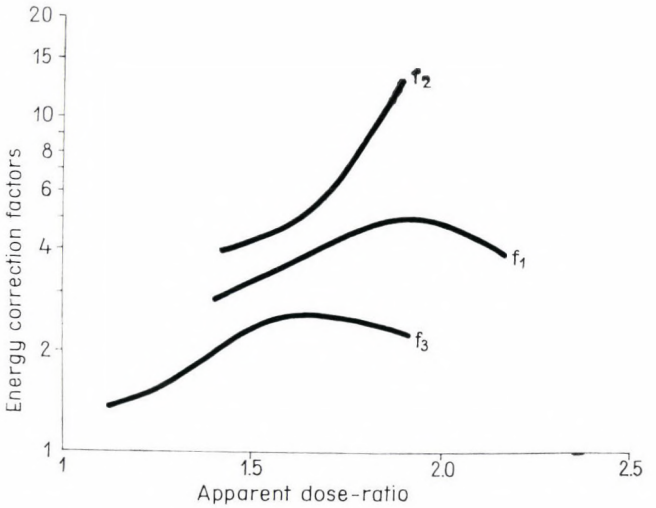

Fig. 1. Curves of the energy correction factors f_1, f_2, f_3, calculated by Dresel's system at different E_{eff} values contained in Table 1, as a function of the apparent dose-ratios:

$$\frac{D^*_{\text{open window}}}{D^*_{0.53\ \text{Al}}} \qquad \frac{D^*_{0.53\ \text{Al}}}{D^*_{1.06\ \text{Al}}} \qquad \frac{D^*_{1.06\ \text{Al}}}{D^*_{1.5\ \text{Al}}}$$

The authors thank Éva Bobok and L. Kiss for their technical assistance.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (1), pp. 113–115 (1970)

Physical Conditions and Dosimetry Problems of Experimental Irradiations in Radiobiology

(Short Communication)

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(Received July 26, 1969)

In compliance with our idea exposed in a previous paper (Koczkás, Dósay, 1966) we continued experimental work in order to achieve that maximum accuracy in biological experimental irradiations which we considered as realizable with the facilities at our disposal. This has been our intention when attempting to improve the technical conditions of irradiations.

First of all, we re-examined and modified the rods ensuring adequate distance between focus and irradiation object. They have to be developed so as to ensure at least mm accuracy in measurement even if the distance between the source and the irradiation object runs to 60 or 100 cm. This mm accuracy may be achieved as follows. A plastic disc, into which we had previously built-in a tape measure out of steel, was fitted and fixed onto the applicator holder on the X-ray tube (Stabilivolt-200 THX-250). An extension, not longer than the shortest conventional distance, is used for directional control while pulling out the tape. The whole device serves for adjusting the distance and is removed prior to irradiation. Such a complete arrangement is shown in Fig. 1.

To increase the accuracy of dose delivery, i.e. to eliminate line voltage variations, a stabilizer was coupled to the dosimeters used.

While performing these experiments, a further requirement has arisen, namely, to be able to irradiate certain regions of the animal's body, without exposing the other regions to significant exposure. For this purpose, special shapes have been developed to suit the region to be irradiated. For other cases shields were developed (out of 4 mm thick Pb) which let through only 2 per cent of radiation excited by 220 kV. These shields do not press the animal since they are not in direct contact with its body. Naturally, there is no way of sparing the other parts of the animal's body from scattered radiation that necessarily arises in its body as a consequence of irradiation.

Two ways are known for eliminating inhomogeneous dose distribution for certain irradiation types. One of these alternatives recommends the slow rotation of the object (ICRU, 1962). According to the other alternative a method developed by ourselves – the distribution of isodoses below the tubes of the respective X-ray units is examined in several planes determined by various distances from the focus. As described in our previous paper (Koczkás, Dósay, 1966), these isodoses are then turned into concentric circles.

These two alternatives are not equivalent. A further series of experiments would be required to decide whether slow rotating – 4 rotations per minute – is not upsetting the biological equilibrium conditions of the animal. Such a rotation irradiation is shown in Fig. 2. In addition to this still unelucidated problem, also the assurance of continuous dose measurement represents a further difficulty if the object is rotated during irradiation.

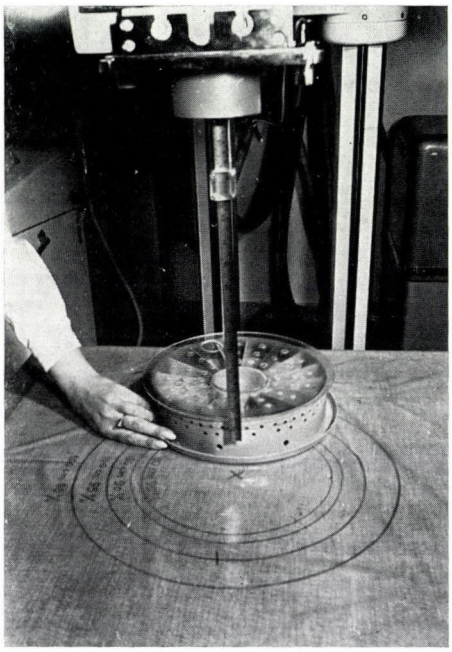

Fig. 1. Irradiation arrangement with the device to adjust distance

The unilateral irradiation of guinea-pigs and rats with X-rays excited in the conventional dose range is considered as inhomogeneous (ICRU, 1962).

Bilateral irradiation can be performed in two different ways. The simpler one is when irradiation is performed by one unit and it is the laterally irradiated animal that is turned round as soon as irradiation-half-time is over. Simultaneous irradiation with two X-ray units is a more elaborate, but better method. Also in the latter case one may choose between two alternatives for the consideration of the various dose rates: 1. Provided the distances between the focus and the irradiation object are adjusted so as to be identical, the time of dose delivery will differ. In an experiment of ours one of the X-ray units was operating for a 6 per cent longer period which represents a minimum divergence from the criterion of homogeneity (ICRU, 1962). 2. On the other hand, if the animal is put into the plane where the respective dose rates are identical, the focal distances will be different, but the criterion of homogeneous irradiation will be fully satisfied. No alteration

in X-ray quality that could have been ascribed to the different distances from the foci, could be demonstrated in that latter case, which proves that this way of irradiation has been correct.

Finally, we have to discuss a problem hardly dealt with hitherto in the field of X-ray technics. Namely, when performing X-ray irradiation, a continuous X-ray spectrum, determined by the exciting energy and by the filters, is used.

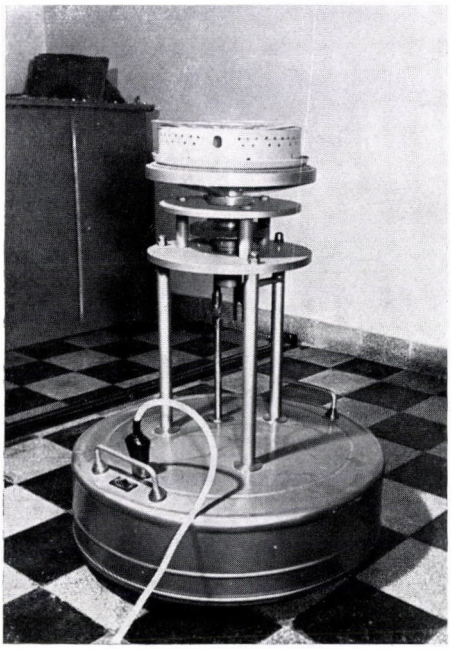

Fig. 2. Irradiation with the object rotated

Accordingly, X-ray photons of various energies will also be included into the absorbed dose. So, it would be difficult to decide, which component and to what extent, eventually through which specific action, should be held responsible for the radiation-induced alteration of some biological factor. This problem can be studied only if a sufficiently *thick* filter is applied before the aperture of the X-ray tube that renders delivered radiation homogeneous. It goes without saying that this will reduce dose intensity and at the same time will considerably increase irradiation time. Thus, in many cases, no *in vivo* irradiations may be performed and one may think of irradiating only homogenates and bacterial cultures *in vitro*.

In our opinion, the irradiation terms reported represent a further contribution to the standardization of animal irradiations.

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Book Review

Transport and Distribution of Matter in Cells of Higher Plants. International Symposium Abhandlungen der deutschen Akademie der Wissenschaften zu Berlin, 1968 Nr. 4a, pp 215.

In recent years an increasing number of publications in biochemistry, biophysics, plant and animal physiology as well as microbiology has been devoted to a study of artificial and natural membranes and to transport problems. This increasing interest and demand for publication is reflected in the appearance of the new series Biomembranes of Biochimica et Biophysica Acta and of the new periodical Journal of Membrane Biology whose first volume has been published last year. All papers on uptake by higher plants are of particular interest, because due to technical difficulties, there are only a few data available about these processes, compared to similar activities in microorganisms and animal cells or tissues.

Studies of the correlations between uptake and energy supplying systems of the cell yield valuable information on transport mechanisms. Molecular biology which has furnished such spectacular results with microorganisms is only of limited use by higher plants in this particular case. At the same time investigations of the electron and energy transport in oxidative phosphorylation and photophosphorylation, together with studies on mitochondrial and chloroplast membrane structures, have focused the attention to the problem of ion uptake. Photophosphorylation can be studied only in plant cells which possess chloroplasts. It is quite possible that data obtained with cell organelles will finally help to solve the problems of transport processes in plant tissues. This trend has given beyond all doubt a great impetus to this more or less neglected field of research. This general tendency and interest was duly reflected by the program of the GDR.

The papers presented at the Symposium were grouped around five main subjects:

1. Passive permeability and water, models;
2. Different aspects of transport;
3. Transport by chloroplasts and mitochondria;
4. Cell potential and transport;
5. Vital dying and secondary plant products.

In the 1st Section D. Woermann dealt with the problems of transport through synthetic membranes. J. D. Kholodova and Z. A. Sorokina reported on the ion binding capacity of polyelectrolytes as one of the possible causes of ion accumulation in the cells. In their opinion the value of the exchange constant K^+/Na^+ is so high in the cells that it can hardly be explained by the selective action of the absorber. A highly topical question was dealt with by W. P. Anderson concerning the diffusional or convective movement of water in roots. Despite the controversial results of recent years it seems that the xylem of the root is not accessible to diffusional water and it may be assumed that the THO molecules reach the exudational compartment by convective flow. Anderson believes the walls of the xylem to be hydrophobic. The question of cell wall permeability was approached from a different aspect by R. K. Salyaev who prepared an ion exchange column from isolated cell walls and confirmed thereby their ion exchange properties. He suggested that plasma membranes contain contractile proteins which regulate their permeability. A. L. Kursanov dealt with the transport of organic substances between the parenchymatic and

meristematic cells, including the role of symplasts and free space. He thinks that the bulk of sugar is accumulated in the free spaces (FS) of the leaf and is in equilibrium with the photosynthetic products of the cells. Hence, the transport of the assimilates towards the phloem would be the cell wall rather than the symplast.

In his lecture on the regulation of transport E. Müller surveyed the results obtained with microorganisms. He discussed the mechanism of transport at the molecular level with an emphasis on genetic regulation.

U. Lüttge dealt with the relation of ion transport with the plant cell compartment and W. J. Cram with the control of cytoplasmic and vacuolar ion contents in cells of higher plants. The first surveyed the experimental material and the debates on the role of absorbing systems functioning at low and high external concentrations in the cell. W. J. Cram was dealing with the problems of influx and efflux through the plasmalemma and the tonoplast and with changes in the cytoplasm and vacuole content. It is a very interesting and surprising finding that in contrast to all expectation, an increase in vacuole content is accompanied by a decrease in protoplasm content. The author discussed the assumption of a possible distinction between two phases in the cytoplasm.

The 3rd Section of the Symposium dealt with energetic of transport problems. This is one of the fields which raises the hope of a clarification of the problems of transport mechanism in higher plants, within a reasonable short time. W. D. Jeschke in his lecture on the connection between electron transport and ion transport reported on possible correlations between photosynthetic and respiratory electron transport, as well as the intermediates with high energy content (or charge separation), ATP formation and ion transport. J. A. Raven presented a paper on photosynthesis and light stimulated ion transport in the green alga Hydrodictyon africanum. The energy for potassium uptake in the light can be supplied by cyclic photophosphorylation alone and in the dark by oxidative phosphorylation. Chloride influx is associated in the dark with respiratory electron transport and in the light with the function of the photosystem II. Ion transport in the light is of course influenced primarily by the processes taking place in the chloroplasts (L. Packer: Relation of light induced electron flow to ion transport, photophosphorylation and ultrastructure of chloroplasts). In the dark, chloroplasts are relatively impermeable to charged ions. The illuminated plastic is characterized by rapid H^+ influx which alters the degree of dissociation of the other ions, and induces thereby an ion movement leading to ultrastructural changes and to the shrinking or swelling of the plastid. These processes affect photophosphorylation, too.

Promising experimental results have been obtained also by using electrochemical methods for studying ion transport. K. Umrath, N. Higinbotham, E. A. C. MacRobbie, V. K. Andrianov, G. A. Kurella and F. F. Litvin, L. N. Vorobyev and V. V. Polevoy presented papers touching on this field of surface active substances, electric double layers, action potential and excitation substances. V. V. Andrianov, G. A. Kurella and F. F. Litvin presented extensive experimental material on changes in the values of resting and action potentials under the influence of light, temperature and various blocking agents. N. Higinbotham discussed the validity of the Nernst, Goldman and Ussing equations in studies on higher plants. In his opinion K^+, Na^+, Ca^{2+}, Mg^{2+}, NO_3^- and $H_2PO_4^-$ ions are not in equilibrium with the external solution in the case of the plant cell. There was an extremely great difference between the expected and actually obtained E values. The potential difference is supposedly due to an electrogenic anion influx pump rather than to the passive diffusion of potassium. It has been found, however, in the cases of Chara australis and Griffithsia cells (L. N. Vorobyev: Electrophysiological peculiarities of plant cells) that there is an electrochemical equilibrium with respect to K^+ between the artificial lake water and the cytoplasm (Chara australis), and between artificial sea water and the cytoplasm (Griffithsia). There was, however, a considerable difference in the electrochemical potential of potassium ions between the vacuole of Griffithsia and the cytoplasm. A very interesting question was raised in E. A. C. MacRobbie's lecture on intracellular compartments. In giant algal cells the composition of the flowing cytoplasm is not homogeneous. Active accumulation may be imagined to occur through the membrane of the plasmalemma, the tonoplast and the chloroplast. The question now arises whether or not with respect to

transport the cell behaves as two intracellular phases in series. According to new experimental data this is not probable, because the activity of the vacuole changes linearly with time. From a certain phase of the cytoplasm a rapid transport into the vacuole must take place. In MacRobbie's opinion it is quite possible that accumulation occurs together with the formation of minivacuoles in the endoplasmic reticulum. These minivacuoles later merge in the central vacuole. The fact that the Nitella cells cannot be regarded as a "two phases in series" model raises doubts as to the validity of calculations based on the analysis of wash-out curves in higher plant systems.

In the 5th Section H. Kinzel presented a paper on the vital staining of plant cells as a model of biological transport and accumulation phenomena.

We are looking forward with great interest to Volume B of the Symposium which will contain the discussions and comments on the papers.

EDIT CSEH

ANNOUNCEMENT OF AN IAEA SYMPOSIUM

Title: Dynamic Studies with Radioisotopes in Clinical Medicine and Research

Date: 31 August – 4 September 1970

Location: Rotterdam, Netherlands

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

Scientific Secretaries: Dr. T. Nagai and Dr. E. H. Belcher Medical Applications Section

The Symposium will be concerned with all applications of radioisotopes in clinical medicine and research which involve measurements of the temporal patterns of uptake, metabolism, clearance or excretion of administered radioactive materials. Topics to be covered include cardiac, gastrointestinal, hepatic, pulmonary, renal and thyroid function studies, regional blood flow studies, calcium, copper, iron, protein and vitamin B_{12} turnover studies and studies of red cell destruction. The Symposium will give emphasis to new instruments, techniques and methods of data analysis. Studies based on scintigraphic techniques will be excluded except insofar as they are concerned with dynamic situations.

Further information and forms to accompany abstracts of papers intended for presentation at the Symposium may be obtained from national authorities for atomic energy matters. Abstracts must be submitted through these authorities so as to reach the International Atomic Energy Agency before 20 April 1970.

Journal of Steroid Biochemistry

Editors-in-Chief
Dr. J. R. Pasqualini *Foundation for Hormone Research*
Dr. R. Scholler *Paris France*

Important developments in the steroid field have resulted in the foundation of a new journal in this field of endocrinology. The journal of Steroid Biochemistry is international in scope and its articles on fundamental research will make a notable contribution to experimental and theoretical knowledge in this field. This new journal is intended to meet the growing interest in steroid biochemistry by providing an international forum for workers concerned with any aspect of the subject; as for instance, the structure and physico-chemical properties of steroids of biological interest, the methodology of their detection and measurement, their biosynthesis and catabolism. Other subjects covered are the mechanism of action of steroid hormones at cellular and molecular level, their interaction with other classes of compounds, microbiological transformation of steroids, clinical investigations concerned with the biochemistry of steroids, and comparative endocrinology of steroids.

The journal will publish such clinical papers as would advance the knowledge of steroid biochemistry in man, and will be published quarterly.

Please write for a free inspection copy of this journal

A selection of papers from the first issue

K. Schubert, F. Ritter, T. Sorkina, K. -H. Böhme und C. Hörhold. Abbau von Steroiden — VIII. Bildung von (1,4-^{14}C) Bernsteinsäure aus (1,3a-^{14}C) 7a-Methyl-5, 6, 7, 7a-Tetrahydroindan-1, 5-dion-4-(3-Propionsäure) durch *Nocardia opaca.*

A. Machino, H. Inano and B. Tamaoki. Studies on enzyme reactions related to steroid biosynthesis — 1. Presence of the cytochrome P-450 in testicular tissue and its role in the biogenesis of androgens.

G. W. Oertel, P. Menzel und D. Wenzel. Über Steroid-konjugate in Plasma — XX1. Zur Isolierung von Steroid-sulfatiden aus biologischem Material.

J. R. Pasqualini. Corticosterone and corticosterone-21-sulfate production in man.

P. I. Jørgensen. Influence of corticosteroids on the excretion of oestrogens in pregnancy.

R. Hampl and L. Stárka. Epimerisation of naturally occurring ^{19}C-steroid allylic alcohols by rat liver preparations.

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Pergamon Press
Headington Hill Hall Oxford OX3 0BW England
Maxwell House Fairview Park Elmsford New York 10523 USA

Hoppe-Seyler's Zeitschrift für

Physiologische Chemie

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Vol. 351 No. 2 February 1970

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

A kézirat nyomdába érkezett: 1970. I. 7. – Terjedelem: 10,75 (A/5) ív, 63 ábra

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Acta

VOLUME 5
NUMBER 2
1970

biochimica et biophysica

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (2), pp. 121—129 (1970)

Viscometric Investigation of Actin G and Actin F

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(Received November 12, 1969)

The results of viscometric studies of actin G and its derivative, actin F, show that the number of monomers of actin F increases with purification. Gel filtered actin G has an elongated ellipsoidal shape with an axis ratio of about 10; the axis ratio of the second fraction is $\simeq 14$, that of non-purified actin F $\simeq 500$ and that of actin F in the third fraction of the gel filtrate >1000. Accordingly, the length of non-purified actin F is 2.5 μ, while that of actin F in the third fraction >5 μ. For the estimation of this least length measurements have to be performed at shear stresses not lower than 10^{-4} τ.

Introduction

Since the discovery of G–F transformation in actin solutions several papers have been published on the viscometric behaviour of actin G and actin F (Straub, 1943; Straub, Feuer, 1950; Szent-Györgyi, 1951). A viscometer with appropriately sized capillary has been found suitable for the observation of the transformation of actin G into actin F. According to Carsten and Mommaerts (1963) the viscometric parameters of a so-called "good" actin are as follows: in a concentration of 1 mg/ml, at pH 7.2 when measured with the Oswald viscometer in a mixture of 0.1 M KCl and 1 mM $MgCl_2$ the value of 0.63 η_{sp} is reached in the 7th minute.

There are several data in the literature on the intrinsic viscosity of actin. This value is according to Kassai et al. (1962) 0.16 dl/g for actin G-ATP, according to Drabikowski and Gergely (1962) 0.14—0.21 dl/g, according to Katz and Hall (1963) 0.18 dl/g. Mihashi (1964) found a value of 0.10 dl/g for the intrinsic viscosity of actin G-ADP and 0.055 dl/g for actin containing some tropomyosin (Cohen, 1966).

Viscometric data have been used to calculate the axis ratios of actin G and actin F. Tsao (1953) found a value of 12, Mommaerts a value of 5 for the axis ratio of actin G. It should be mentioned that lately most authors seem to prefer viscometers with longer flow-times which seems absolutely justified in view of the fairly small effects which have been recorded (Katz, Hall, 1963; Mueller et al., 1964; Rees, Young, 1967).

It was one of the aims of the present work to make viscosity data for actin G more reliable by means of a capillary viscometer with longer flow-time

and to study the dependenceof the intrinsic viscosity of actin F on shear stresses. It was expected to obtain thereby more exact parameters of pure actin F in solution and to estimate delayed polymerization under the effect of various muscle factors.

Materials and Methods

The actin used in the experiments was prepared from the acetone powder of the leg and back muscles of not more than four-month-old rabbits. Actin of the lowest molecular weight (46 000) was obtained by the method of Rees and Young (1967) and Fazekas et al. (1966). After centrifugation and depolymerization of the polymerized actin the denatured part was removed and the actin separated into four fractions on a Sephadex G 200 gel column. The third fraction exhibited exceptionally good polymerization properties, and this actin was studied in detail. The Sephadex column was always of the same size (50×2.3 cm). With these columns the elution volumes and the equilibrium constants (k_D) of the four fractions had always a characteristic value.

The k_D value of Fraction III on the standardized column was 0.82 ± 0.05.

Actin G was polymerized at 25 °C, with a mixture containing 0.1 M KCl, 1 mM $MgCl_2$, 0.05 M Tris-HCl buffer. Polymerization was considered completed after one hour. The actin solutions used in the experiments were stored at 0 °C and used within about 48 hours after their preparation.

Viscosities were measured in three types of viscometers. The main data of the Ubbelohde type viscometer used in the case of actin G were as follows: length of capillary 20 cm, radius of capillary 0.02 cm, flow-time for water at 25 °C, 333.0 sec.

The shear stress dependence of viscosity was studied in an apparatus working on a similar principle at six different values of shear stress (0.43, 0.85, 1.28, 1.93, 2.55 and 3.37 dyne/cm^2). The flow-times of the six bulbs for water varied between 230 and 105 sec, the length of the capillary was 100 cm, its diameter 0.03 cm.

At shear stresses below 0.43 dyne/cm^2 a modified Zimm—Crothers type (Zimm, Crothers, 1962) rotation viscometer was used in which the shearing force was varied by moving the rotor in an inhomogeneous magnetic field. The concentration range was chosen so as to obtain a reliable extrapolation $(\eta_{sp}/C)_{c \to 0}$. Accordingly, the measurements were performed in the capillary viscometer when actin F was present in a concentration of a few hundredth g/dl and in the rotation viscometer when the concentration of actin F was a few thousandth g/dl.

In the case of the capillary viscometer the shear stress at the capillary wall, in the case of the rotation viscometer the average shear stress was calculated. In our estimation the error not greater than 10 per cent may be involved in this procedure.

The intrinsic viscosity was always determined at 25 ± 0.01 °C.

Gel filtration. Polymerizing actin was prepared as described in Methods in a buffer which contained ATP. Our best preparations reached and even surpassed the 0.63 η_{sp} value mentioned in the Introduction. The other fractions did not poly-

merize. The k_D values of the fractions were the following: first fraction 0.1—0.18, second fraction 0.57, third fraction 0.82, fourth fraction 1.10. The fourth fraction and the other accompanying substances could be removed by dialysis. Under adequate conditions and by a complete elimination of Ca and Mg ions it could be achieved that there was no second fraction in the chromatogram, or that only the second fraction was obtained and the third fraction was missing.

The second fraction eluted from the Sephadex column did not polymerize and had no ATP content despite the fact that it had been isolated by means of an ATP-containing buffer and gel filtration.

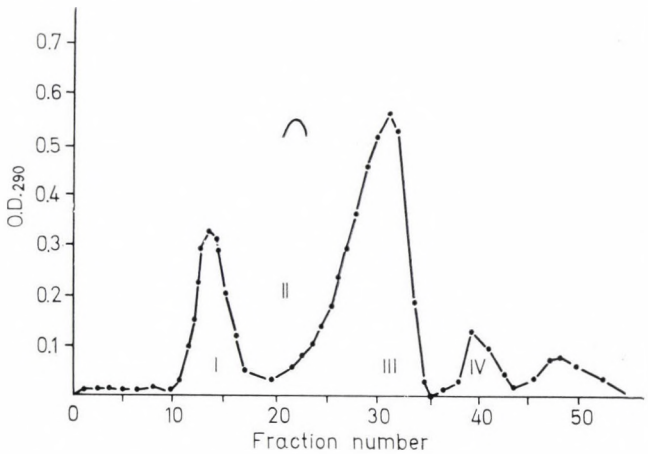

Fig. 1. Chromatography of actin on a Sephadex G 200 (52 × 2.3 cm) column. Actin was isolated from acetone-dried powder with 2.5×10^{-4} M ATP, and 2.5×10^{-4} M 2-mercaptoethanol, pH 7.2. Volume of fractions 6 ml

Viscosity measurements. The following general correlation is valid for the intrinsic viscosity and the molecular parameters of compact rigid particles of the solute (Cvetkov et al., 1964):

$$[\eta] = \frac{N_A \overline{xv}}{M} xv(p) \tag{1}$$

where N_A is the Avogadro number, $\bar{v}$ the volume of the solute particle, M the molecular weight, $v(p)$ the viscosity increment (p is the axis ratio). $v(p)$ is related to morphological factors, and for rotational ellipsoids its dependence on the axis ratio is well known (Kuhn, Kuhn, 1945; Simha, 1945). For a sphere $v(p) = 2.5$. If the solute particle is anisometric due to the orientating effect of flow, intrinsic viscosity will be a function of the velocity gradient or of the shear stress. The effect will also depend on the asymmetry of the particle. In this case Eq. (1) will be valid only at $[\eta]$ values extrapolated to zero velocity gradient or shear stress. In the investigation of the properties of actin G and actin F molecules two fundamental problems arose. On the one hand the intrinsic

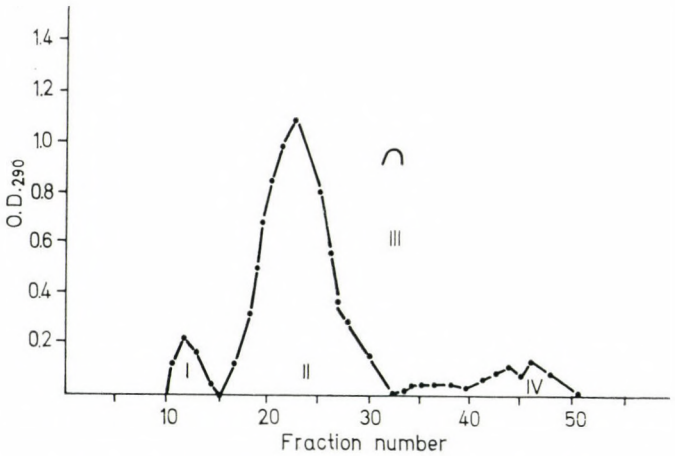

Fig. 2. Chromatography of actin on a Sephadex G 200 (2.3 × 50 cm) column. Actin was isolated from acetone-dried powder with 2.5×10^{-4} M ATP, and 2.5×10^{-4} M 2-mercaptoethanol, and 2.0×10^{-4} M $CaCl_2$ at pH 7.2. Volume of fractions 6 ml

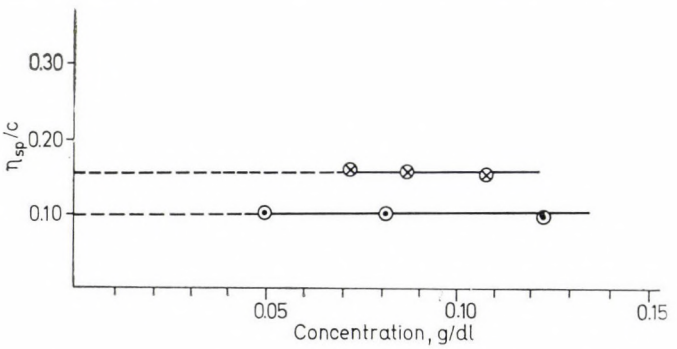

Fig. 3. Intrinsic viscosity of Fraction II (⊗) and Fraction III (⊙) eluted from a Sephadex column

viscosities, on the other hand the dependence of the latter on the shear stress had to be determined.

Data relating to the second and third fractions are shown in Fig. 3. The intrinsic viscosity value without polymerizing salt is 0.10 dl/g for the third fraction and 0.15 dl/g for the second fraction.

Fig. 4 shows the concentration dependence of the specific reduced viscosity of purified actin F (third fraction) at various shear stresses. Fig. 5 is the same for non-purified actin F.

The dependence of the relative viscosity on the shear stress for various concentrations of non-purified actin F is shown in Fig. 6. It can be seen that the non-Newtonian behaviour is more and more pronounced as concentration in-

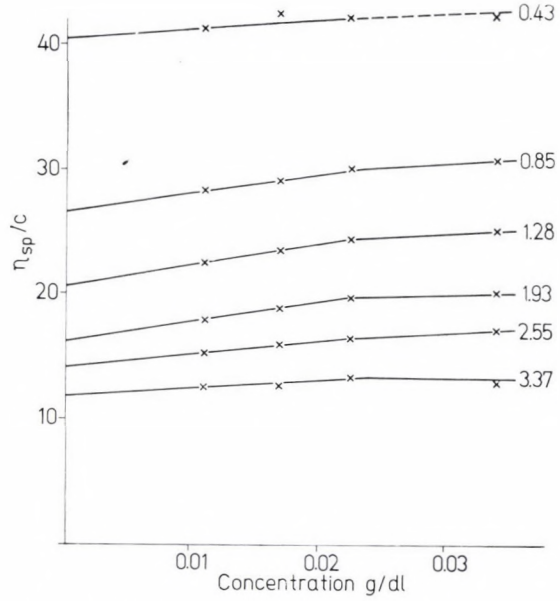

Fig. 4. Dependence of the reduced specific viscosity of purified actin F (Fraction III) on concentration at different shear stresses in dyne/cm^2

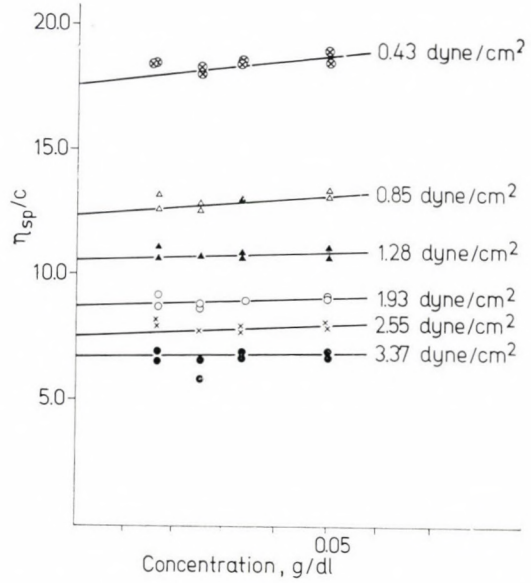

Fig. 5. Dependence of the reduced specific viscosity of unpurified actin F on concentration at different shear stresses. The numbers are the same as in Fig. 4

creases. The specific reduced viscosities shown in Fig. 6b were calculated from the data shown in Fig. 6a. The concentration dependence of the specific reduced viscosities is surprisingly low in the concentration range used. In Fig. 7 the results are summarized and intrinsic viscosity is plotted vs. shear stress.

The figures clearly show that in the characterization of the actin F the dependence on the shear stress must always be taken into account because if this is not done the results will vary to a high extent depending on the visco-

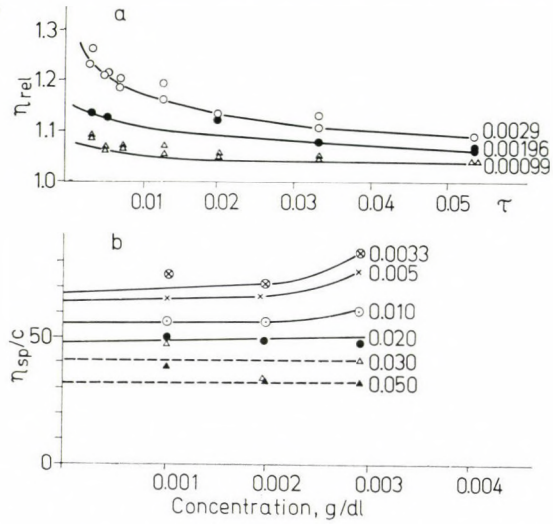

Fig. 6. Viscosity of unpurified actin F as determined with the rotation viscometer. *a)* Dependence of the relative viscosity on shear stress; *b)* Dependence of the reduced specific viscosity on concentration at different shear stresses. The numbers stand for shear stress

meter used. With non-purified actin F the intrinsic viscosity value pertaining to zero shearing force can be estimated from the curve and is about 73 dl/g. Actin F prepared from the third fraction shows a far more marked dependence on the shear stress.

Because viscosity increases with time, of the values determined by means of the rotational viscometer only that measured at 0.002 g/dl was accepted. Experience has shown that in this concentration range η_{sp}/C hardly depends on the concentration, so that the η_{sp}/C values pertaining to 0.002 g/dl were taken as being identical with the values of the intrinsic viscosity. The points obtained in this way fit well the points on the lower section of the curve as measured with the capillary viscometer. With respect to the $[\eta]_{\tau\to 0}$ value of the actin prepared from the third fraction only rough estimations can be made, since so far it has not been possible to carry out the measurements in an appropriate range of shear stresses.

The character of the curve seems to indicate that in this case $[\eta]_{\tau\to 0}$ is higher than 400 dl/g.

Assuming the solute molecules to be rigid and compact the axis ratios and the approximate values of the length of molecules can be determined from Eq. (1). The related $p - \nu(p)$ values needed for the calculation of the axis ratios were taken from the table reported by Simha (1945). From these data the axis ratio of the second fraction (actin G) is about 14, that of the third fraction is 10. For non-purified actin F about 500, for the actin F of the third fraction a value greater than 1000 was obtained for the axis ratio. If the diameter of the double filament

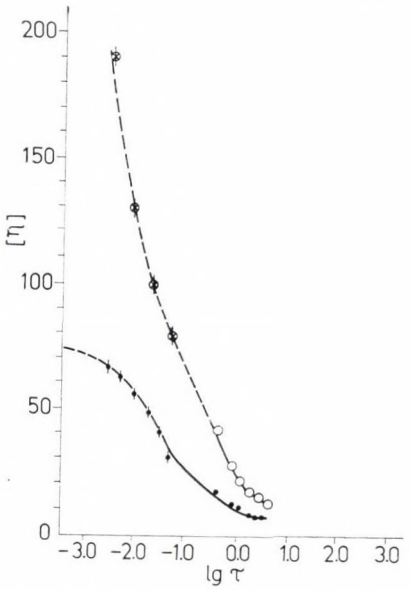

Fig. 7. Dependence of the intrinsic viscosity of unpurified (●), and purified actin F (Fraction III) (○) on shear stress, at 25 °C. The symbol (⊗) refers to data measured with the rotation viscometer, η_{sp}/C at 0.002 dl/g conc.

is taken as 50 Å in actin F (Gergely, 1966), the lengths will be as follows: non-purified actin F 2.5 μ, actin F of the third fraction 5 μ. Actin F is not formed by a simple tail to tail attachment, but is a helically arranged double filament with a certain periodicity (Gergely, 1966). However, according to Hanson (1968) the axial periodicity of actin F is absent in the twice purified actin filament and periodicity may therefore be associated with some other proteins. The actin F filament polymerized from the chromatographed actin fraction of a molecular weight of 46 000 has no periodicity.

To assume a perfect rigidity of the particles is obviously but a rough approximation and the true length of the actin F filament is greater than the value calculated from the simplified model. Furthermore, it is quite obvious that in the case of purified actin F the intrinsic viscosity values at low shear stresses increase rapidly and the molecule is much longer than the assumed 5 μ. With our apparatus

it is possible to measure the viscosity values shown in Fig. 7 for lower shear stresses, but because of a lack of accurate theoretical correlations the axis ratio and molecular length values calculated from the data obtained in the above manner are not considered satisfactory.

Discussion

It is interesting to compare our data with those obtained by other authors from electron microscopic pictures. According to Mommaerts, when purified actin F, or a concentration of 0.2 mg/ml or 0.5 mg/ml, was polymerized in KCl an average length of 0.6 μ was obtained (Takahashi, Yagi, 1968).

Other authors calculated an average length of 1.6 μ and found actin F filaments to have a maximum length of 18 μ (Kawamura, Maruyama, 1967).

Our measurements permit the conclusion that actin F polymerized from gel filtered actin has an average length well over 5 μ. The viscosity (and hence the degree of polymerization) of actin F originating from non-purified actin was found to be much lower. These results explain unequivocally the data on the viscosity and axis ratio of actin G found by various authors as mentioned in the Introduction, provided they are compiled from the viscosity data of the second and third fraction in the gel filtered actin and of the data of minor components detected by gel filtration.

Two conclusions can be drawn from these experiments: first that for the calculation of the exact length of the molecule viscosity values determined at shear stresses of 10^{-2}—$10^{-3}\,\tau$ are not sufficient but under standardized conditions it is nevertheless possible to investigate the mechanism of action of muscle factors at low shear stresses; second that the second fraction eluted from the Sephadex column is probably nothing else but some conformational modification of the polymerizing third fraction eluted from the gel column. Denaturation is here out of question, since this was prevented by using an appropriate pH, reducing agents and ATP. This form has no ATP and the method of reversion into polymerizing actin is not known.

The first fraction eluted from the Sephadex column is biuret negative and consists almost entirely of lecithin with some protein contamination.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (2), pp. 131—136 (1970

Studies on Carboxyl Modified Proteins and their Enzymatic Hydrolysates*

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(Received December 15, 1969)

Protein derivatives obtained by coupling amines with the free carboxyl groups of proteins were either split into chains or hydrolyzed by proteolytic enzymes. The possibility of fractionating the resulting mixtures was studied by gel filtration, paper electrophoresis and ion exchange chromatography. It has been found that previous modification resulted in an enhanced solubility of the proteolytic digests. As a consequence, fractionation by gel filtration could be carried out under mild conditions. Modification also facilitated the removal of urea after ion exchange chromatography in concentrated urea solutions. As an illustration, the isolation of four different peptides (with 16 to 69 residues), including the carbohydrate-containing tryptic peptide of ovalbumin, is reported. Other properties of the fragments of modified proteins and possibilities of the application of these properties are also discussed.

Introduction

Hoare and Koshland (1967) developed a method for the quantitative coupling of amino acid esters with the free carboxyl groups of proteins. The modification reaction was carried out in aqueous solution, the coupling reagent was a water soluble carbodiimide. In order to enhance the solubility of S-alkylated proteins, their procedure was adapted to couple arginine methylester, or dimethylamino ethylamine, or methylamine with the free carboxyl groups of different carboxymethylated or aminoethylated proteins (Furka, Sebestyén, 1969a). The modified proteins showed good solubility, and it seemed interesting to study the properties of the enzymatic hydrolysates of these protein derivatives.

* The conclusions based on the experimental work reported in this paper were in part shortly reviewed at the VIIth Congress of the Polish Biochemical Society, Wroclaw, 1969. Abbreviations: AE-: aminoethyl-, CM-: carboxymethyl-. ARG-, DMAEA- and MEA-protein: proteins coupled with L-arginine methylester, 2-dimethylamino ethylamine and methylamine, respectively. CMCI: N-cyclohexyl-N'-[2-(4-β-morpholinyl)-ethyl]-carbodiimide methyl-*p*-toluene sulphonate. TPCK: tosyl-L-phenylalanyl-chloromethylketone.

Experimental

Materials

Chymotrypsin-A and chymotrypsin-B were commercial products (Fluka AG, Buchs and Wilson Laboratories, Inc., Chicago, resp.). The commercial (Fluka AG) trypsin was previously treated with TPCK according to Kostka and Carpenter (1964). Carboxypeptidase-A and B were products of Serva, Heidelberg, and Schwarz, Orangeburg, respectively. Ovalbumin was prepared from fresh chicken eggs, six times crystallized (Sorensen, Hoyrup, 1915—17), then fractionated on DEAE-cellulose (Furka, Sebestyén, 1969b). The main (A_1) component was used. The 1–28 fragment of human ACTH was a synthetic peptide (Kisfaludy, Lőw, 1968) supplied by Gedeon Richter Chemical Works, Ltd., Budapest. Arginine methylester dihydrochloride was synthesized from L-arginine according to Boissonnas et al. (1958). Methylamine hydrochloride, 2-dimethylamino ethylamine, guanidine hydrochloride and CMCI were Fluka AG, Buchs, reagents.

Methods

S-carboxymethylation and S-aminoethylation of the proteins were carried out according to the procedure developed by Canfield and Anfinsen (1963) and Raftery and Cole (1966), respectively.

For the isolation of the carboxymethylated B chain of chymotrypsin-B, the procedure described by Parkes and Smillie (1966) was followed.

Coupling reactions (Furka and Sebestyén, 1969a): In a typical experiment 100 mmoles of amine component (6.75 g methylamine hydrochloride, or 26.12 g L-arginine methylester dihydrochloride, or 11.9 ml 2-dimethylamino ethylamine) were dissolved in 80 ml 7.5 M guanidine hydrochloride and the pH of the solution was adjusted to 4.5. Then 0.022 mmole (1 g) AE-ovalbumin, and — with stirring — 10 mmoles (4.23 g) of CMCI were dissolved. The pH was adjusted to 4.8 and left at 27° C for 5 hrs. The pH was then adjusted to 3 and the excess of the reagents was removed by either dialysis or gel filtration on Sephadex G 25.

Digestions by both α-chymotrypsin and TPCK-trypsin were carried out in a Radiometer pH-stat at pH 8.0 and 37° C for 4 hrs, the enzyme–substrate ratio being 1 : 100.

For hydrolysis of chymotryptic fragments of MEA-ACTH 1–28 with carboxypeptidase the eluted samples were freeze-dried, dissolved in 150 μl 0.2 M N-ethyl morpholine buffer at pH 8.0, carboxypeptidase-A and carboxypeptidase-B (0.015 μmole of each) added, then left overnight at 37° C. The digests were directly spotted on Whatman 3MM paper.

Paper electrophoresis was carried out on a horizontal cooled plate apparatus (Labor MIM, Hungary) at 31 V/cm for 2 hours, using volatile buffers.

Amino acid analysis. Samples were hydrolyzed in 6 N hydrochloric acid at 105° C for 16 hours, then analyzed on an EEL automatic analyzer.

The carbohydrate content of the fractions of the digests of ovalbumin derivatives was estimated by the orcinol method (Weimer, Moshin, 1952).

For the analysis of the fractions eluted from the Dowex 50X2 column, 0.7 ml samples were removed from each tube and subjected to alkaline hydrolysis. The ninhydrin colour was measured at 570 mμ (Hirs et al., 1956).

Results and Discussion

1. Possibility of fractionation of protein subunits

The cleavage of protein molecules into subunits or chains is very often accompanied by the formation of subunits or chains of low solubility. Thus, the fractionation of these mixtures has to be carried out either at extreme pH values or in concentrated urea solutions. As it has been found earlier (Furka, Sebestyén,

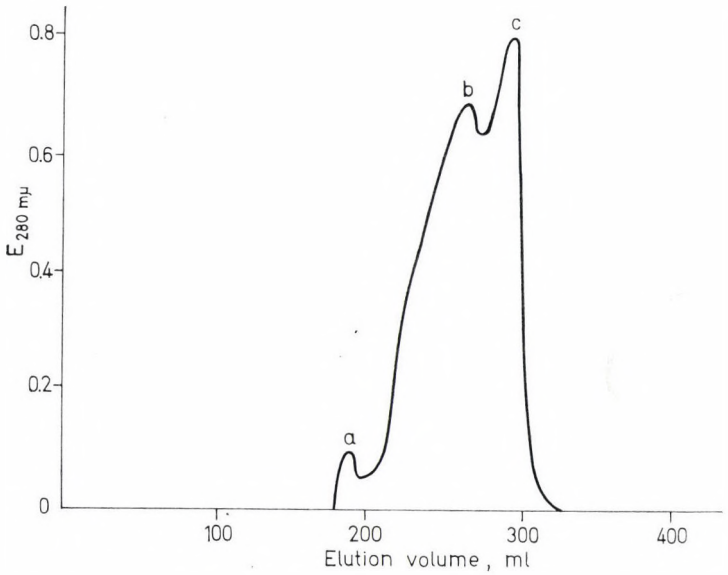


Fig. 1. Gel filtration of the mixture of B and C chains of ARG-CM-chymotrypsin-A on Sephadex G 50. 42 mg protein mixture was eluted from the column (150 × 2.2 cm) with 0.2 N acetic acid. Flow rate, 26 ml/hour; fraction size, 4 ml

1969a) the solubility of S-alkylated proteins can be enhanced by coupling different amines with their free carboxyl groups. Since the modified proteins showed good solubility under mild conditions, the possibility of fractionation of previously modified chain-mixtures was investigated.

Chymotrypsin-A was reduced and carboxymethylated in 8 M urea, then dialyzed. A mixture of the carboxymethylated B and C chains was treated with arginine methylester in the presence of CMCI, then the product was chromatographed on a Sephadex G 50 column, in 0.2 M acetic acid (Fig. 1). Fractionation into two main components (b and c) was observed with a recovery of 90 per cent,

as calculated from the weights of the freeze-dried materials applied to the column and eluted.

In another experiment chymotrypsin-B was first coupled with dimethylamino ethylamine, then reduced and aminoethylated. The product was chromatographed on a Sephadex G 75 column (Fig. 2). Based on optical density units, all material applied to the column was recovered. On the chromatogram three components were distinguishable.

The number of detected components in both cases corresponded to earlier findings, since in the first case the B and C chains (Hartley, 1964), and in the

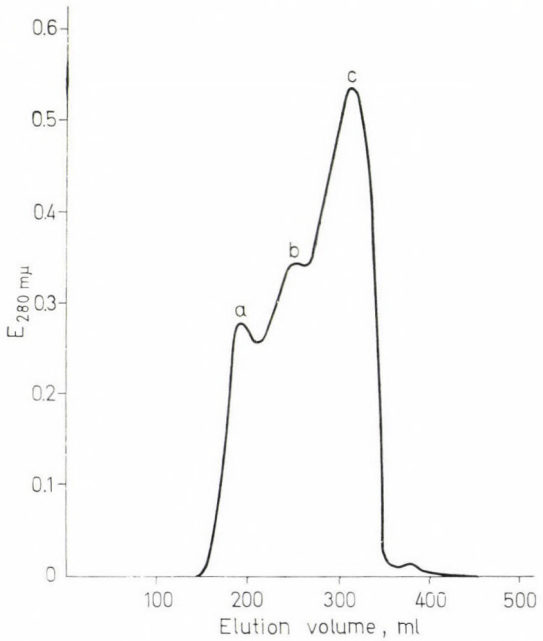


Fig. 2. Gel filtration of the reduced and aminoethylated derivative of DMAEA-chymotrypsin-B on Sephadex G 75. 44 mg protein mixture was eluted from the column (94 × 2.5 cm) with 0.2 N acetic acid. Flow rate, 26 ml/hour; fraction size, 4 ml

second run three components (B and C chains and the B + C fragment) (Parkes, Smillie, 1966) were expected to occur in the mixture. Thus, it can be concluded that modification of the proteins at their free carboxyl groups may facilitate the separation of their subunits under mild conditions and with good recoveries, whenever the differences in chain length make it possible to apply gel filtration.

2. Expected general properties of the enzymatic hydrolysates of modified proteins

Enzymatic hydrolysis of denatured or S-alkylated proteins in many cases leads to the formation of a mixture of fragments with low solubility. This fraction of the digest which is very difficult to handle and fractionate often comprises a

large part of the hydrolysate. It is reasonable to suppose that modification of the carboxyl groups of the proteins will alter the solubility of their enzymatic digests. For this reason, the solubility and possibility of fractionation of the enzymatic hydrolysates of the modified proteins were studied in some detail.

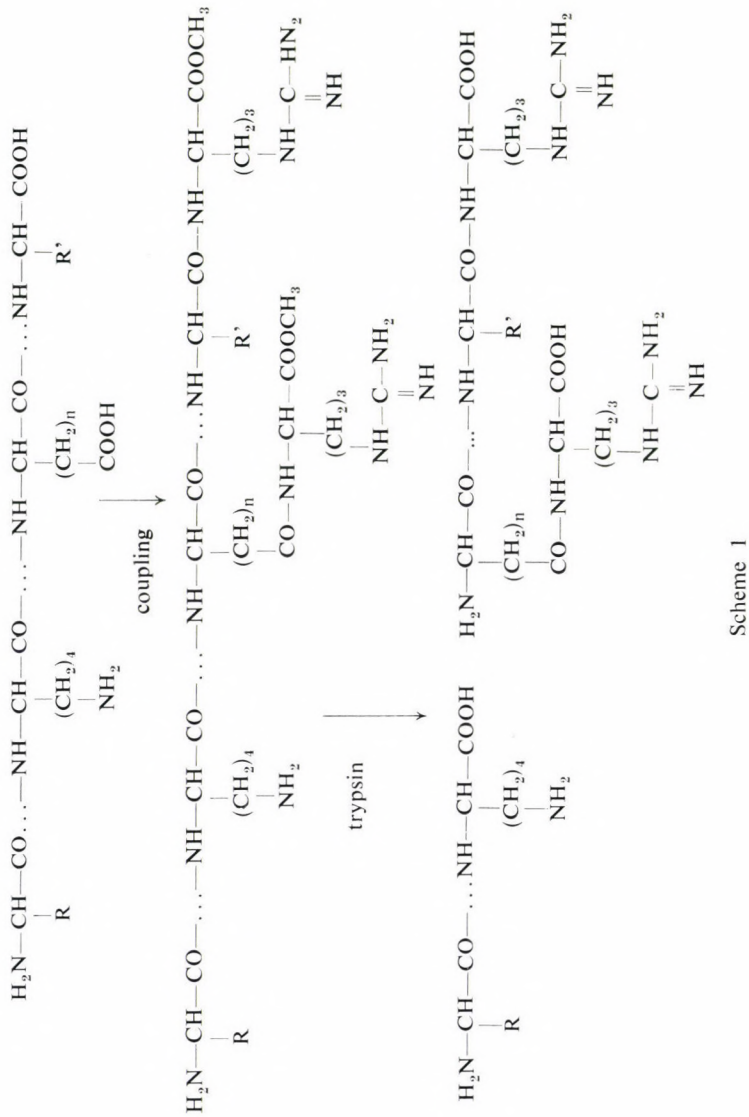
2.1. Arginine methylester derivative. Upon coupling with arginine methylester the free carboxyl groups of the protein are transformed into amide ones, the acylation taking place at the α-amino group of the arginine methylester. Since the terminal α-carboxyl group is also coupled with an arginine methylester molecule, the peptide chain is lengthened with one residue. It has to be kept in mind, however, that in the course of the tryptic digestion, in addition to the cleavages in the main peptide chain, the methyl groups are also removed from the arginine residues in the side chain by the enzyme. A comparison of the peptides derived from the unmodified and modified protein, respectively, shows that each carboxyl group in the side chain is replaced by a new free carboxyl group and a basic guanidine group (Scheme 1).

From the above findings it may be concluded that, while the acidic groups of the protein are transformed into basic ones by coupling, resulting in a strongly basic protein, the acidic groups in the side chains of the tryptic fragments are practically neutralized by coupling followed by tryptic digestion, with a simultaneous increase in the number of ionizable groups. All tryptic peptides are expected to be basic. Other proteolytic enzymes do not hydrolyse the ester groups in the side chains and so the properties of the peptides may be expected to be similar to those derived from the protein modified with dimethylamino ethylamine (see the next paragraph).

2.2. Dimethylamino ethylamine derivative. By the modification of the protein with dimethylamino ethylamine, the acidic side chains are transformed into basic ones. The C-terminal of the peptide chain is also coupled with a basic group. The number of basic groups in the modified protein is identical to that of an arginine methylester derivative.

Since proteolytic enzymes do not affect the side chains, the peptides in a hydrolysate of the DMAEA-protein contain basic groups instead of acidic side chain carboxyls. All tryptic peptides are expected to be basic, some of them strongly basic (containing more than two basic groups besides the single acidic α-carboxyl). Hydrolysis by other proteolytic enzymes can lead to the formation of neutral peptides in addition to the basic ones. It is worth-while to note that the C-terminal fragment is also expected to be basic and contains no free carboxyl group.

2.3. Methylamine derivative. The modification of the protein by methylamine results in the transformation of the terminal and side chain acidic groups into neutral amide groups. The number of the ionizable groups of the protein is reduced. All tryptic peptides can be supposed to be basic, since they contain two (or, in the case of histidine-containing peptides, more) basic groups and only one carboxyl group. The C-terminal fragment is also basic, since it contains one α-amino group and no free carboxyl (except in case when the undigested peptide chain ends in lysine or arginine; in this case, the terminal N-methyl-amide group is hydrolysed but the peptide still remains basic). Digestion by other proteolytic



Scheme 1

enzymes results in the formation of a mixture of basic and neutral peptides. All peptides containing one or more basic amino acid residues are basic. The C-terminal fragment is also basic even if no basic amino acid residue is present in it. This is not true when the hydrolyzing enzyme, due to its specificity, removes the amide group from the C-terminal. All other peptides are neutral. It can be established that the number of ionizable groups present in the peptides of the digest is less than that of the ionizable groups found in the peptides of the hydrolysate of the unmodified protein.

The above considerations indicate that only in the case of arginine methylester or dimethylamino ethylamine modifications can a favourable influence be expected on the solubilities of enzymatic hydrolysates. Furthermore, the modification of the protein can only affect the solubility of fragments containing free carboxyl groups.

3. Studies on the solubility and gel filtration of the enzymatic digests of modified proteins

The solubilities of tryptic digests of three modified derivatives of AE-ovalbumin were compared qualitatively. It was found that while the digests of ARG-AE-ovalbumin and DMAEA-AE-ovalbumin were readily soluble in water, a large amount of the hydrolysate of the MEA-protein remained insoluble. Similar results were obtained with the hydrolysates of modified derivatives of several other proteins. In some cases, enzymes other than trypsin were also used.

The possibility of fractionation by gel filtration was also tested. The tryptic digest of ARG-AE-ovalbumin was chromatographed on a Sephadex G 50 column using 0.2 M acetic acid for elution (Fig. 3). The recovery, based on the weights of the freeze-dried material applied to the column and eluted, was 88 per cent. Carbohydrate was detectable in peak c.

The fractionation of the tryptic digest of DMAEA-AE-ovalbumin was carried out under similar conditions (Fig. 4). Recovery based on optical densities was 96 per cent.

3.1. Isolation of large fragments. In the course of sequence studies of chymotrypsinogen-B, not published in detail so far, the tryptic digestion of CM-zymogen was found to lead to the formation of a large quantity of poorly soluble fraction comprising about two thirds of the molecule. The soluble part of the tryptic digest of the B chain of CM-chymotrypsin-B contained only one fifth of the molecule. Therefore, we attempted to fractionate the tryptic digest of arginine methylester derivative of the B chain. Chromatography on Sephadex G 50 column resulted in good fractionation (Fig. 5). The amino acid analysis revealed that peaks c and d represent tryptic peptides containing 69 and 36 residues, respectively, comprising all the remainder of the molecule, so far insoluble. The complete amino acid analysis and other experimental details will be published elsewhere, in connection with the sequence studies of chymotrypsinogen-B.

It is worth-while mentioning another aspect of this experiment. None of the two peptides derived from the unmodified chain contains arginine, lysine being the C-terminal residue in both fragments. The arginine content of the isolated

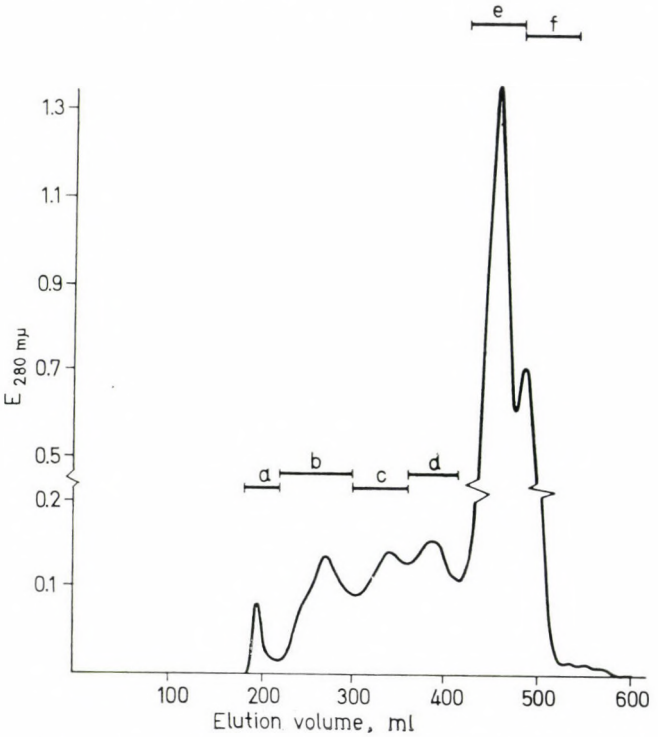


Fig. 3. Fractionation of the tryptic digest of ARG-AE-ovalbumin on Sephadex G 50. 250 mg digest was eluted with 0.2 N acetic acid from the column (150 × 2.2 cm). Flow rate, 40 ml/hour; fraction size, 5 ml

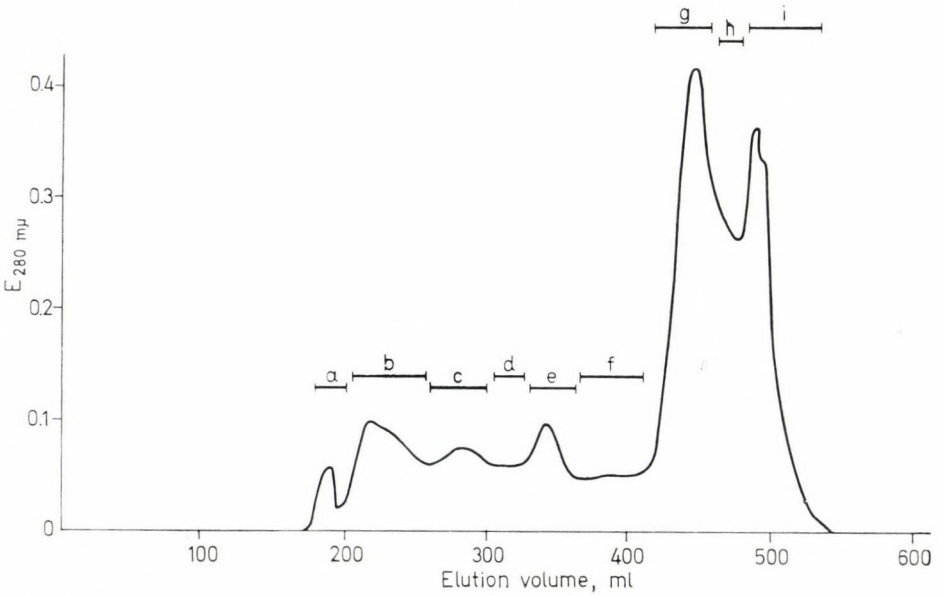


Fig. 4. Fractionation of the tryptic digest of DMAEA-AE-ovalbumin on Sephadex G 50. 80 mg digest was eluted from the column (150 × 2.2 cm) with 0.2 N acetic acid. Flow rate, 26 ml/hour; fraction size, 3.8 ml

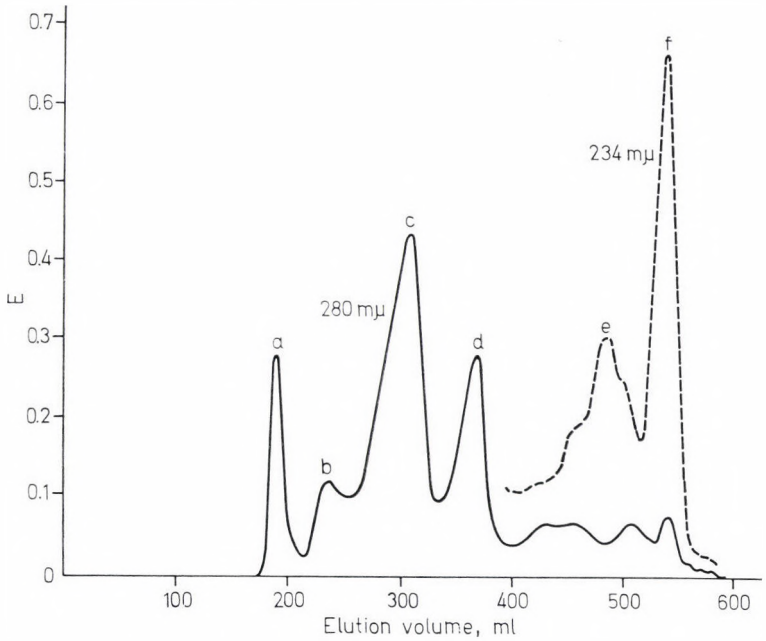


Fig. 5. Fractionation of the tryptic digest of the B chain of ARG-CM-chymotrypsin-B on Sephadex G 50. 60 mg digest was eluted from the column (145 × 2.4 cm) with 0.2 N acetic acid. Flow rate, 40 ml/hour; fraction size, 5 ml

peptides as compared to the number of free carboxyl groups calculated from the known sequence (Smillie et al., 1968) gave information about the yield of the coupling reaction in the sections of the molecule overlapped by the peptides. It is seen from the data in Table 1 that the analysis of both peptides shows con-

Table 1

The number of possible arginine-binding residues and the found arginine content of the two large peptides isolated from the tryptic digest of the B chain of ARG-CM-chymotrypsin-B

	Peak c fragment 16—84*	Peak d fragment 108—143*
Glutamic acid	5	2
Aspartic acid	6	2
CM-cysteine	2	2
Total free carboxyl	13	6
Arginine found	12	5.5
Conversion, per cent	92	91

* Numbers refer to the amino acid sequence of the zymogen.

versions over 90 per cent. If the sequence of the isolated peptide is unknown, the number of free carboxyl groups can be estimated from the arginine content.

The studies on the enzymatic digests of modified proteins suggest that the modification of the free carboxyls by coupling with either arginine methylester or dimethylamino ethylamine before submitting the protein to digestion, may facilitate the fractionation of the hydrolysates under mild experimental conditions and be useful for the isolation of large peptides.

4. Fractionation by paper electrophoresis

As it has been discussed above, deductions can be made regarding the basicity and, as a consequence, the electrophoretic properties of peptides derived from modified proteins. Some of the deductions have been tested experimentally.

4.1. Behaviour at pH 6.5. One general conclusion is that in the hydrolysate of any of the three types of derivatives, regardless of the proteolytic enzyme used, no acidic peptides are found, at least when the conversion of the free carboxyl groups to amide groups is almost quantitative. Several peptide and protein derivatives were hydrolyzed with either trypsin or chymotrypsin (see Table 2), then

Table 2

Enzymatic digests of modified proteins subjected to paper electrophoresis

		Trypsin	Chymotrypsin
MEA-AE-ovalbumin		+	
ARG-AE-ovalbumin		+	
DMAEA-AE-ovalbumin		+	
ARG-AE-chymotrypsin-A	B, C chain	+	+
ARG-CM-chymotrypsin-A	B, C chain		+
MEA-CM-chymotrypsin-B	C chain		+
DMAEA-AE-chymotrypsin-B	B, C chain	+	+

subjected to paper electrophoresis at pH 6.5. No acidic component was detected. In all cases, however, a spot was observed in the neutral region even with digests in which no neutral fragment was expected to occur (for example in the tryptic digest of DMAEA-AE-chymotrypsin-B). This may be due to impurities (e.g. formed by non-specific cleavages), or to the presence of some non-reacted side chain carboxyls.

The fractions obtained by gel filtration from a tryptic digest of DMAEA-AE-ovalbumin (see Fig. 4) were subjected to paper electrophoresis at pH 6.5. The resulting picture is shown in Fig. 6. It is seen that only the fragments of medium size are strongly tailing, the shorter peptides and even the two fractions of the highest molecular weight migrate properly and are well resolved.

4.2. Detection of peptides with no free carboxyl group by diagonal paper electrophoresis. It has also been mentioned that the C-terminal fragment of a

modified protein has no free carboxyl group at all if digestion is done by a suitable enzyme. An attempt was made to detect this fragment in the chymotryptic digest of the 1–28 synthetic fragment of human ACTH previously modified with methylamine. The method is based on the fact that neither carboxypeptidase-A nor carboxypeptidase-B is able to hydrolyze peptides which have no free terminal α-carboxyl.

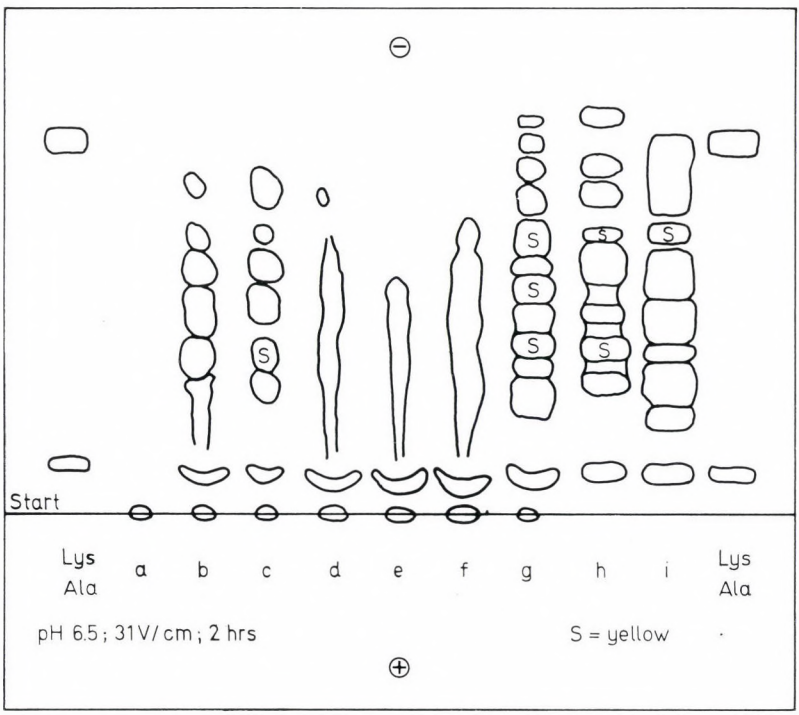


Fig. 6. Paper electrophoresis at pH 6.5 of the fractions of the tryptic digest of DMAEA-AE-ovalbumin

The chymotryptic hydrolysate of the modified product was subjected to electrophoresis on a wide paper sheet at pH 6.5. The sheet was cut into 13 strips parallel to the bands, then eluted. Each sample was distributed into two parts. One half of each sample was spotted to a second paper sheet according to its position on the first sheet after the first run. The second half of each sample was first hydrolyzed by a mixture of the two carboxypeptidases, then spotted to a third sheet. Both the second and the third sheets were rerun at pH 6.5. The results are shown in Fig. 7. As expected, the undigested samples gave a diagonal pattern. Digestion with carboxypeptidase resulted in the disappearance of all spots located in the diagonal except those belonging to samples 5 and 6. Thus, it can be concluded that a fragment with blocked α-carboxyl group was really present in the

chymotryptic digest and its band after the first pH 6.5 run was probably cut into parts (strips No. 5 and No. 6).

Based on the assumption of the blocked C-terminals and the findings described above, a simple electrophoretic method was developed for the isolation of the C-terminal fragment of proteins (Furka et al., 1970). This method gave consistent results as to the electrophoretic mobility of the C-terminal peptide of the

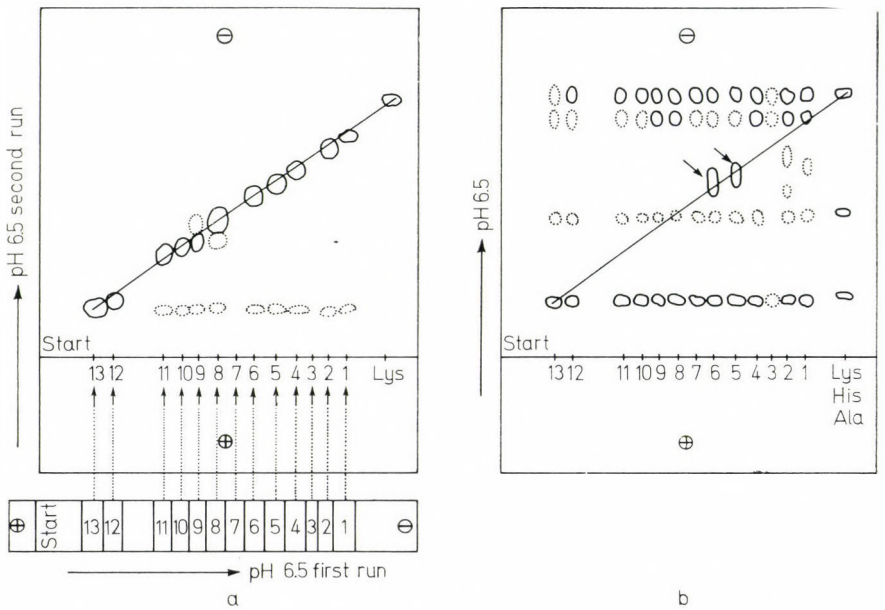


Fig. 7. Diagonal paper electrophoresis at pH 6.5 of the chymotryptic digest of fragment 1–28 of MEA-ACTH. *a)* Without carboxypeptidase treatment; *b)* with carboxypeptidase treatment

1–28 fragment of MEA-ACTH. Amino acid analysis revealed that this fragment is a tripeptide containing alanine, glycine and glutamic acid.

There is no basic amino acid residue in the molecule and still it is a basic peptide. This also indicates that its carboxyl groups are blocked.

4.3. Some other possibilities of application. Sequence studies on proteins usually start with the isolation and sequencing of the tryptic peptides. Then the protein is hydrolyzed by another enzyme, for example chymotrypsin, to get overlapping peptides. Leaving histidine peptides out of consideration, obviously all basic chymotryptic peptides are overlapping ones. But overlapping fragments can be found among the neutral and acidic peptides, too. Thus, in order to find all overlapping fragments, the acidic and neutral regions of an electrophoretogram obtained at pH 6.5 also have to be processed.

This procedure may well be expected to become simpler when looking for overlapping fragments in a chymotryptic digest of a MEA-protein. In addition to the C-terminal and histidine peptides all overlapping fragments will be basic

and found — if they are soluble — in the basic region after paper electrophoresis at pH 6.5. All other components, not important for overlapping, will accumulate in the neutral band and so will become negligible.

Another problem is that the N-terminal of numerous proteins is acylated, and so the determination of the N-terminal residue or N-terminal sequence of

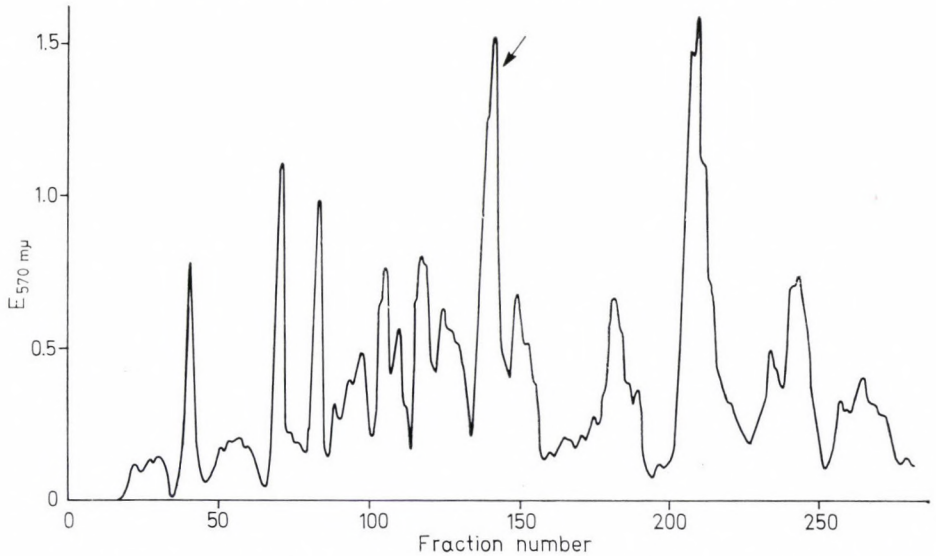


Fig. 8. Chromatography of the low molecular weight fraction of the tryptic digest of ARG-AE-ovalbumin on a Dowex 50X2 column. 70 mg fraction B was applied to the column (73 × 1 cm) previously equilibrated with 0.2 M pyridine-acetate buffer at pH 5. Gradient I: 500 ml 0.2 M pyridine-acetate, pH 5, and 500 ml 2.0 M pyridine-acetate, pH 5. Gradient II (started at fraction No. 205): 220 ml 2.0 M pyridine-acetate, pH 5, and 220 ml 4.0 M pyridine-acetate, pH 5.5. Flow rate, 16 ml/hour; fraction size, 4 ml; temperature, 40 °C

these proteins is difficult. But, modification with arginine methylester offers a possibility to solve this problem. It can be deduced that in each tryptic fragment of an ARG-protein the number of basic groups exceeds by only one the number of acidic groups. If the terminal amino group of the protein is acylated, the number of the basic and acidic groups of the N-terminal fragment is the same. As a consequence, the N-terminal fragment is neutral while all other peptides are basic.

Studies for experimental realization of the possibilities mentioned are in progress.

5. Fractionation by ion exchange

On the fractionation of hydrolysates of modified proteins by ion exchange chromatography, only preliminary studies were made. ARG-AE-ovalbumin was hydrolyzed by trypsin, then fractionated on Sephadex G 50 (see Fig. 3). The high molecular weight part (fraction A, pooled fractions under peaks a, b, c and d) and the fraction of shorter peptides (fraction B, pooled fractions under peaks e and f) were freeze-dried and studied separately.

5.1. Fractionation of the low molecular weight part of the digest. Further fractionation of fraction B was studied on a Dowex 50X2 column equilibrated at 40 °C with 0.2 M pyridine-acetate buffer at pH 5. Elution was started with a concentration gradient of the same buffer (from 0.2 to 2.0 M) and finished with a concentration and pH gradient from 2.0 M, pH 5 to 4.0 M, pH 5.5. Good resolution was obtained (Fig. 8). The pooled fractions of the peak indicated by

Table 3

Amino acid analysis of two peptides isolated from ARG-AE-ovalbumin

	TB 13a	TA 11
Lysine	—	1
Arginine (total)*	4	2
Aspartic acid	2	3
Threonine	1	2
Serine	1	6
Glutamic acid	3	1
Glycine	2	2
Alanine	3	3
Valine	1	2
Methionine	—	1
Isoleucine	1	2
Leucine	1	4
Tyrosine	—	1
Phenylalanine	—	1
Number of residues**	16	29
Glucosamine	—	+

* 3 and 2 arginine residues are supposed to be found in the side chains of peptides TB 13a and TA 11, respectively.

** From the total number of residues the number of side chain arginines was subtracted.

an arrow on Fig. 8 were freeze-dried, then subjected to paper electrophoresis at pH 1.8. The fastest moving component (TB 13a) was hydrolyzed by acid and then analyzed.

The data of the amino acid analysis are summarized in Table 3. Three of the four arginine residues are supposed to be found in the side chain of the analyzed hexadecapeptide. The peptide migrated on the paper during electrophoresis without tailing.

5.2. Fractionation of the high molecular weight part of the digest. In contrast to the high molecular weight fraction of the tryptic digest of the unmodified AE-ovalbumin which was not completely soluble even in 8 M urea, fraction A readily dissolved in distilled water. It was tested, therefore, whether chromatography can

be successful with eluants containing no urea. Fractionations on CM-cellulose, DEAE-Sephadex and DEAE-cellulose columns using Tris-HCl buffers were tried without success. The samples precipitated at the top of the columns and the fractionations failed, at least under the conditions tested. After these failures fractionations were tried in 6 M urea solutions. The separations were carried out on CM-cellulose columns using gradient elution. Successful fractionations were obtained (Fig. 9). Urea could be easily removed from samples on Sephadex

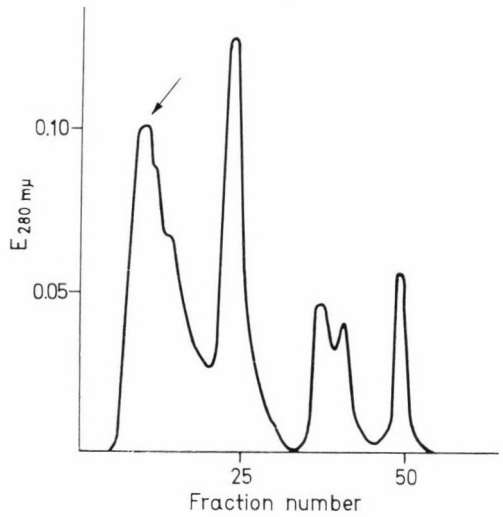


Fig. 9. Chromatography of the high molecular weight fraction of the tryptic digest of ARG-AE-ovalbumin on CM-cellulose column. 17 mg fraction A was applied to the column (39 × 1 cm) previously equilibrated with 0.02 M sodium acetate in 6 M urea at pH 5. Gradient: 250 ml 0.02 M sodium acetate, pH 5, and 250 ml 2.0 M sodium acetate, pH 5, both in 6 M urea. Flow rate, 26 ml/hour; fraction size, 3.6 ml

G 25 column. Again 0.2 M acetic acid was used as eluant. One of the components (indicated by an arrow in Fig. 9) was rechromatographed on DEAE-cellulose column equilibrated at pH 8 with 0.01 M Tris-HCl buffer containing 8 M urea. The amino acid composition of the purified, carbohydrate containing peptide (TA 11) is indicated in Table 3. The presence of the two arginine residues in the molecule can probably be attributed again to the modification of the protein.

Although, as the results of this study show, the application of concentrated urea solution in the fractionation of the high molecular weight fraction by ion exchange chromatography could not be avoided, the ease and the mild conditions in the removal of urea and salts due to enhanced solubility can be considered as a real advantage.

Thanks are due to Prof. V. Bruckner for his interest in this work, to Dr H. Medzihradszky for amino acid analysis as well as to Mrs I. Császár and Mrs Zs. Gémes for technical assistance. Financial assistance of the Hungarian Academy of Sciences is also acknowledged.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (2), pp. 147—157 (1970)

Altered Repression Behaviour in a Feedback Insensitive Mutant of Escherichia coli K12

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(Received January 6, 1970)

One-step mutants showing multiple alterations in the biosynthesis and regulation of the histidine operon were isolated from Escherichia coli K12 on the basis of their resistance to 2-thiazolealanine. The first enzyme of histidine biosynthesis in one of the mutants has lower activity and is partially resistant to inhibition by histidine. The decreased activity of this enzyme necessitates the derepression of the histidine operon in order to produce histidine at a normal rate. Derepression by the mutant, however, can be effected only by severe histidine starvation. This feature is more pronounced at lower temperatures, resulting in cold sensitivity of growth. Thiazolealanine renders derepression and growth normal. The different features of the mutant behaviour may be pleiotropic effects of the mutation in the gene for the first enzyme.

Introduction

The pathway of histidine biosynthesis in Salmonella typhimurium consists of a series of ten enzymatic steps. The rate of formation of histidine is controlled by two different mechanisms. Feedback inhibition controls the activity of the first enzyme (phosphoribosyladenosinetriphosphate : pyrophosphate phosphoribosyltransferase) specific for the pathway, by the end product, histidine (Ames et al., 1961; Moyed, 1961; Martin, 1963a). The other regulatory mechanism is repression-derepression which controls the level of the enzymes involved in the biosynthesis of histidine. A cluster of the structural genes for these enzymes and an operator gene, known as the histidine operon, are regulated as a unit (Ames, Garry, 1959; Ames et al., 1960; Ames, Hartman, 1962; Ames, Hartman, 1963; Ames et al., 1963). The fact that derepression occurs when the histidine biosynthetic pathway is intact, but the formation of histidyl-tRNA is defective (Schlesinger, Magasanik, 1964; Roth et al., 1966a; Roth, Ames, 1966; Silbert et al., 1966), suggests that histidyl-tRNA rather than histidine is important in the repression of the histidine operon in both Escherichia coli and Salmonella typhimurium. Studies on Escherichia coli revealed a high degree of similarity to Salmonella typhimurium with respect to the histidine system and its regulation (Shedlovsky, Magasanik, 1962a, b). Although it has been assumed that the two regulatory mechanisms — feedback inhibition and repression — are unrelated processes, several findings suggest that the feedback sensitive enzyme may play some unknown role in the repression

process also (Sommerville, Yanofsky, 1965; Berberich, Gots, 1965). For the histidine operon some recently published results strongly support this suggestion (Kovach et al., 1969a, b).

In this paper we report that some histidine feedback insensitive mutants of Escherichia coli K12 are hypersensitive to repression and that the two regulatory processes may be strongly connected to each other.

Materials and Methods

L-histidinol · 2 HCl was prepared chemically (Bauer et al., 1955). 5-Phosphoribosyl-1-pyrophosphate was synthesized enzymatically using a partially purified synthetase of the mutant his-G70 of Salmonella typhimurium (R. F. Goldberger, personal communication). All the other chemicals used in the experiments were commercial products.

Cells were grown in minimal medium A (Davis, Mingioli, 1950) containing 0.2 per cent glucose and the required growth factors. The cultures were aerated in a gyratory shaker at 37 °C and the growth was followed photometrically by sampling and reading the absorbance at 490 mμ in a 10 mm cuvette.

For the preparation of cell-free extracts 50—100 ml samples containing approximately 5×10^8 cells per ml were centrifuged and the sedimented cells were washed and resuspended in 7 ml of buffer to be used in the enzyme assay. The cells were disintegrated by sonic treatment and after centrifugation enzyme activities in the supernatant were determined immediately. All procedures preceding the enzyme assays were carried out at 0 °C. Protein content of the samples was determined by the Folin-phenol method (Lowry et al., 1951).

The activities of phosphoribosyladenosinetriphosphate : pyrophosphate phosphoribosyltransferase (PR-ATP pyrophosphorylase) and histidinol dehydrogenase (EC 1.1.1.23) were determined according to described methods (Ames et al., 1961; Shedlovsky, Magasanik, 1962b).

The amount of histidine excreted into the medium by the isolated mutants was determined microbiologically using the histidine-requiring mutant his-152 of Salmonella typhimurium. The histidine excreted was also identified by thin layer chromatography.

The organisms employed in this study were Escherichia coli Y10 ($F^-thr^-leu^-B_1^-$), a derivative of the strain K12, and the histidine-requiring mutants his-G70 and his-152 of Salmonella typhimurium. The Salmonella typhimurium mutant strains were kindly supplied by Dr R. F. Goldberger.

Results

2-Thiazolealanine resistant mutants. The histidine analogue, 2-thiazolealanine, mimics histidine in its action on the feedback-sensitive first enzyme, PR-ATP pyrophosphorylase, of histidine biosynthesis (Moyed, Friedman, 1959). The inhibition of this enzyme activity by 2-thiazolealanine results in histidine starvation.

Using this property of the analogue, resistant mutants could be selected by plating 10^8 cells of Escherichia coli Y10 on minimal medium at 37 °C containing 2.5 mM DL 2-thiazolealanine. This concentration of the analogue inhibited the growth of the sensitive parent strain strongly, while several resistant colonies appeared without delay. Some of these resistant mutants showed histidine excretion, as identified by thin layer chromatography, and stimulation of growth of his-152 mutant of Salmonella typhimurium. Ten independent 2-thiazolealanine-resistant and histidine-excreting mutants were thus isolated, purified and numbered serially.

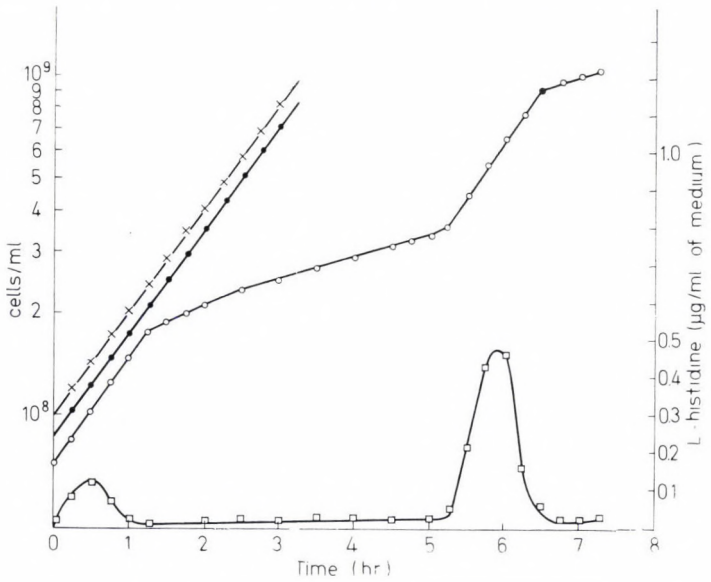

Fig. 1. Growth of Y10-6 on minimal -○-○-, DL 2-thiazolealanine (10^{-4} M) -×-×- and L-histidine-(10^{-4} M) -●-●- containing media at 37 °C. The histidine content of the culture-fluid during growth on minimal medium, as determined microbiologically -□-□-

Eight of the ten mutants showed normal growth; Y10-6 and Y10-7, however, displayed a strange growth pattern on minimal medium at 37 °C. Phases of rapid growth (doubling time identical with that of the wild type Y10, i.e., 57 minutes) were alternating with slow ones (doubling time about 200 minutes). Both histidine (0.1 mM) and DL 2-thiazolealanine (0.1 mM) restored wild type growth (Fig. 1).

The subsequent work was concentrated on Y10-6.

Since the mutant excreted histidine and required histidine for constant rapid growth, it was interesting to measure the level of L-histidine in the medium during growth. The result of an experiment is shown in Fig. 1. As can be seen, the rapid growth phase coincides with the accumulation of L-histidine in the medium; after its exhaustion, slow growth follows.

Alteration in phosphoribosyl-ATP pyrophosphorylase. The 2-thiazolealanine resistance and histidine excretion of the mutant suggested that it might have an altered first enzyme. Assay of the enzyme in extracts of derepressed Y10 and Y10–6 showed that the activity of this enzyme in the latter was damaged. A comparison of the activities of the first enzyme and histidinol dehydrogenase in the parent and the mutant strains showed that the mutant had some eight times lower activity of the first enzyme as compared to histidinol dehydrogenase (Table 1).

Table 1

Activities of PR-ATP pyrophosphorylase and histidinol dehydrogenase in crude extracts of derepressed Y10 and Y10–6 strains of E. coli

Y10 was derepressed by growing the parent for 2—3 hours in the presence of 10^{-4} M DL 2-thiazolealanine. Derepression of Y10–6 was obtained by growing the mutant for 3—4 hours in the absence of histidine. The enzyme activities are expressed as μmoles of product formed per hour per mg of protein

	Y10	Y10—6
PR-ATP pyrophosphorylase	1.27	0.15
Histidinol dehydrogenase	1.46	1.32

Table 2

Comparison of the feedback sensitivity of PR-ATP pyrophosphorylase in Y10 and Y10–6 strains of E. coli

For experimental conditions see Table 1

	Y10	Y10—6
50 per cent inhibition of the enzyme by L-histidine	1.2×10^{-4} M	20×10^{-4} M

Since repression of the histidine operon is coordinate, activity of an enzyme in two strains can be compared only if another enzyme activity of the histidine biosynthetic pathway is used as reference (Ames, Garry, 1959).

The mutant enzyme requires about ten times higher concentration of L-histidine for 50 % inhibition than the wild type enzyme (Table 2).

Study of derepression behaviour. Preliminary experiments indicated that the mutant contained high levels of histidinol dehydrogenase during slow growth in minimal medium. When the mutant was grown on exogenous histidine the enzyme was repressed. This behaviour is typical of leaky auxotroph mutants.

In control experiments derepression of the parent strain was obtained by the addition of 2-thiazolealanine. Starting point of derepression is the time of DL 2-thiazolealanine addition. The initial growth rate of Y10 was identical with the leaky growth of Y10–6.

Derepression of histidinol dehydrogenase in the parent Y10 at 37 °C is shown in Fig. 2. Addition of DL 2-thiazolealanine at a final concentration of

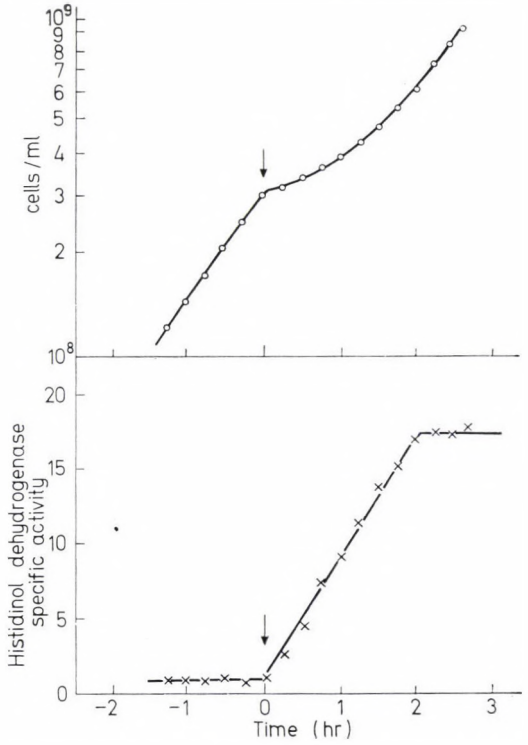

Fig. 2. Growth and derepression of histidinol dehydrogenase in Y10 parent strain after addition of DL 2-thiazolealanine (10^{-4} M) at 37 °C. The arrows indicate the addition of DL 2-thiazolealanine. One unit is the specific activity in repressed bacteria, corresponding to 78 mμmoles NADH formed per hour per mg protein

0.1 mM caused a leaky growth, and the enzyme started to derepress at once. The increasing enzyme levels compensate for the lower (inhibited) activity of the first enzyme and this is reflected in more rapid growth. When a certain level of the histidine biosynthetic enzymes is reached, the inhibition by 2-thiazolealanine is fully overcome and the enzyme levels are regulated at this level, and the growth rate is normal.

Derepression of Y10–6 was studied on minimal medium. Exhaustion of histidine is reflected by the onset of slow growth. This point is defined as 0 minute of derepression.

Derepression behaviour of Y10–6 was abnormal under these conditions (Fig. 3). Derepression started at a very low rate, and this lack of normal derepression aggravated the histidine starvation further, as reflected by an even slower growth. At this growth rate, however, derepression started at a more rapid rate.

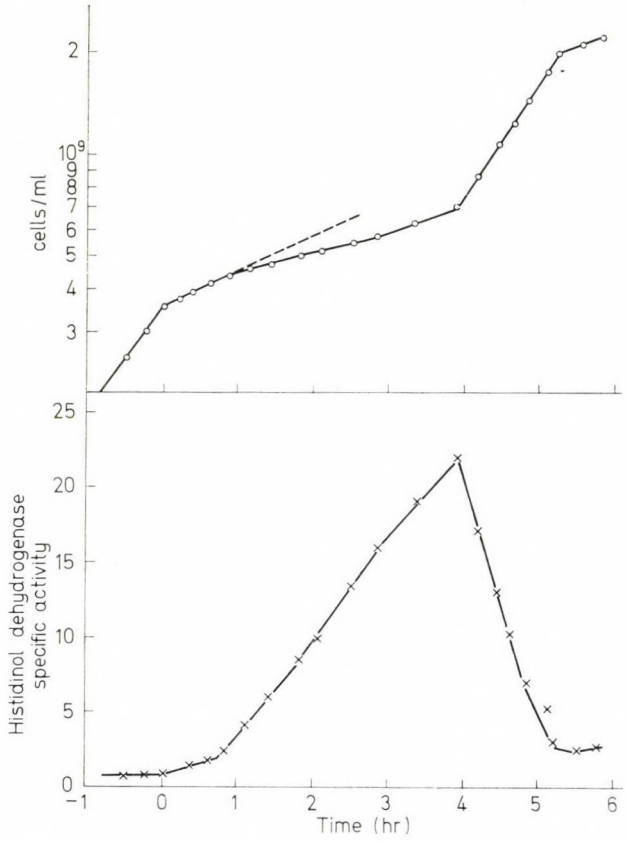

Fig. 3. Growth and derepression of histidinol dehydrogenase in mutant Y10–6 after exhaustion of L-histidine at 37 °C

In further contrast with the parent, derepression does not result in a continuous improvement of growth. Upon reaching a certain enzyme level, growth becomes abruptly normal, histidine appears in the medium (Fig. 1), and the operon becomes repressed: the specific activity of the enzyme decreases.

Study of the derepression behaviour of the mutant at lower temperatures revealed that its derepression is heavily retarded. The lack of derepression leads to slower growth (Fig. 4). At 25 °C the mutant is completely incapable of derepression, although Y10 under the same conditions shows normal derepression.

Since at all temperatures tested the initial growth rate of Y10 was identical with that of the mutant, the differences in derepression behaviour may be due to an alteration in the regulatory system of the mutant.

Plotting derepression rate against doubling time at 31 °C, 34 °C and 37 °C, it becomes clear that at a given temperature the derepression rate increases with

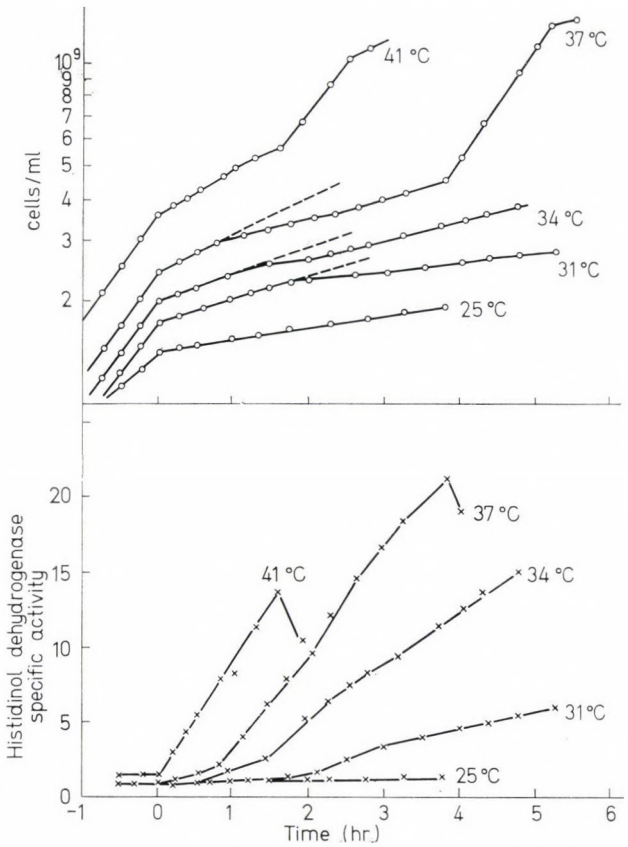

Fig. 4. Growth and derepression of histidinol dehydrogenase in Y10–6 at different temperatures

increasing histidine starvation. On the other hand, a given doubling time allows higher rate of derepression at higher temperatures (Fig. 5).

Addition of DL 2-thiazolealanine (0.1 mM) rendered the growth of the mutant normal. Derepression behaviour was normal in all respects; the mutant derepressed at a rate identical with that of the parent and after reaching a level of enzymes at which growth was of the wild type, the enzyme level remained constant (Fig. 6).

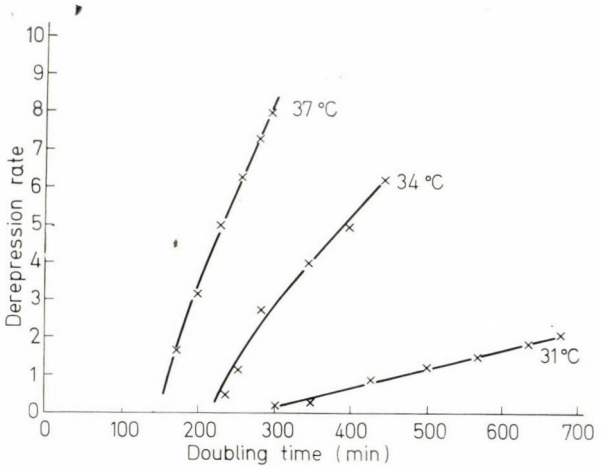

Fig. 5. Dependence of derepression rate on doubling time at various temperatures. Derepression rate is changed in histidinol dehydrogenase specific activity units per hour

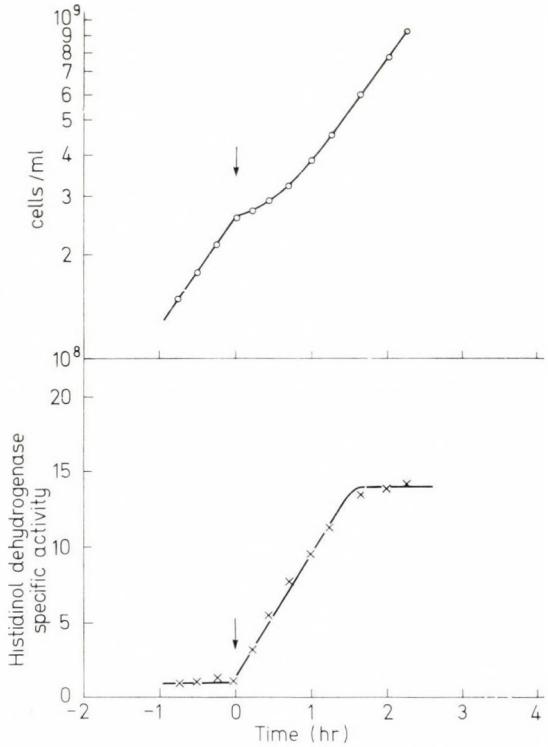

Fig. 6. Growth and derepression of histidinol dehydrogenase in Y10–6 after addition of DL 2-thiazolealanine (10^{-4} M) at 37 °C. The arrow indicates the exhaustion of L-histidine and addition of DL 2-thiazolealanine

Discussion

Assay of PR-ATP pyrophosphorylase revealed a low level of activity in the mutant and this activity showed partial resistance to inhibition by L-histidine. Parallel with these changes the repression behaviour of the mutant was also altered. The histidine operon remained repressed at a histidine starvation where the parent strain derepressed normally. Derepression of the mutant started only later when the slower growth indicated a more severe histidine starvation. On the basis of these data the unusual behaviour of the mutant may be explained in the following way.

a) Minimal medium (Figs 1 and 3). Due to the alteration in the structure of the first enzyme of histidine biosynthesis the mutant grows slowly because of the impaired production of histidine, like any leaky auxotroph mutant. Nevertheless, derepression is very slow since the mutant has a hypersensitive repression mechanism. Because of a lack of normal derepression, growth is further impaired and now derepression can proceed at a higher rate. The growth rate, however, is not improved. It may be assumed that the first enzyme is inactive at this stage. An enzyme level is reached which is potentially higher than necessary for wild type growth when the enzyme becomes functional at once. The lack of feedback inhibition allows the manifestation of all synthesizing potential, and it leads to excretion of histidine. At the same time repression occurs. Histidine is excreted until the enzyme level is lowered by repression to a level equivalent to the requirements of the mutant. The accumulated histidine, however, causes further repression until a complete exhaustion of histidine occurs, thus annihilating the enzymatic basis of normal growth. At this point a new period is started.

It would be premature to speculate on the molecular mechanism which keeps the first enzyme inactive during the period of slow growth, and which causes its sudden activation, leading to rapid growth. It seems possible that the appearance of histidine, similarly to 2-thiazolealanine (see below), activates the accumulated enzyme.

b) Histidine containing medium. Since the mutant is a leaky histidine auxotroph, addition of histidine restores normal growth rate at all temperatures, and represses the operon.

c) 2-Thiazolealanine-containing medium (Fig. 6). 2-Thiazolealanine exerts a double effect on the mutant. On the one hand it normalizes the derepression of the mutant (derepression at maximal rate without delay) and on the other hand it renders the synthesized enzymes functional. Therefore, near normal growth rate is assumed at a relatively low level of derepression and this level is reached in a short time. "Overshoot" of histidine production is avoided since the enzymes are functioning as soon as produced. Thus when the enzyme level is reached which can produce histidine at the appropriate rate the enzyme level is kept constant. The growth rate is of wild type afterwards.

The biosynthesis of histidine is catalyzed by ten enzymatic steps. The structural genes for these enzymes are clustered in the histidine operon. All genes are transcribed on a large polycistronic mRNA (Martin, 1963b; Venetianer et al.,

1968), and are expressed coordinately: the ratio of different enzymes is constant for a given organism independent of the degree of derepression (Ames, Garry, 1959). In bacteria with normal regulatory mechanism derepression is elicited by histidine starvation. Studies on mutants with high repressed enzyme levels revealed a number of genetic loci involved in the regulation of the histidine system.Although some of the elements which underwent mutation could not be assigned a clear role, it seems to be sure that histidyl-tRNA rather than free histidine plays a central role in regulation, and a normal operator locus at the end of the operon is essential for regulation (Roth et al., 1966b). It is not clear, however, whether this operator is independent of the G gene encoding the feedback sensitive first enzyme of histidine biosynthesis (Loper et al., 1964).

In the case of three amino acids (histidine, tryptophane, leucine) it has been found that the feedback sensitive first enzyme is determined by the first gene of the respective operon (Ames, Hartman, 1963; Matsuhiro et al., 1962; Margolin, 1963). It seems likely that this correlation between first gene and first enzyme has some evolutionary significance. Perhaps the fact that the newly synthesized first enzyme is located near the operator end of the mRNA ensures a selectional advantage. Mechanisms utilizing this position of the feedback sensitive enzyme were discussed (Maas, McFall, 1964; Gruber, Campagne, 1965; Cline, Bock, 1966; Koshland, Kirtley, 1966).

The finding that the functional state of the feedback site of the feedback sensitive enzyme influences the way of repression (Kovach et al., 1969b) in the histidine operon, supports this notion.

In our work on a mutant with a partially feedback insensitive first enzyme it has been found that the mutant shows an altered repression behaviour. Although the position of the mutation is genetically not mapped, the relatively frequent occurrence of such mutants, and the fact that 2-thiazolealanine influences the activity of the first enzyme as well as the repression behaviour of the mutant, suggests that it is the mutation in the first enzyme which brings about the altered repression behaviour.

The repression behaviour of the mutant is such as would be expected of mutants with hypersensitive repression mechanisms. Derepression occurs only at a high degree of histidine starvation and the differential rate of synthesis of the histidine biosynthetic enzymes increases with increasing histidine starvation. No such correlation can be observed with the wild type bacterium.

The repression hypersensitivity of the mutant is more pronounced at lower temperatures and this keeps the growth rate at even lower values. This cold sensitivity of the mutant is similar to that resulting from increased feedback inhibition (O'Donovan, Ingraham, 1965; St. Pierre, 1968). O'Donovan et al. found that cold sensitivity of different systems resulted from stronger binding of an allosteric modifier at low temperatures. Supposing that repressor molecules are at least in part allosteric proteins they have predicted that there may be cold sensitive mutants whose cold sensitivity results from alteration of the repressor molecule.

If our conclusion that the altered element is the first enzyme of histidine biosynthesis is right it would reinforce the assumption that its unique position in the operon reflects its unique role in regulation.

Valuable discussion with Dr P. Venetianer is gratefully acknowledged. Thanks are due to Dr R. F. Goldberger for sending us his manuscript prior to publication.

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Factors Affecting Freezing-Induced Transient Dissociation of Lactate Dehydrogenase Tetramers

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(Received January 3, 1970)

Three test procedures were developed for studying the factors which affected freezing-induced transient dissocation of lactate dehydrogenase (LDH) tetramers. The three characteristics of dissociated LDH on which the tests were based were (a) recombination of the four polypeptide chains, (b) increased rate of "spontaneous" denaturation, and (c) increased sensitivity towards iodoacetamide. Estimation of the relative availability of the dissociated (probably monomeric) form of the enzyme was possible with the help of the iodoacetamide test. Sodium chloride, potassium phosphate, ammonium sulphate, Tris buffer and substrates were added to the enzyme solution both before freezing and at the commencement of thawing. While the salts only affected the transient dissociation of LDH tetramers if added to the enzyme solution before freezing, substrates also produced marked effects if added in the course of thawing. When substrates were present, the rate of re-formation of non-dissociating tetramers in the thawed solution was increased. The presence of sodium chloride during freezing favoured subsequent dissociation, while the presence of potassium phosphates, ammonium sulphate or Tris buffer were inhibitory. Both the temperature and the duration of freezing affected the transient dissociated state of LDH which was obtained upon thawing. Results obtained in the three tests support the view that there is a common step, namely transient dissociation, in the development of the measured responses. The possible nature of freezing-induced transient changes in the steric structure of LDH is described by a simple four-step scheme.

Introduction

Freeze-thaw procedures have been originally applied to LDH (Markert, 1963; Chilson et al., 1965) for bringing about a recombination of the polypeptide chains which apparently form non-dissociating tetramers under ordinary laboratory conditions (Markert, Massaro, 1968; Jaenicke, 1969; Südi, Khan, 1970). The available evidence supports the view that hybridization by freezing and thawing involves the temporary dissociation of LDH tetramers. Accordingly, the extent of dissociation, i.e. the properties of a postulated transient dissociated state, should determine the extent of hybridization.

We have recently shown (Südi, Khan, 1970) that pig lactate dehydrogenase isoenzymes H_4 and M_4 are reversibly dissociated by freezing and thawing under appropriate conditions. It was demonstrated that in the transient dissociated state the protein was enzymatically inactive, and that the rate and final extent of

reactivation was dependent on protein concentration. The same standard freeze-thaw treatment also made the two proteins more reactive towards iodoacetamide (Südi, Khan, 1970).

The present communication is based on the working hypothesis that a certain dissociated form of LDH is transiently obtained by an appropriate freeze-thaw treatment. Transient inactivation of the protein, its increased reactivity towards iodoacetamide, and a recombination of the polypeptide chains were assumed to be three phenomenological consequences of this transient dissociation. We modified the standard freeze-thaw procedure by varying the composition of the solution in which the protein(s) were frozen and thawed and by varying the duration and temperature of freezing. These modifications were carried out parallel in three standard tests based on the above three phenomena. The responses obtained support the view that the three phenomena are all based on the transient dissociation of tetramers. Differences in the extent of transient dissociation of subunits could be described in quantitative terms with the help of the iodoacetamide test. A simple model is presented which describes the main events which take place during freezing and in the transient state obtained upon thawing.

Materials and Methods

Enzymes and reagents. Two to four times recrystallized and chromatographically homogeneous preparations of pig heart lactate dehydrogenase (LDH-H_4) and pig muscle lactate dehydrogenase (LDH-M_4) were prepared by the procedure of Straub (1940), and that of Jécsay (1961), respectively, with slight modifications (Khan, Südi, 1968). Enzyme stock solutions were stored at most for a few days, and before use they were always passed through a column of Sephadex G-25 equilibrated with the buffer to be used.

Iodoacetamide (Fluka A.G.) was recrystallized from carbon tetrachloride. Tris-hydroxymethyl aminomethane (Tris buffer) was recrystallized from aqueous ethanol. All other reagents were analytical grade commercial preparations.

Protein content and enzyme activity. Protein was routinely measured at 280 mμ and the concentration of both LDH-H_4 and LDH-M_4 was calculated from $E^{1\ cm}_{1\ mg/ml} = 1.29$ (Khan, Südi, 1968). Enzyme activity was determined at room temperature by measuring the rate of decrease of optical density at 340 mμ with 0.01—0.20 μg/ml of enzyme protein, 0.15 mM NADH and 1 mM pyruvate in 0.2 M Tris/HCl buffer, pH 7.5. Under these conditions, the molecular activity of the five isozymic forms of pig lactate dehydrogenase is, in moles NADH oxidized per minute per mole of LDH tetramer (M. wt. = 140 000): 44 100 (M_4), 37 000 (HM_3), 29 900 (H_2M_2), 22 800 (H_3M), and 15 600 (H_4), respectively. "Active enzyme protein" content of chromatographically separated isoenzymes was calculated from these specific activities and from the experimentally found enzyme activity of the samples.

DEAE-cellulose chromatography. DEAE-cellulose (Serva DEAE-SH) was used for the separation and quantitative determination of the amount of LDH isoenzymes present in a mixture. A 12-ml column of DEAE-cellulose was equilib-

rated with 0.025 M Tris/HCl buffer, pH 8.0. The column was loaded with 10—40 mg of enzyme protein dissolved in the same buffer. Elution was done by the application of a linear gradient of NaCl (0 to 0.3 M) in 0.025 M Tris/HCl, pH 8.0. The eluate was monitored with Uvicord (LKB) at 280 mμ. The five peaks corresponding to the five isoenzymes were pooled, and LDH activity was determined under the standard assay conditions. The peaks were identified on the basis of their chromatographic behaviour, checked occasionally by co-chromatography with a purified isoenzyme, or by acrylamide gel electrophoresis (Südi, 1969). From the known specific activity of the isoenzymes the amount of "active enzyme protein" was finally calculated (see above).

Standard test 1: freeze-thaw hybridization. Stock solutions of LDH-M_4 and LDH-H_4 in 0.1 M sodium phosphate buffer, pH 7.0, were mixed to yield a solution which contained the two homotetramers in known and roughly equal amounts. The final concentration of each protein was 1—3 mg/ml. 5-ml aliquots of such solutions in test tubes were transferred to an ethyleneglycol-water bath thermostated at -18 ± 1 °C. The samples were frozen in less than 2 minutes, and incubated in the bath for 2 hours. At the end of the freeze-treatment the tubes were transferred to a water bath at room temperature, and from this to an ice-water bath just before the disappearance of ice from the test tube. Thawing was completed in about 2 minutes. Following a 30—60-min incubation of the thawed solution at 0 °C, the protein was transferred from phosphate into 0.025 M Tris/HCl buffer, pH 8.0, by Sephadex filtration. This solution was finally chromatographed on DEAE-cellulose (see above) for a quantitative analysis of the resulting isoenzyme pattern. Recovery of LDH activity in the eluate was routinely determined and found to be 85 to 105 per cent of the LDH activity loaded onto the column.

Standard test 2: freeze-thaw inactivation at low protein concentration. The final concentrations in the solutions to be frozen were 0.1 M sodium phosphate, pH 7.0, and either 4 μg/ml of LDH-M_4, or 6 μg/ml of LDH-H_4. 1-ml aliquots in test tubes were frozen for 2 hours at —18 °C, then allowed to thaw for 30—60 minutes at 0 °C, after which residual LDH activity was determined under the standard assay conditions.

Standard test 3: reaction of dissociated LDH with iodoacetamide. Solutions were prepared at 0 °C. They contained, in a final concentration, 0.1 M sodium phosphate buffer, pH 7.0; 1 mg/ml of either LDH-M_4 or LDH-H_4; and 0.1 M iodoacetamide. 1-ml aliquots in test tubes were frozen for 2 hours at —18 °C. For thawing, the tubes were transferred first to a room-temperature water bath, and then to an ice-water bath. Following incubation of the thawed solution for 30 to 60 minutes, the samples were diluted and residual LDH activity was determined under the standard assay conditions.

Freezing at non-standard temperatures. In some experiments the temperature of freezing was different from the standard —18 °C. For freezing at —8 °C an eutectic solution of $MgSO_4$ was used. The temperature of this bath was constant for days when placed into a refrigerator. The standard sodium phosphate solution could be frozen in this bath in a few minutes, by seeding with small ice crystals

in a capillary tube. Freezing could not be achieved in the presence of 1 M NaCl. Thawing was carried out as in the standard treatment. Freezing at temperatures varying from —70 to —76 °C was achieved by immersion of stainless steel tubes into an acetone–dry ice bath. For thawing, the tubes were transferred first to a water bath at room temperature, and then to an ice-water bath.

Results and Discussion

1. Standard test conditions. Above we have described three standard tests for transient dissociation of LDH by freezing and thawing. When the three standard tests are compared it is seen that the buffer in which the protein(s) are dissolved and the freeze-thaw treatment itself are the same. In test 1 both homotetramers are simultaneously present in high concentration, in test 2 a low concentration of one of the homotetramers is frozen and thawed, and in test 3 a high concentration of one homotetramer is frozen and thawed in the presence of 0.1 M iodoacetamide.

In test 1 the extent of molecular hybridization between homotetramers M_4 and H_4 was determined. With regard to hybridization as a test for transient dissociation of LDH the following considerations should be kept in mind: (a) Hybridization is only obtained if both homotetramers become sufficiently dissociated. (b) Even if both homotetramers are sufficiently dissociated for some time during the freeze-thaw cycle, hybridization might be inhibited by preferential, as opposed to random, reassociation (Freier, Bridges, 1964; Markert, Whitt, 1968). (c) The test is being carried out at a high protein concentration (>2 mg/ml) to prevent any permanent loss of enzyme activity which may result from freezing and thawing (Südi, Khan, 1970).

Tests 2 and 3 are also based on earlier findings (Südi, Khan, 1970). In test 2 we measured the loss of enzyme activity which occurred when LDH was frozen and thawed at a low protein concentration. It should not be forgotten that the extent of reactivation is determined not only by the extent of dissociation, but also by the operation of two opposing consequent reactions, i.e. the rate of re-formation of non-dissociating tetramers, and the rate of "spontaneous" inactivation of dissociated LDH.

In test 3 LDH in the dissociated state was reacted with 0.1 M iodoacetamide, and residual enzyme activity was determined after sufficient time was allowed for the reactivation of unreacted LDH to occur. The concentration of iodoacetamide was sufficiently high to bring about 95 to 99 per cent loss of enzyme activity when frozen with LDH under the standard conditions, and sufficiently low to make the loss of activity negligible in unfrozen controls kept at 0 °C. On the other hand, protein concentration was high (1 mg/ml), in order to prevent any significant loss of activity in controls frozen in the absence of iodoacetamide. With regard to the iodoacetamide test as a test for transient dissociation of LDH, the predictions of our working hypothesis are as follows: The extent of inactivation by iodoacetamide depends on iodoacetamide concentration, and on the availability of dissociated (probably monomeric) enzyme. By availability we mean a certain

period of time during which the protein exists in a dissociated state. While it would be difficult to determine the absolute value of this period of time, it is demonstrated in Fig. 1 that the idea is basically right. The molar ratio of iodoacetamide (10^{-3} M) to enzyme protein (0.7×10^{-6} M of the tetramer) was high and, therefore, one had to expect the reaction of enzyme inactivation to follow a first-order time course. Indeed, an apparently first-order time course was obtained by carrying the same solution through a number of freeze-thaw cycles, and equating time with the number of completed cycles (Fig. 1).

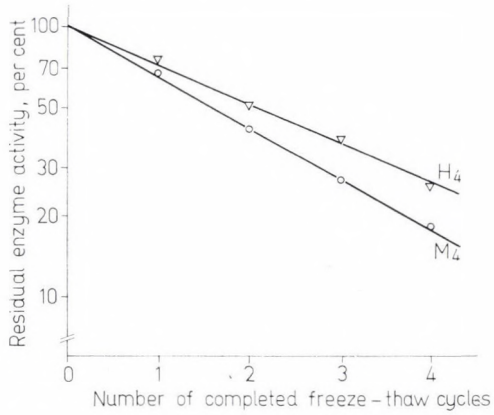

Fig. 1. Inactivation of a 1 mg/ml solution of LDH by repeated freezing and thawing in the presence of 0.001 M iodoacetamide. Each freeze-thaw cycle involved freezing for 2 hours at −18 °C and thawing for 30 minutes at 0 °C. Both LDH-M_4 (○) and LDH-H_4 (▽) were dissolved in 0.1 M sodium phosphate buffer, pH 7.0. The logarithm of per cent residual activity which has been determined at the end of each freeze-thaw cycle is plotted against the number of previously completed cycles

We suggest that even a quantitative estimation of the availability of the dissociated form of LDH is possible by an application of the iodoacetamide test. Quantitation seems to be fully justified when the reaction between protein and iodoacetamide takes place under the same conditions, as in Fig. 2. Since it is known (Südi, Khan, 1970) that the reaction between enzyme and reagent is initiated by thawing, the three treatments in Fig. 2A and the two treatments in Fig. 2B can only differ in the freezing-induced structural modifications of the protein. Therefore, the reciprocal of the iodoacetamide concentration with which 50 per cent inactivation was obtained by freezing and thawing (Fig. 2) could be taken as a measure of the extent of dissociation. Accordingly, we conclude that e.g. the availability of dissociated LDH-H_4 following freezing at —8 °C and at —76 °C is about 5, and 60 times lower, respectively, than after the standard freeze-treatment at —18 °C (Fig. 2A). Fig. 2B shows similar results obtained in the presence of 1 M NaCl. The marked difference between Figs 2A and 2B may be due to a specific effect of NaCl on the chemical reaction between iodoacetamide and

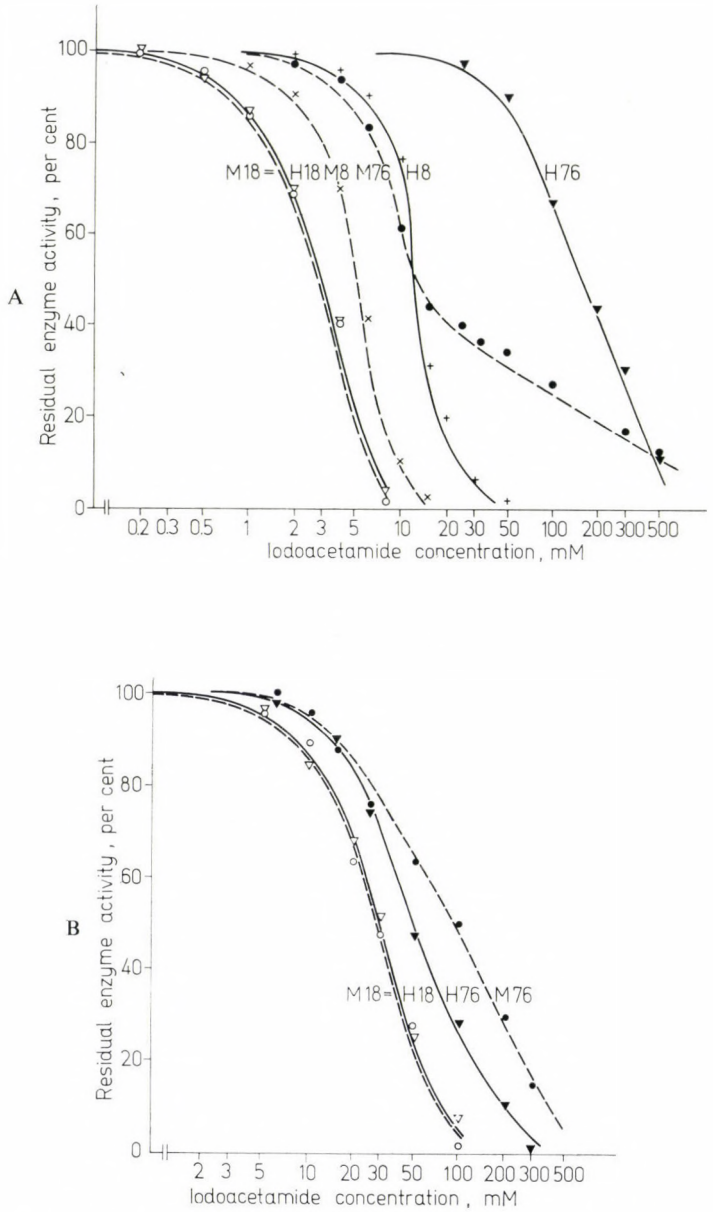

Fig. 2. Dependence on iodoacetamide concentration of the inactivation of frozen and thawed LDH. 1 mg/ml of LDH-H_4 (solid lines) or LDH-M_4 (broken lines) was dissolved in 0.1 M sodium phosphate buffer, pH 7.0, in the absence (A) or in the presence (B) of 1 M NaCl. Iodoacetamide was added in the indicated final concentration and 1-ml aliquots were frozen at $-8\,^{\circ}C$(+, ×), $-18\,^{\circ}C$ (▽, ○) or $-76\,^{\circ}C$(▼, ●). The duration of freezing was 24 h ($-8\,^{\circ}C$), 2 h ($-18\,^{\circ}C$), and 30 min ($-76\,^{\circ}C$), respectively. Residual enzyme activity was determined following thawing for 30 min at $0\,^{\circ}C$

enzyme protein, and not necessarily to a difference in the availability of dissociated LDH.

If the concentration of enzyme protein is raised above the standard 1 mg/ml level, the rate of re-formation of non-dissociating tetramers should be increased (Südi, Khan, 1970), thereby reducing the availability of the dissociated form of LDH for the iodoacetamide reaction. This prediction of our working hypothesis is substantiated by the results shown in Fig. 3. It is seen that the apparent availability of both LDH-s is markedly lowered by an about 3.5-fold increase in protein concentration. A comparison of Figs 3A and 3B again shows a marked effect of 1 M NaCl. These effects of sodium chloride will be further discussed in sections 2.6 and 3.

2. Ionic milieu. The experimental results described in this section were obtained by carrying out the three standard tests for transient dissociation with modifications of the ionic milieu in which the enzyme(s) were frozen and thawed. The ionic milieus chosen were known to have a marked effect on freeze-thaw hybridization (also as a source of further references, see Chilson et al., 1965; Markert, Massaro, 1968). We wanted to decide whether reduced hybridization was as a rule accompanied by higher residual activities in the low protein concentration test and in the iodoacetamide test. The results in Tables 1 to 3 and Fig. 4 show a satisfactory agreement between results obtained in the three tests. This supports the view that the three phenomena have a common basis in the transient dissociation of tetramers.

Most experiments were carried out both in the absence and in the presence of 1 M NaCl (Tables 1 to 3). For this we had two reasons. Firstly, we wanted to have a comparison with results obtained in other laboratories, in which about 1 M NaCl is an invariable component of the standard solution for freeze-thaw hybridization. Secondly, because of the possible involvement of eutectic temperatures in the freezing-induced modifications of protein structure; since the eutectic temperature for an aqueous solution of a mixture of NaH_2PO_4 and Na_2HPO_4 is —9.9 °C (Van den Berg, Rose, 1959), while that for NaCl is —21.5 °C (Lovelock, 1957).

2.1. pH of the sodium phosphate buffer. It has been suggested (Chilson et al., 1965) that sodium phosphate buffer favoured freeze-thaw hybridization because the pH of the eutectic solution was 3.6, in contrast to an eutectic pH of 7.5 for potassium phosphate buffers. Experiment 1 in Tables 1 to 3 shows that there must be additional effects of initial pH, which are most probably manifested after thawing, in the critical transient state. While at the standard initial pH value of 7.0 hybridization was not affected by the presence or absence of sodium chloride, both at pH 6 and pH 8 the omission of sodium chloride markedly reduced the extent of hybridization (Table 1). By variation of the pH of the solution over a pH range of 6 to 8, a marked variation was obtained in residual enzyme activity both in the low protein concentration test (Table 2) and in the iodoacetamide test (Table 3). However, since the residual activities in Tables 2 and 3 might be greatly affected by such specific factors as pH-dependence of the rate of inactivation by iodoacetamide, or of the "spontaneous" inactivation of the dissociated form of

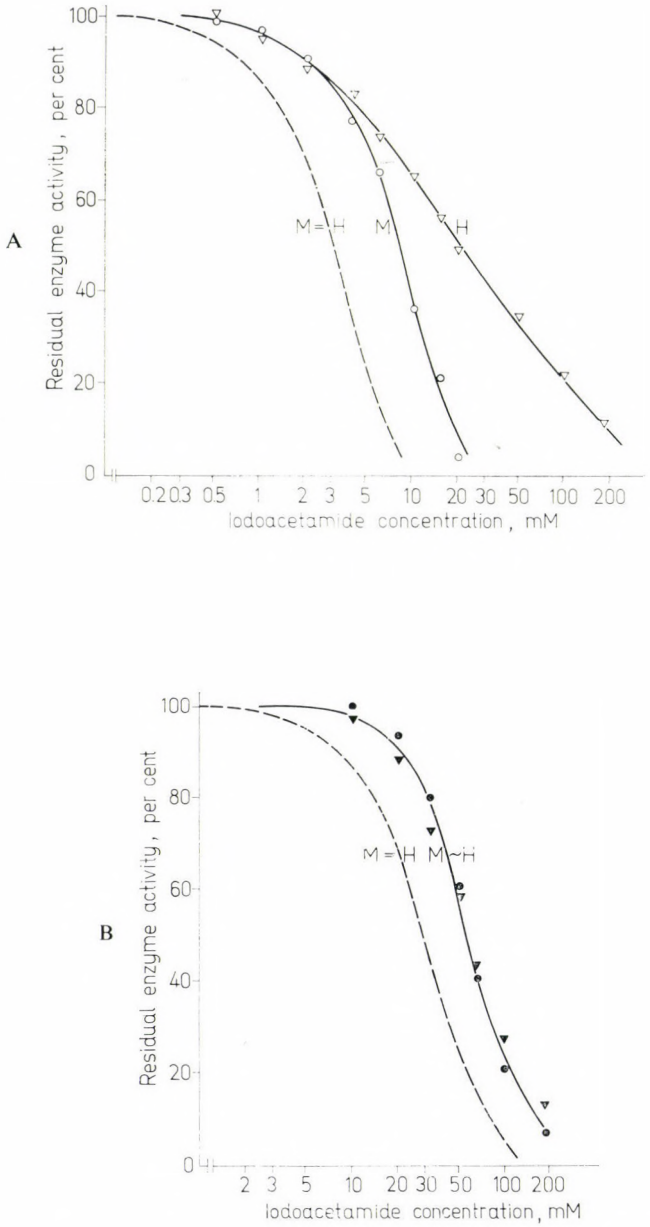

Fig. 3. Effect of protein concentration on the inactivation of LDH frozen and thawed in the presence of iodoacetamide. LDH was dissolved in the presence of the indicated concentration of iodoacetamide in 0.1 M sodium phosphate buffer, pH 7.0, in the absence (A) or in the presence (B) of 1 M NaCl. Protein concentration was 3.5 mg/ml (solid lines) of LDH-H_4 (▽ ▼), or of LDH-M_4 (○, ●); and 1 mg/ml (broken lines), respectively. Freezing and thawing were carried out as in standard test 3 (Materials and Methods)

the enzymes, one must not try to interpret the observed effects in terms of enzyme dissociation.

2.2. Potassium phosphate. As discussed above the extent of transient dissociation was expected to be reduced by substituting potassium phosphate for the standard sodium phosphate buffer. Experiment 2 (Tables 1—3) supports this assumption, and demonstrates that results obtained in the three tests show a satisfactory agreement. It is further seen that the apparent inhibition of dissociation could not be overcome by increasing ionic strength through the addition of 1 M KCl to the solution.

2.3. Tris buffer. It has been shown in different laboratories (Chilson et al., 1965; Massaro, 1967) that Tris buffer inhibited hybridization even in the presence of phosphate. It has also been pointed out (Südi, Khan, 1970) that neither transient inactivation nor increased reactivity towards iodoacetamide could be obtained by freezing and thawing in Tris buffer. Tables 1—3 show that similar, negative results were obtained in all three tests, irrespective of the presence or absence of 1 M NaCl. We should like to add that addition of an equal volume of Tris buffer at the commencement of thawing to solutions frozen in phosphate was ineffective in any of the tests. Conversely, no indication of dissociation was observed by adding sodium phosphate to thawing enzyme which had been frozen in Tris. Therefore, we suggest that whereas dissociation of LDH cannot be brought about by freezing in Tris buffer, the presence of Tris buffer in the thawed enzyme solution does not affect transient dissociation.

2.4. Ammonium sulphate. The inclusion of not more than 0.3 M $(NH_4)_2SO_4$ into the standard medium was found to inhibit transient dissociation in all three tests (Expt 4, Tables 1—3). Inactivation of LDH-M_4 by iodoacetamide is an apparent exception (Table 3). However, we should like to recall that a considerable reduction in the availability of M-subunits due to the presence of 0.3 M $(NH_4)_2SO_4$ could have passed undetected, since even 0.01 M iodoacetamide would have been sufficient for full inactivation in the absence of ammonium sulphate (Fig. 2). It should be added that, just like Tris buffer, ammonium sulphate had no effect if added at the commencement of thawing.

2.5. Substrates (and analogs). In the presence of 10 mM NAD plus 10 mM oxalate the ternary complex LDH-NAD-oxalate is the predominant form of the enzyme (unpublished observations). Table 1 shows that 10 mM NAD plus 10 mM oxalate completely inhibits hybridization, however, the inhibition is overcome by 1 M NaCl. From Table 3 it is seen that 10 mM NAD plus 10 mM oxalate markedly protect both enzymes against inactivation by iodoacetamide and that protection is reduced in the presence of 1 M NaCl. In contrast, in the low protein concentration test no effect was obtained either in the presence of 1 mM NAD plus 1 mM oxalate or in the presence of 5 mM NADH (Table 2).* The apparent contradiction between Table 2 and Tables 1 and 3 disappears when we consider the findings shown in Fig. 4. It is seen that both the rate and the final extent of reactivation are markedly increased if 100 μg/ml protein is frozen in the presence

* 10 mM NAD plus 10 mM oxalate could not be sufficiently diluted when measuring residual enzyme activity in this test.

Table 1

Freeze-thaw hybridization of LDH-M_4 and LDH-H_4 in different media

Deviations from the standard freeze-thaw hybridization treatment (Materials and Methods) are given under the heading Experimental conditions. The values give percentage distribution of the total protein among the five isozymic forms, as recovered from the DEAE-cellulose column

Experimental conditions	No NaCl added					
	Ratio M : H*	M_4	HM_3	H_2M_2	H_3M	H_4
Expt 1: 0.2 M Tris/HCl buffer, pH 7.5, substituted for sodium phosphate	48/52 (50/50)	48.2	1>***	1>	1>	51.8
Expt 2: pH of the 0.1 M sodium phosphate buffer adjusted to						
(a) pH 6.0	60/40 (60/40)	60.1	1>	1>	1>	39.9
(b) pH 7.0	63/35 (60/40)	18.8	6.4	52.4	11.0	11.3
(c) pH 8.0	50/50 (60/40)	45.5	0.9	4.6	4.5	44.5
Expt 3: +0.3 M $(NH_4)_2SO_4$	43/57 (50/50)	42.8	1>	1>	1>	57.2
Expt 4: +10 mM NAD +10 mM oxalate	48/52 (50/50)	47.5	1>	1>	1>	52.5
Expt 5: 0.1 M potassium phosphate, pH 7.0, substituted for sodium phosphate	59/41 (60/40)	59.0	1>	1>	1>	41.0

Experimental conditions	+1 M NaCl					
	Ratio M : H*	M_4	HM_3	H_2M_2	H_3M	H_4
Expt 1: 0.2 M Tris/HCl buffer, pH 7.5, substituted for sodium phosphate	43/57 (50/50)	41.3	0.9	0.8	0.9	56.2
Expt 2: pH of the 0.1 M sodium phosphate buffer adjusted to						
(a) pH 6.0	60/40 (60/40)	14.6	36.6	28.8	15.2	4.8
(b) pH 7.0	61/42 (60/40)	33.9	6.2	44.5	2.3	13.1
(c) pH 8.0	60/40 (60/40)	12.1	36.9	34.6	10.4	6.0
Expt 3: +0.3 M $(NH_4)_2SO_4$	—	—	—	—	—	—
Expt 4: +10 mM NAD +10 mM oxalate	65/35 (50/50)	9.7	41.9	43.4	5.0	1>
Expt 5: 0.1 M potassium phosphate, pH 7.0, substituted for sodium phosphate **	51/49 (60/40)	51.0	1>	1>	1>	49.0

* Ratio of "active enzyme proteins" calculated from the isoenzymes separated by chromatography; the ratios before freezing are given in brackets.

** NaCl was also replaced by 1 M KCl.

*** Traces; not determined precisely and neglected in the calculations.

Table 2

Inactivation of LDH at low protein concentration by freezing and thawing in different media

In the standard treatment protein in 0.1 M sodium phosphate buffer, pH 7.0, is frozen for two hours at −18 °C and thawed at 0 °C. Deviations from the standard treatment are indicated under the heading Experimental conditions. Values in the Table give per cent residual activity. There was no significant loss of activity in any of the unfrozen controls

Experimental conditions	4 μg/ml LDH-M_4		6 μg/ml LDH-H_4	
	No NaCl added	+1 M NaCl	No NaCl added	+1 M NaCl
Expt 1: 0.2 M Tris/HCl buffer, pH 7.5, substituted for sodium phosphate	100	100	100	100
Expt 2: pH of the 0.1 M sodium phosphate adjusted to				
(a) pH 6.0	45.4	—	70.2	—
(b) pH 7.0	35.7	16.0	5.8	0.0
(c) pH 8.0	17.9	—	0.0	—
Expt 3: +0.3 M $(NH_4)_2SO_4$	81.1	—	76.0	—
Expt 4: addition of substrates (analogs)				
(a) +1 mM NAD +1 mM oxalate	25.0	26.3	4.2	4.8
(b) +5 mM NADH	33.2	27.2	6.3	5.6
Expt 5: 0.1 M potassium phosphate, pH 7.0, substituted for sodium phosphate	57.8	—	89.6	—

Table 3

Inactivation of LDH due to freezing and thawing of 1 mg/ml enzyme protein in the presence of 0.1 M iodoacetamide

Variations in the composition of the solution in which the protein was frozen (for the standard treatment see Materials and Methods) are indicated under the heading Experimental conditions. Values in the Table give per cent residual activity. There was no significant loss of activity in any of the controls which had been frozen in the absence of iodoacetamide

Experimental conditions	LDH-M_4		LDH-H_4	
	No NaCl	+1 M NaCl	No NaCl	+1 M NaCl
Expt 1: 0.2 M Tris/HCl buffer, pH 7.5, substituted for sodium phosphate	100.0	98.5	105.2	97.6
Expt 2: pH of the 0.1 M sodium phosphate buffer adjusted to				
(a) pH 6.0	15.8	0	15.6	0
(b) pH 7.0	1.1	6.5	7.5	1.2
(c) pH 8.0	0.8	15.4	—	3.1
Expt 3: +0.3 M ammonium sulphate	13.0	—	103.5	—
Expt 4: +10 mM NAD +10 mM oxalate	33.0	9.0	67.2	13.0
Expt 5: 0.1 M potassium phosphate, pH 7, substituted for sodium phosphate	68.1	66.0*	75.5	75.5*

* 1 M KCl was substituted for NaCl.

of 5 mM NADH (Fig. 4A). It is further seen from Fig. 4B that NADH is also effective if it is only present during thawing. Similar results were obtained with both enzymes, but only above a threshold protein concentration of 20 to 40 μg/ml. Therefore, we suggest that the rate of re-formation of non-dissociating tetramers is increased in the presence of substrates. This would account for both inhibition of hybridization (Table 1) and protection against inactivation by iodoacetamide

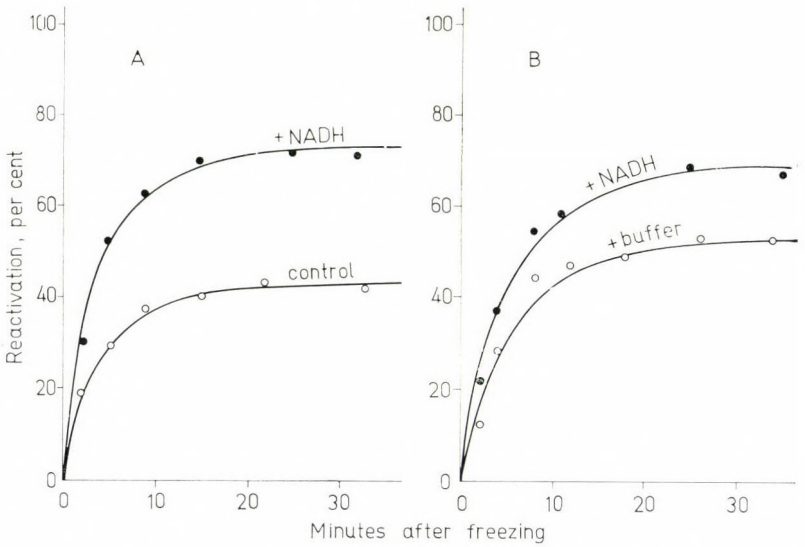

Fig. 4. Time course of reactivation of LDH in the presence of 5 mM NADH. To a 1-ml aliquot of a 200 μg/ml solution of LDH, 1 ml of a 10 mM solution of NADH (●), or 1 ml of buffer (○) was added; either before freezing (A) or at the termination of freezing (B). All solutions contained 0.1 M sodium phosphate, pH 7.0. Freezing was carried out by a 2-hour incubation at −18 °C. Initial activity was taken for 100 per cent, and the termination of the freeze-treatment for 0 minute

(Table 3). It is further conceivable that at very low protein concentrations (Table 2) reassociation is the limiting step in the re-formation of non-dissociating tetramers, and that the rate of this elementary reaction is not affected by the presence of substrates.

2.6. Sodium chloride. In Tables 1 to 3 experimental results obtained both in the absence and in the presence of 1 M NaCl are shown. It is seen that in most cases sodium chloride has a marked effect in each test. We should like to remark that in no experiment did we get a significant NaCl-effect by adding a concentrated solution of NaCl to frozen enzyme at the commencement of thawing. Consequently, we suggest that the observed effects of sodium chloride on the transient form of the protein are due to its presence during freezing. The registered manifestation of these effects seems to be an increased dissociation of the protein upon thawing. This is indicated by an apparent counteraction of the inhibitory effects

of both non-optimal pH (Expt 1, Tables 1 and 2) and of substrates added to the standard phosphate buffer (Expt 5, Tables 1 and 3).

Some of the observed effects of sodium chloride might be specific to a given test. This suggestion has been made above (Section 1) to account for the marked effect of added NaCl on the apparent availability of dissociated LDH in the iodoacetamide test (Fig. 2). It is further possible that the clear-cut hybridization-promoting effects of added NaCl at non-optimal pH values and in the presence of

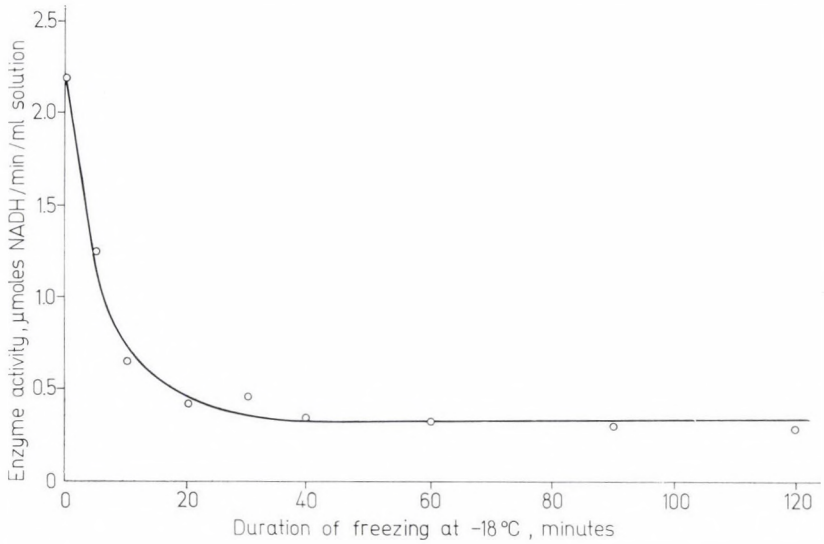

Fig. 5. Maximal reactivation of 12 μg/ml of LDH-H_4 following freezing for 5 to 120 minutes. Freezing and thawing were carried out under the standard conditions (Materials and Methods), except that the duration of freezing at —18 °C was varied

substrates are at least partly due to non-preferential reassociation (Freier, Bridges, 1964; Markert, Whitt, 1968) which is being favoured by freezing in the presence of 1 M NaCl.

Results obtained by freezing LDH in the presence and in the absence of 1 M NaCl at temperatures lower than —70 °C are discussed in more detail in Section 3. We should like to note in advance that freezing-induced dissociation of LDH is markedly affected by the presence of 1 M NaCl even if the temperature of freezing is much lower than the eutectic temperature of aqueous sodium chloride.

3. Duration and temperature of freezing. Table 4 and Figs. 5 and 6 demonstrate that transient dissociation of LDH is also affected by the duration of freezing at —18 °C. Results obtained in the three tests were very similar in that maximal effect was obtained following freezing for 30 to 60 minutes. It should be added that the duration of freezing required for a maximal effect did not detectably vary when the ionic milieu was varied over the range indicated in Tables 1—3.

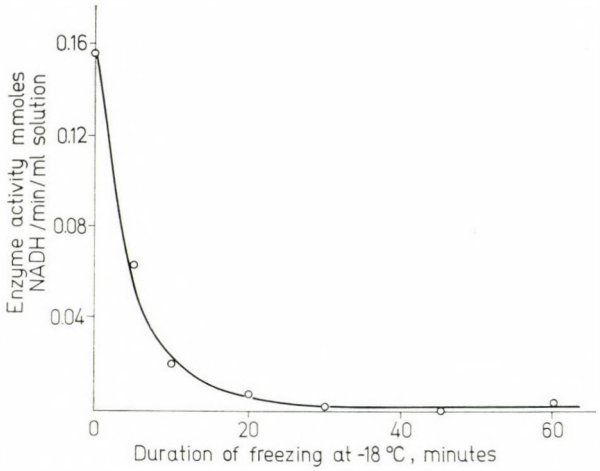

Fig. 6. Inactivation of 1 mg/ml LDH-H_4 by freezing in the presence of 0.1 M iodoacetamide for 5 to 60 minutes. Freezing and thawing were carried out under the standard conditions (Materials and Methods), except that the duration of freezing at −18 °C was varied

Table 4

Freeze-thaw hybridization of LDH-M_4 and LDH-H_4 with the standard procedure except that the duration of the freeze-treatment was varied

The solutions, 5-ml aliquots of which were frozen, contained 2 mg/ml LDH-M_4 + 2 mg/ml LDH-H_4 and 1 M NaCl in 0.1 M sodium phosphate buffer, pH 7.0. Values in the Table give per cent distribution of the total protein among the five tetrameric forms, as recovered from the DEAE-cellulose column

Duration of the freeze-treatment	Ratio M/H*	Tetrameric form				
		M_4	HM_3	H_2M_2	H_3M	H_4
0 minute	48.2/51.8	48.2	0	0	0	51.8
5 minutes	49.5/50.5	49.5	0	1>**	0	50.5
10 minutes	47.7/52.3	42.3	0.9	7.9	3.0	45.9
30 minutes	49.2/50.8	15.7	5.4	47.8	22.5	8.6
60 minutes	47.7/52.3	4.0	10.0	62.5	19.7	3.8

* Calculated from the separated isoenzymes; the initial ratio of "active enzyme proteins" was 50.0/50.0.

** Traces; not determined precisely and neglected in the calculations.

Figure 7 supplies additional proof that sufficiently prolonged uninterrupted freezing is not equivalent to the repeated application of a number of short freeze-treatments. It is seen that hybridization is negligible after ten freeze-thaw cycles involving freezing for 10 × 5 minutes (Fig. 7A), while almost random reassociation of the polypeptide chains can be achieved by freezing for 50 minutes without interruption (Fig. 7B).

Some results obtained in the iodoacetamide test by freezing at —8 °C and at —76 °C have already been shown in Fig. 2. We should like to add the following remarks to the foregoing discussion of these results:* (a) The duration of freezing required for maximal effect depended on the temperature of freezing. For instance, when freezing was carried out in an acetone–dry ice bath, 5 minutes were sufficient to bring about a maximal effect in the three tests. In complete agreement with the iodoacetamide test (Fig. 2), both the low protein concentration

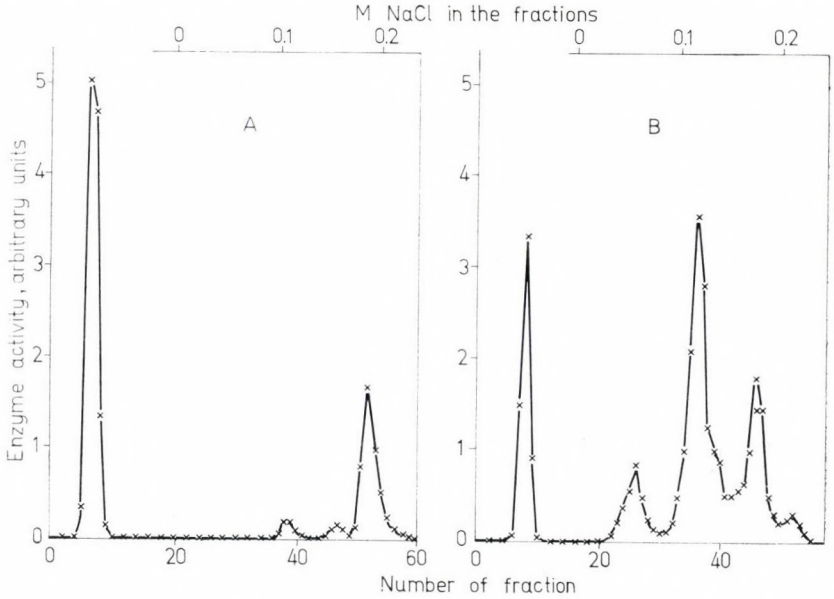

Fig. 7. Chromatographic isoenzyme patterns obtained by the repeated application of a number of short freeze-thaw cycles (A), and by only one, but prolonged freeze-treatment (B). The solutions contained 1.84 mg/ml of LDH-M_4 plus 1.51 mg/ml of LDH-H_4 in 0.1 M sodium phosphate buffer, pH 7.0. One 5-ml aliquot of this solution was carried through 10 freeze-thaw cycles, each involving freezing for 5 min at —18 °C followed by thawing for 5 min in a room-temperature water bath (A). Another 5-ml aliquot of the solution was frozen for 50 min at —18 °C and then thawed (B). The upper abscissa shows the concentration of NaCl in the effluent from the column

test and the hybridization test also indicated reduced dissociation following freezing at —8 and —76 °C. It should be noted that our freeze-thaw treatments in acetone–dry ice are equivalent to the "quick freeze–quick thaw" treatment of Chilson et al. (1965), and that these authors have also reported reduced hybridization under such conditions. (b) The relative effectiveness of freezing at —8, —18 and —76 °C was very similar in the three tests, both in the absence and in the presence of 1 M NaCl. For instance, in the absence of sodium chloride no hybridization was obtained by freezing either at —8, or at —76 °C.

* A more detailed description of these results will be published elsewhere.

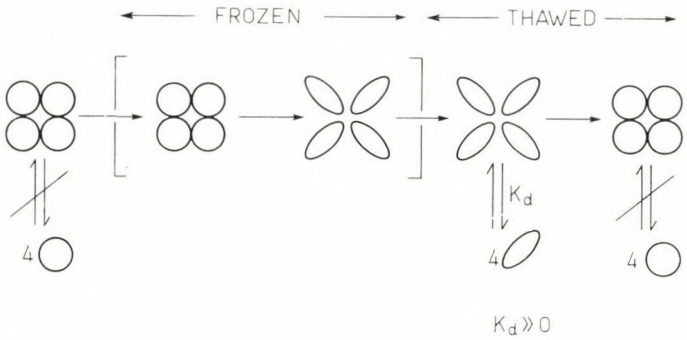

Fig. 8. Postulated changes in protein structure in the course of freezing and thawing

Conclusions

These results substantiate our working hypothesis according to which LDH tetramers are transiently dissociated by appropriate freeze-thaw treatments. The evidence presented is necessarily indirect, but the predictions of this hypothesis are satisfactorily fulfilled by observations made in three independent tests.

However complex the effects of any modification of the standard freeze-thaw treatment may be, a simple model can be easily constructed which would account for the presented observations (Fig. 8). According to the model, the freeze-thaw cycle starts from, and leads to, the non-dissociating tetramer. During incubation in the frozen state (shown between brackets in Fig. 8) the steric structure of the polypeptide chains is slowly altering. It is this altered protein which will go into solution upon thawing. By thawing, a transient state of the protein is established which can be described by a real dissociation-association equilibrium. However, this equilibrium is not a stable one, because thermodynamic considerations again favour the non-dissociating tetrameric state, and this will be re-formed with a characteristic half-time.

The main features of this model can be summarized in the following postulates: (a) In the normal tetramer, dissociation is a prohibited structural transition (Südi, Khan, 1970). (b) A structural change (distortion) does occur in the protein while the solution is frozen, however, dissociation itself only takes place upon thawing. (c) In the transient dissociated state the distorted tetramers can be characterized by an equilibrium between an associated and a dissociated (probably monomeric) form.

The final state to which the postulated structural rearrangement in the frozen solution leads would be determined by the ionic milieu. We have shown above that in this way one can account for the effects of sodium chloride, potassium phosphates, Tris buffer, and ammonium sulphate. We have also shown that when the temperature of freezing is —18 °C, about 30 to 60 minutes are required for the completion of the postulated structural rearrangement. It is suggested that this time requirement is a consequence of the slow rearrangement of the structure

of frozen water. Similarly, the final state of the protein in the frozen solution would be dependent on the temperature of freezing because of differences in the equilibrium structure of the frozen solution.

The postulated dissociation constant of the transiently "distorted" tetramers is, accordingly, determined by the distortion which has taken place. Clearly, the apparent availability of the dissociated (probably monomeric) form of the protein would be determined by both this dissociation constant and the rate of re-formation of non-dissociating tetramers. The overall rate of this structural rearrangement was shown to be characterized by a half-time of about 5 to 10 minutes (Südi, Khan, 1970). In this paper we have shown that in the presence of substrates the rate of reversal of the structural distortion is increased with no apparent effect of substrates on the "forward" structural changes which take place in the frozen state.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (2), pp. 177—182 (1970)

Simulation by Analogue Computer of the Reaction Catalyzed by Alcohol Dehydrogenase

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(Received February 3, 1970)

The Theorell–Chance model of the reaction catalyzed by alcohol dehydrogenase is simulated in both directions with analogue and digital computer techniques. In the pre-steady state there is a short period during which the concentrations of the enzyme-reduced coenzyme and the enzyme-oxidized coenzyme complexes, respectively, rise abruptly. At the end of the pre-steady state the values of both complexes are constant. The advantages of this simulation are discussed.

The mathematical modelling of enzymatic reactions offers a possibility to simulate processes which vary in time. With a given mechanism it is possible to examine by the use of differential equations the changes in the concentrations of the materials which participate in the reactions during a given time period. The analogue computer can be applied for studying processes described by differential equations.

Hommes (1962) was the first to describe the simulation of simple enzyme-substrate changes in the steady and pre-steady state. Walter and Morales (1964) reported the simulation of enzyme reactions with more than one substrate. Theorell et al. (1967) determined the rate constants of the NADH*–LADH binding reaction with a "stopped flow" apparatus. They used a digital IBM 1401 computer for finding the combination of the four rate constants that fitted best into the average of the experimental curves.

The purpose of the present investigation was to simulate the whole ADH catalyzed reversible reaction as a first step towards further studies on effectors and modulators. The simulated reaction was the following:

$$E + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ER \tag{1}$$

$$ER + ald \underset{k_3}{\overset{k_3}{\rightleftharpoons}} alc + EO \tag{2}$$

$$EO \underset{k_1}{\overset{k_2'}{\rightleftharpoons}} E + O \tag{3}$$

* Abbreviations: ADH = alcohol dehydrogenase; LADH = liver alcohol dehydrogenase; NADH or R = reduced coenzyme; NAD or O = oxidized coenzyme; E = enzyme; ald = aldehyde; alc = alcohol.

For this reaction mechanism differential equations can be written with seven variables:

$$\frac{dR}{dt} = - k_1[E][R]\, k_2[ER] \tag{4}$$

$$\frac{dO}{dt} = - k_1'[E][O] + k_2'[EO] \tag{5}$$

$$\frac{dER}{dt} = + k_1[E][R] - k_2[ER] - k_3[ER]\,[ald] + k_3'[EO][alc] \tag{6}$$

$$\frac{dEO}{dt} = + k_1'[E][O] - k_2'[EO] + k_3[ER][ald] - k_3'[EO][alc] \tag{7}$$

$$\frac{dald}{dt} = - k_3[ER][ald] + k_3'[EO][alc] \tag{8}$$

$$\frac{dalc}{dt} = + k_3[ER][ald] - k_3'[EO][alc] \tag{9}$$

$$E = E_0 - ER - EO \tag{10}$$

Methods

The numerical values of the reaction velocity coefficients were measured and calculated by Theorell and Chance (1951) and Theorell and McKinley-McKee (1961). According to their data we used the following values given for pH 9:

$$k_1 = 5.7 \ \ sec^{-1}\ \mu M^{-1};\ k_2 = 4.9\ sec^{-1};\ k_3 = 0.062\ sec^{-1}\,\mu M^{-1};$$
$$k_1' = 0.51\ sec^{-1}\ \mu M^{-1};\ k_2' = 8.1\ sec^{-1};\ k_3' = 0.01 \ \ sec^{-1}\,\mu M^{-1}.$$

The initial coenzyme and substrate concentrations R_0 and ald_0, respectively, as well as O and alc_0 were 10^{-4} M and the total enzyme concentration was 10^{-8} M.

The analogue computer was a model, constructed by the Chair of Electric Power Plants and Networks. The digital computer was a MINSK model.

Results and Conclusions

Fig. 1 illustrates the circuit diagram of the analogue computer elements concerned in the process which starts with the binding of the reduced coenzyme (R). Beginning the reaction from the reversed side, i.e. from the binding of the oxidized coenzyme (O), the system of the differential equations does not change

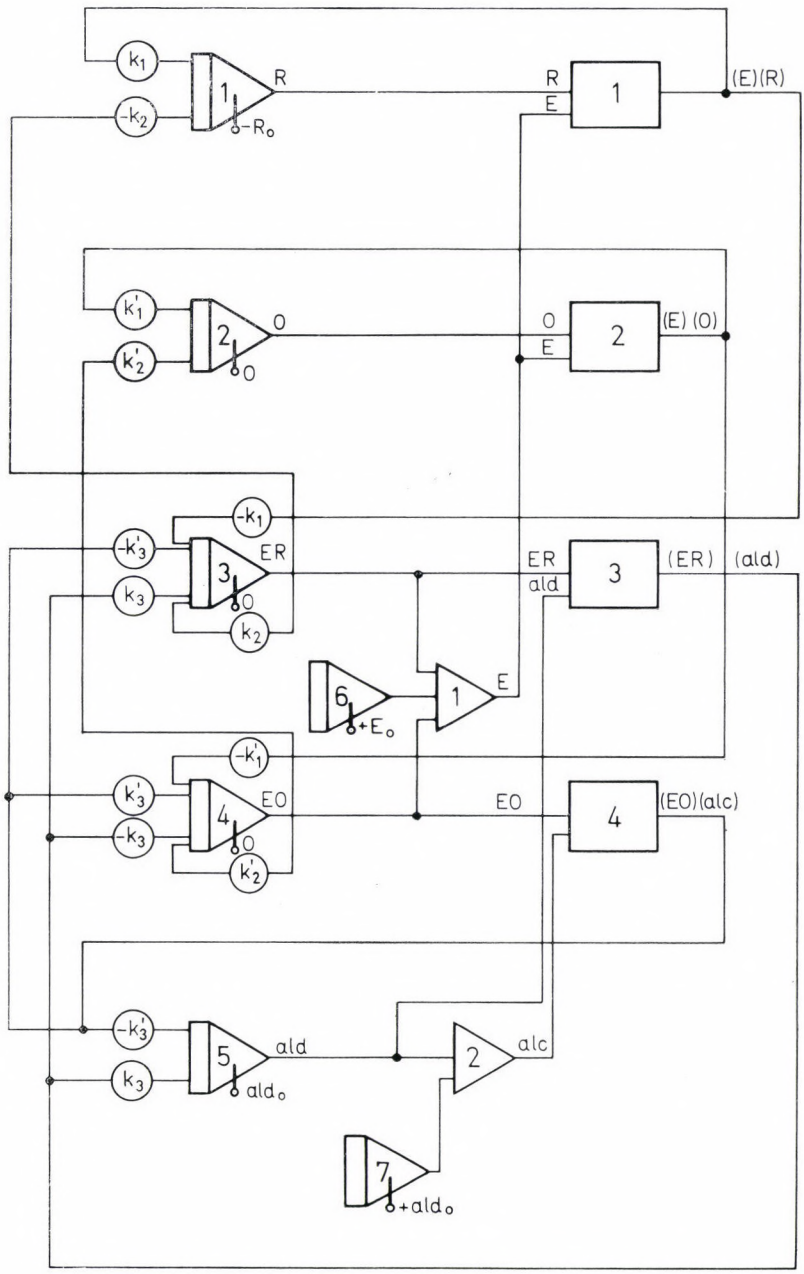

Fig. 1. Circuit diagram of the analogue computer for the reaction equations (1)—(3)

at all, the programming of the analogue computer differs only in the input of the initial conditions. Instead of the initial values R_0 and ald_0, O_0 and alc_0 are to be put in, while the values of R_0 and ald_0 are zero. In the solution of this total switching program difficulties were encountered, which originated from the characteristics of the analogue computer and the numerical values of the constants.

In the pre-steady state, the concentrations of the reduced and oxidized coenzyme as well as those of the aldehyde and alcohol can be regarded practically

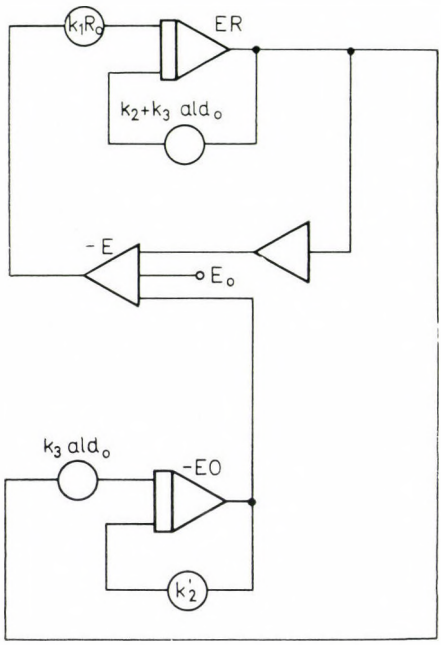

Fig. 2. Circuit diagram for the pre-steady state, starting from the ER side. If starting from the EO side read EO instead of ER, $k_1'O_0$ instead of k_1R_0; $k_2' + k_3'\,alc_0$ instead of $k_2 + k_3\,ald_0$; $k_3'\,alc_0$ instead of $k_3\,ald_0$; $-ER$ instead of $-EO$; k_2 instead of k_2'

constant and therefore the reaction equations will be more simple. Starting the sequence of the reaction equations from the reduced coenzyme and aldehyde $R = R_0$, $ald = ald_0$, alc and O are zero. Therefore only three equations with three variables remain:

$$\frac{dER}{dt} = k_1[E]R_0 - k_2[ER] - k_3[ER]ald_0 \tag{11}$$

$$\frac{dEO}{dt} = k_2'[EO] + k_3[ER]\,ald_0 \tag{12}$$

$$E = E_0 - ER - EO \tag{13}$$

The simulation of the equations on the analogue computer was performed according to the circuit diagram illustrated in Fig. 2, the recorded curves are shown in Fig. 3. The reversed reactions in the pre-steady state under similar conditions,

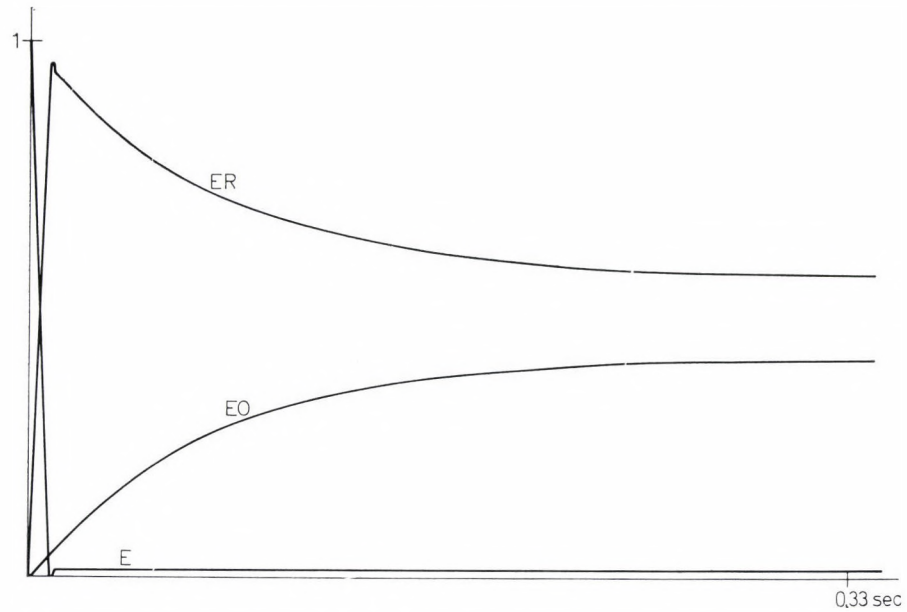

Fig. 3. Pre-steady-state curves of the process, starting from the ER side. $E_0 = 10^{-8}$ M; $R_0 = 10^{-4}$ M; $ald_0 = 10^{-4}$ M

i.e. when $O = O_0$, $alc = alc_0$, R and ald are zero, are described by the following equations:

$$\frac{dEO}{dt} = k_1'[E]O_0 - k_2'[EO] - k_3'[EO]alc_0 \tag{14}$$

$$\frac{dER}{dt} = -k_2[ER] + k_3'[EO]alc_0 \tag{15}$$

$$E = E_0 - ER - EO \tag{16}$$

The circuit diagram and the curves obtained on the analogue computer are illustrated in Figs 2 and 4. From the graphical illustrations it is evident that also in the pre-steady state there is a short period during which the concentration of one complex rises abruptly to a high value. At the end of the pre-steady state the values for both complexes (ER and EO) are constant. The same results are obtained with a digital computer.

To sum up the advantages of the simulation of the reaction catalyzed by ADH it can be established that with the aid of the analogue computer, parts of the reaction can be illustrated, which are not directly measurable. It is possible to follow the changes of more than one substance and also the changes of the supposed intermediates. The effects of changing the concentration of any param-

eter on the enzyme activity can be directly investigated. The aim of the simulation is to prove or to disprove a supposed mechanism. According to the above observations the changes of the individual parameters in the graphical illustrations are in accordance with the Theorell—Chance mechanism. The aim of our further work in modelling is to elucidate the effects of activators and inhibitors on dehydrogenase activity.

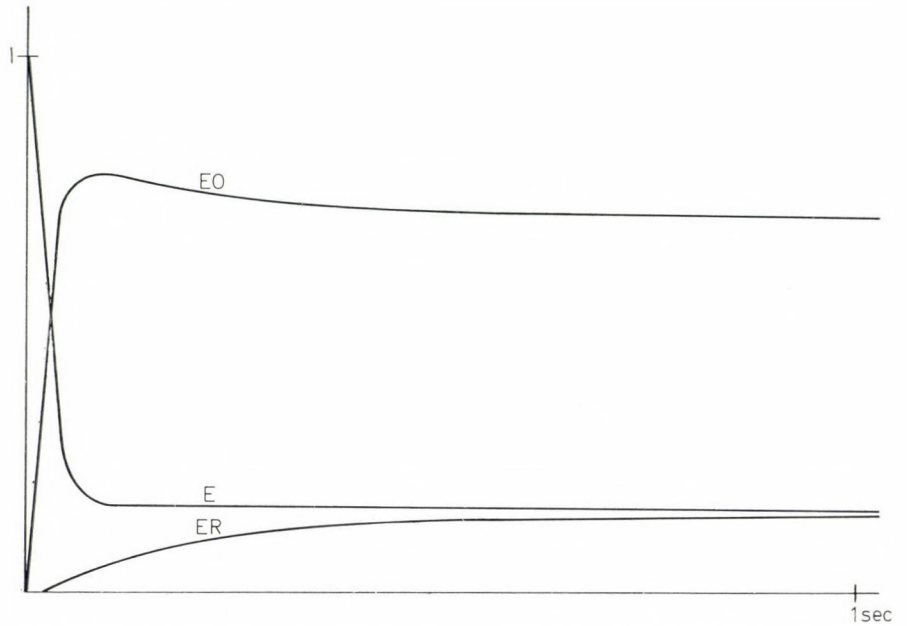

Fig. 4. Pre-steady-state curves of the process starting from the EO side. $E_0 = 10^{-8}$ M; $O_0 = 10^{-4}$ M; $alc_0 = 10^{-4}$ M

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (2), pp. 183—190 (1970)

Effect of Ribonuclease on Pigeon Liver Ribosomal Ribonucleic Acids

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(Received February 11, 1970)

Pigeon liver ribosomes were treated with various amounts of pancreatic RNase, and the state of their RNAs was studied by sucrose density gradient centrifugation. We have found that a complete inhibition of RNase is necessary before the extraction of RNAs, because even minute quantities of RNase may result in degradation of rRNA when it is liberated from ribosomal structure. Two types of macromolecular products arising from *28S* RNA were found on RNase treatment: *a)* A degradation product with a sedimentation constant between *26S* and *27S*. This product is formed if intact ribosomes are treated with RNase. *b)* Another product with a sedimentation constant of about *18S*, which is formed only or preferentially when the enzyme acts on RNAs which are liberated from ribosomal structure.

Introduction

The size and complexity of ribosomes make their structural investigation difficult. While the role of some protein components has recently been established, comparatively little is known about the contribution of RNA components to ribosomal structure and specific function.

One way to obtain information concerning the structural and functional role of rRNA* is to examine the changes following nucleolytic digestion of ribosomes. In the present work the effect of RNase treatment on pigeon liver ribosomes has been studied. One of the methodological problems of such investigations is the removal of RNase at the desired time. In addition it is very difficult to prove that RNase had been completely removed.

Attempts were made to remove exogenously added RNase by precipitating ribosomes with magnesium acetate. It will be shown that although this method can be used to remove the bulk of RNase from ribosomes, small quantities of residual RNase may cause further degradation of rRNA during and after extraction of rRNA from ribosomes. The effect of RNase on ribosomes can only be followed if all traces of RNase are inactivated before liberating rRNA from the ribosomal structure. In the present paper it will be demonstrated that diethyl pyrocarbonate, a very potent inhibitor of RNase, can be used for this purpose.

* Abbreviations used: DEP: diethyl pyrocarbonate, RNase: pancreatic ribonuclease (EC.2.7.7.16), rRNA: ribosomal RNA, SDS: sodium dodecylsulfate.

Materials and Methods

All reagents used were reagent grade and were purchased from Reanal, Budapest, Hungary, except sodium dodecyl sulfate which was a preparation of Fluka and DEP which was a product of the Bayer Werke, Leverkusen, Germany.

Four times crystallized bovine pancreatic RNase (Reanal, Budapest) was used in this work without further purification. The nominal activity of this enzyme preparation was 1900 units/mg protein.

Ribosomes were prepared in the following way: Pigeons were killed by decapitation and their livers were removed and immersed immediately into ice cold physiological saline solution. The excised livers were homogenized with three volumes of standard buffered sucrose solution (0.05 M tris-HCl, pH 7.6; 0.025 M KCl; 0.005 M magnesium acetate, 8.5 per cent sucrose) in a teflon-glass Potter—Elvehjem type tissue grinder. After the removal of nuclei and mitochondria by centrifugation in the Spinco rotor No. 30 at 11 000 rpm for 15 minutes, 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. 40 or No. 50 at maximum speed for 120 or 90 minutes, respectively, using the Spinco ultracentrifuge Model L or L–2. The ribosomal pellets were suspended in the standard buffered solution and sedimented again. The washed pellets were suspended in the same solution. The resulting suspension was either used immediately or stored at —4 °C and used within a week.

RNase activity was measured by the method of Schucher and Hokin (1954), using purified commercial yeast RNA as substrate.

Determination of RNA content was carried out essentially by the method of Littlefield et al. (1955). Aliquots of samples were hydrolyzed for one hour at room temperature in 1 N NaOH and then acidified by the addition of 3 N HCl to a final concentration of 1 N HCl. The optical density of the acidified solution was measured in a Beckman DU spectrophotometer and RNA content was calculated on the basis of the extinction coefficient $OD_{260}^{mg} = 34.2\ ml^{-1}\,cm^{-1}$.

RNA was prepared for sedimentation analysis by the method of Kurland (1960). To the samples containing ribosomes corresponding to about 0.7 mg rRNA, EDTA and SDS were added to a final concentration of 0.01 M and 1 per cent, respectively. The mixtures were incubated at 37 °C for 5 minutes and were diluted to a final concentration of 0.15—0.3 per cent SDS with 0.01 M sodium acetate buffer, pH 5.2. The samples were layered on a 5—20 per cent (w/w) linear sucrose gradient containing 0.01 M sodium acetate buffer, pH 5.2 and 0.15 per cent, SDS and centrifuged for 16 hours at 24 000 rpm in the rotor No. SW–25.1 of the Spinco ultracentrifuge model L or L–2. After centrifugation the tubes were punctured and 30—33 fractions were collected from each tube. The optical density of the fractions was measured in a Beckmann DU spectrophotometer. Some experiments were evaluated by the ISCO model UA–2 density gradient analyzer.

RNase treatment of ribosomes was performed at 0 °C for 10 minutes. The composition of the reaction mixture was the following: 0.05 M tris-HCl, pH 7.6; 0.025 M KCl; 0.005 M magnesium acetate; 8.5 per cent sucrose; ribosomes cor-

responding to 8 mg rRNA/ml; 0.4—0.8 μg crystalline pancreatic RNase per ml. The reaction was stopped by precipitating ribosomes with magnesium acetate essentially by the method of Takanami (1960). The magnesium acetate concentration of the reaction mixture was raised to 0.07 M by the addition of 1 M magnesium acetate. The suspension was centrifuged at 20 000 rpm for 10 minutes. Ribosomes were suspended in a solution containing 0.05 M tris-HCl, pH 7.6; 0.025 M KCl; 8.5 per cent sucrose, and centrifuged at 20 000 g for 20 minutes. Ribosomes were resuspended in the same solution. Throughout these procedures the temperature of the suspensions was kept at 0 °C.

Inhibition of RNase by DEP before the liberation of rRNA from ribosomes was performed in the following way: samples containing ribosomes corresponding to about 0.7 mg rRNA in 0.2 ml were shaken with 0.01 ml DEP for 10 minutes at 0 °C. The removal of non-reacted DEP seemed to be unnecessary.

Results

Pigeon liver ribosomes were treated with pancreatic RNase at 0 °C for 10 minutes and then the ribosomes were precipitated by the addition of magnesium acetate as described in Methods. The RNase activity of the precipitated ribosomes was checked by the method of Schucher and Hokin (1954). RNase activity was either absent or negligible (Table 1).

Table 1

RNase content of ribosomes treated with RNase and precipitated with magnesium acetate

Ribosomes were treated with the indicated amounts of RNase and precipitated with magnesium acetate as described in Methods. The residual RNase activity was measured by the method of Schucher and Hokin (1954), using the following reaction mixture: 20 μmoles of tris-HCl, pH 7.6; 10 μmoles of KCl; 2 μmoles of magnesium acetate; 100 μmoles of sucrose; 2.5 mg RNA as substrate, 20 μmoles of phosphate buffer, pH 7.4; ribosomes corresponding to 0.8 mg rRNA in a final volume of 1 ml. The amount of RNase was calculated from the concentration of acid soluble fraction by means of a calibration curve

Added RNase, pg/ml	Time of incubation, min	Acid soluble fraction, OD_{260}	RNase found after precipitation, pg/reaction mixture
0	0	0.225	1.7
	10	0.270	
	20	0.305	
400	0	0.240	<1.0
	10	0.245	
	20	0.240	
3200	0	0.215	4.0
	10	0.340	
	20	0.415	

In order to study the state of RNA of the ribosomes treated in the above way, rRNA was liberated by the addition of EDTA and SDS according to the method of Kurland (1960).

The action of RNase on rRNA may depend upon the state of rRNA: certain sites of rRNA can be attacked by the enzyme if rRNA is in a free state, but may be protected if rRNA is associated with ribosomal proteins. Therefore, in studies on the action of RNase on rRNA the enzyme must be completely removed or inhibited before rRNAs are liberated for the analyses. If this is not done, subsequent degradation of free RNA may falsify the characteristics of RNA degradation in the ribosomes.

Although it is widely believed (for review see Mc Conkey, 1967) that SDS stops the action of RNase, it has recently been shown that plant RNAs isolated in the presence of SDS as the only nuclease inhibitor were obviously degraded by the ribonucleases present (Solymosy et al., 1968). This contradiction can probably be attributed to the different methods used for the detection of RNase activity. Most of the methods used for measuring the activity of RNase are based on the measurement of the acid soluble fraction. Indeed, using the method of Schucher and Hokin (1954) complete inhibition of RNase activity was found by

Table 2

The inhibitory effect of SDS on RNase

RNase activity was measured by the method of Schucher and Hokin (1954) using the following reaction mixtures: 0.05 μg RNase, 20 μmoles of tris-HCl, pH 7.6; 10 μmoles of KCl; 2 μmoles of magnesium acetate; 100 μmoles of sucrose; 2.5 mg RNA as substrate, 20 μmoles of phosphate buffer, pH 7.4 and SDS in the indicated concentration

SDS mg/ml	OD_{260} in 20 min
0	1.08
10	0.03

SDS (Table 2). When, however, the inhibitory effect of SDS on RNase activity was checked by sucrose density gradient analysis, whereby high molecular weight degradation products can also be detected, very little, if any, inhibition of RNase activity by SDS was found. A very small amount of RNase caused extensive degradation of the *28S* RNA in spite of the presence of SDS (Fig. 1). It should be noted that the peak of *28S* RNA sharply decreases and the quantity of *18S* RNA apparently increases.

As SDS does not inhibit the degradation of RNA into products of high molecular weight, all traces of RNase have to be completely inhibited before any

attempt is made to liberate undegraded RNAs from ribosomes by the use of SDS. Rosén and Fedorcsák (1966) have shown that DEP completely inactivates RNase. This compound was successfully applied by Solymosy et al. (1968) in the isolation of plant RNAs. The inhibitory effect of DEP was tested in our system and used for studying RNA profiles of RNase treated ribosomes. In these experiments

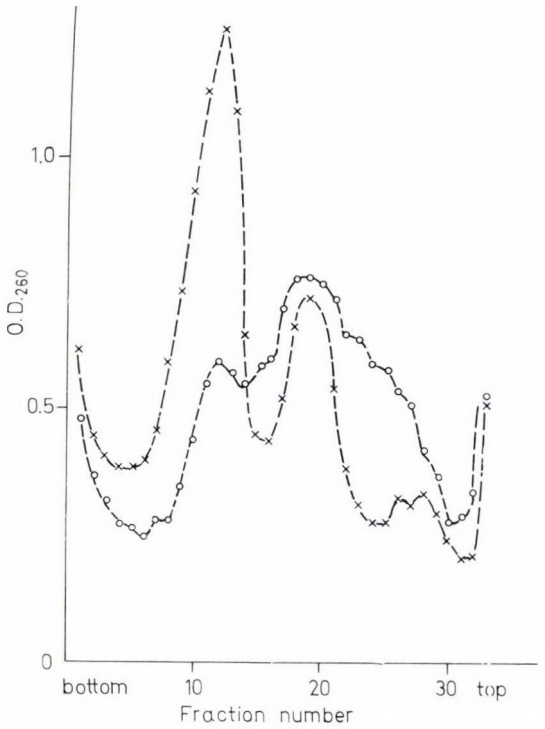

Fig. 1. Effect of RNase on rRNA in the presence of EDTA and SDS. rRNA was liberated from ribosomes by the addition of EDTA and SDS as described in Methods. × — ×: control rRNA; ○ — ○: rRNA treated with 0.04 μg/ml RNase during the liberation of rRNA

RNase action was destroyed by the addition of DEP before liberating RNAs from the ribosomal structure by SDS.

When DEP is used to destroy RNase activity, it becomes clear that RNase treatment of ribosomes results in the degradation of both *28S* and *18S* RNA (Fig. 2). In this case no increase in *18S* material can be detected. Upon RNase treatment of the ribosomes the peak of *28S* RNA is shifted to a zone with a lower sedimentation constant.

Essentially the same RNA profiles as shown in Fig. 2 were obtained when RNase-treated ribosomes were first precipitated with magnesium acetate and DEP was added afterwards.

The results summarized in Table 1 do not prove unambiguously that all RNase was removed by precipitating RNase-treated ribosomes with magnesium acetate. Therefore an attempt was made to determine whether traces of RNase, if any, remaining on the ribosomes after precipitation may cause further degradation of rRNA. Ribosomes were incubated in a buffered solution containing 0.8 μg/ml RNase, precipitated with magnesium acetate and washed. No RNase activity associated with the ribosomes could be detected by the method of Schucher

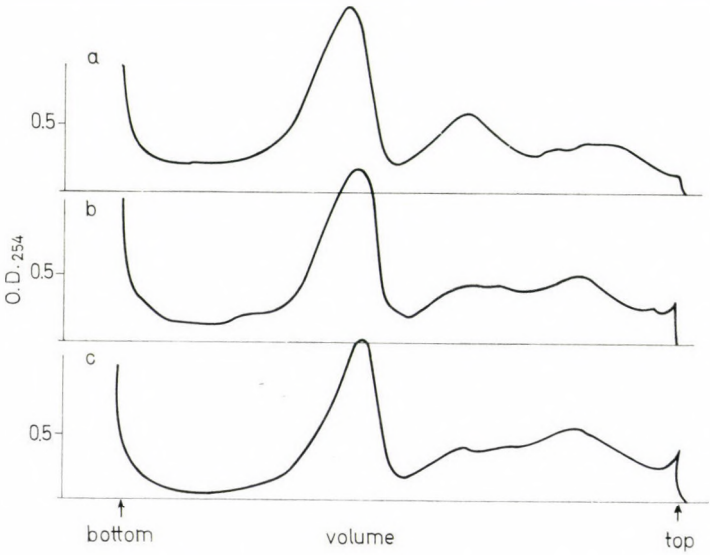

Fig. 2. Sucrose density gradient profile of RNA from ribosomes treated first with RNase and then with DEP. Ribosomes were treated with *a)* 0.0; *b)* 1.4; *c)* 2.8 μg/ml RNase at 0 °C for 10 minutes. DEP was added to destroy residual RNase activity. The liberation of RNA and centrifugation were performed as described in Methods. The samples were evaluated by ISCO gradient analyzer

and Hokin (1954). Significant differences were found, however, between the RNA profiles obtained with or without DEP treatment preceding the liberation of RNAs by SDS (Fig. 3). When DEP treatment was omitted, incubation of ribosomes in the presence of EDTA and SDS resulted in further degradation of the RNA as indicated by an increase of the *18S* material. This is characteristic of the degradation which occurs in the presence of EDTA and SDS.

Thus, incubation in the presence of EDTA and SDS followed by the analysis of high molecular weight degradation products allows the detection of very small quantities of RNase that cannot be detected by measuring the acid soluble fraction. In accordance with Solymosy et al. (1968), we have found that inhibition of RNase by DEP is an essential step for obtaining reliable data concerning the state of rRNA.

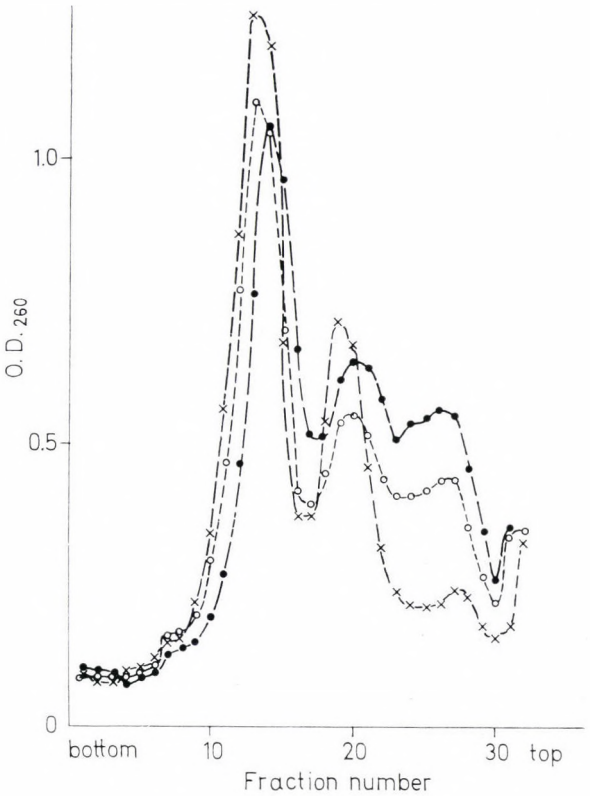

Fig. 3. Sucrose density gradient profile of RNA from ribosomes treated with RNase and precipitated with magnesium acetate, with and without DEP treatment. (1) ○—○ RNA from ribosomes, treated with 0.8 μg/ml RNase for 10 minutes at 0 °C and precipitated with magnesium acetate. DEP was added to destroy residual RNase activity. (2) ●—● The same as (1) but without DEP treatment. (3) ×—× RNA from ribosomes which were not treated with RNase. This sample was also precipitated with magnesium acetate and treated with DEP. RNA was liberated and centrifugation was performed as described in Methods

Discussion

When pigeon liver ribosomes are treated with RNase a qualitative evaluation of the RNA profiles shows that both *28S* and *18S* RNA are degraded. It has been shown (Fig. 2) that after RNase treatment the peak of *28S* RNA was shifted to a zone of lower sedimentation constant. We can calculate the change in the sedimentation constant of *28S* RNA if we assume that

1. the rate of sedimentation is linear and
2. the degradation product is homogeneous.

While 1. is approximately true for our system, there is no evidence that 2. is also true. However, since the position of the shifted peak was not affected by the concentration of the RNase used and the peak was not enlarged in RNase treatment,

the degradation product may be homogeneous. If this is so indeed it probably means that there are only a few sites on the RNA chain which are accessible to RNase attack in the ribosome.

The sedimentation constant of the degradation product of *28S* RNA was estimated to be between *26S* and *27S*. The molecular weight of *28S* RNA is about $2 \cdot 10^6$ daltons (Click, Tint, 1967) and if the decrease of the sedimentation constant reflects a decrease in molecular weight rather than a change in the conformation of the molecule (which seems to be a reasonable assumption), then the degradation product originates from *28S* RNA by the loss of about 3—400 nucleotides. When intact ribosomes were treated with RNase using the DEP technique (Fig. 3), we were not able to detect an increase in the *18S* RNA. Without DEP treatment, however, in the presence of EDTA and SDS the increase in *18S* material, paralleling the decrease of *28S* RNA on RNase treatment seems to be a characteristic phenomenon. This indicates that this sort of degradation of *28S* RNA occurs mainly or at least preferentially after the disorganization of ribosomal structure. It appears that the region the nucleolytic splitting of which results in the "halving" of the *28S* RNA is protected in the intact ribosome, but exposed after EDTA and SDS treatment.

The differential response of RNase to SDS found by the two different methods (i.e. by gradient analysis and by measuring the acid soluble fraction) may be the consequence of using different substrates, or it may reflect differences in the sensitivity of the two methods, the gradient analysis being much more sensitive. This higher sensitivity of the alteration of sedimentation profile can be explained by the fact that a few breaks inside the RNA chain result in the formation of material with a smaller sedimentation coefficient, which is still insoluble in the precipitating medium used in the method of Schucher and Hokin (1954). It is also possible that EDTA which is added before the analysis of RNA, makes rRNA very susceptible to nucleolytic degradation.

The authors are much obliged to Prof. F. B. Straub for his interest and helpful suggestions. The skillful technical assistance of Miss J. Kőmüves is gratefully acknowledged.

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RNase Sensitivity of Messenger and Ribosomal RNA in Pigeon Liver Polysomes

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(Received February 11, 1970)

The effect of mild RNase treatment on pigeon liver ribosomes was studied. Changes in RNA pattern, polysome profile and amino acid incorporating activity of the ribosomes were followed after treatment with low amounts of pancreatic RNase. Partial degradation of both rRNA and mRNA was obtained by similar treatments with RNase. At the same time, decrease in amino acid incorporation was observed.

It is concluded that in pigeon liver sensitivities of rRNA and mRNA towards RNase are not significantly different.

Introduction

It has been shown in an earlier paper from this laboratory (Székely et al., 1967) that RNA in the ribosomes of pigeon pancreas was partially degraded by endogenous nucleases in the course of preparation of the particles. It has also been suggested that this breakdown may be responsible for the low amino acid incorporation found with these ribosomes. A decrease in the incorporation of amino acids by liver ribosomes could indeed be observed when the RNA of these particles was broken down by treatment with cell sap from pancreas. As changes in the structure of RNA in ribosomes and a possible effect of these changes on amino acid incorporation may provide information on both ribosome structure and its role in protein synthesis, it seemed interesting to do a more detailed study on the degradation of ribosomal RNA and on amino acid incorporation by such damaged particles.

As a more convenient system, pigeon liver ribosomes were used and RNA degradation was carried out by digestion with pancreatic RNase.* Changes in polysome profile, rRNA patterns and amino acid incorporation were followed after RNase treatment. The sensitivity of rRNA in the particles and that of mRNA towards pancreatic RNase were found to be similar. RNase treatment considerably reduced amino acid incorporation of ribosomes, the decrease being parallel to the loss of intact *28S* RNA.

* Abbreviations used: DEP: diethyl pyrocarbonate, PEP: phosphoenolpyruvate K salt, rRNA: ribosomal RNA, RNase: pancreatic ribonuclease (EC 2.7.7.16), SDS: sodium dodecylsulfate, TCA: trichloracetic acid, tRNA: transfer RNA.

Materials and Methods

Preparation of ribosomes, treatment of ribosomes with pancreatic RNase and sucrose density gradient analysis of rRNA were performed as described in the preceding paper (Hüvös et al., 1970).

Ribosomal suspensions enriched in polysomes were prepared by the method of Wettstein et al. (1963).

Protein content of ribosome suspensions was measured by the method of Lowry et al. (1951) using ribosomal protein as standard.

Polysome content of ribosomal suspensions was estimated by sucrose density gradient analysis. Aliquots corresponding to 0.5—0.7 mg rRNA were layered on a 10—30 per cent (w/w) linear sucrose density gradient containing 0.05 M tris-HCl, pH 7.6; 0.025 M KCl and 0.005 M magnesium acetate. The gradient was centrifuged for 2 hours at 22 500 rpm in the Spinco rotor No. SW-25.1. The gradients were analyzed either by an ISCO density gradient analyzer model UA-2, or by collecting fractions (30—33 from each tube) and measuring their absorbancy at 260 and 320 nm. The quantity of ferritin co-sedimenting with ribosomes was taken into correction according to Munro et al. (1964) on the basis of the absorbancy of the samples at 320 nm.

The amino acid incorporating system contained ATP, 0.5 μmole; GTP, 0.1 μmole; tris-HCl, pH 7.6, 12.5 μmoles; NH_4Cl, 12.5 μmoles; magnesium acetate, 5 μmoles; KCl, 5 μmoles; 2-mercaptoethanol, 7 μmoles; phosphoenolpyruvate K-salt, 5 μmoles; pyruvate kinase, 0.17 mg; a mixture of tRNAs, 25 μg; 105 000 *g* supernatant protein, 0.5 mg; sucrose, 50 μmoles; ribosomes corresponding to 0.2 mg RNA; unlabelled amino acids (except valine), 0.25 μmoles of each; ^{14}C-valine, 0.25 μCi in a total volume of 0.5 ml. Each sample was preincubated for 3 minutes at 38 °C before addition of radioactive valine. Incubation was at 38 °C for 10 min. The reaction was stopped by the addition of 2 vols of 10 per cent TCA containing 5 nM ^{12}C-valine. Afterwards the samples were kept in a boiling water bath for 15 minutes, washed three times with a solution of 5 per cent TCA containing 0.5 per cent casamino acid and once with 5 per cent TCA on Schleicher—Schüll 2043/a paper.

Radioactivity of the samples was determined in a methane gas flow counter.

ATP, GTP, PEP were the products of Reanal (Budapest).

Pyruvate kinase was prepared by the method of Bücher and Pfleiderer (1955) from rabbit muscle.

tRNA mixture was prepared from E. coli cells by the method of Zubay (1966).

Supernatant proteins were prepared by pelleting microsomes and ribosomes from the post-mitochondrial supernatant. The resulting supernatant solution was passed through a Sephadex G25 column to remove amino acids and peptides.

Results

Some characteristics of RNase-treated ribosomes

The effect of RNase treatment on ribosomes was studied after removing RNase by precipitation of the particles with 0.07 M magnesium acetate (Hüvös et al., 1970). Ribosomes obtained by this treatment were free from the bulk of RNase. They contained, however, traces of bound RNase the activity of which could be observed when in the presence of SDS, RNA was liberated from the particles. In experiments where SDS was used, a potent inhibitor of RNase, DEP, was therefore applied which prevented further changes in RNA profiles.

Recovery of ribosomes after precipitation with magnesium acetate was about 50 per cent in the case of control ribosomes. RNase treatment reduced recovery to about 10—30 per cent. Precipitation resulted in the removal of some contaminating proteins (e.g. ferritin), thus increasing the RNA : protein ratio of control ribosomes to about 0.9. Ribosomes treated with RNase gave lower RNA : protein ratios (0.3—0.8), probably because of losses of RNA from heavily degraded particles and because of coprecipitation of some dissociated ribosomal proteins with the particles.

RNA profile of RNase-treated ribosomes

Degradation of RNA upon treatment of the ribosomes with RNase was followed by sucrose density gradient analysis. Treatment with DEP was applied to ensure that RNase action had been stopped before RNAs were liberated. Fig. 1 shows typical sedimentation profiles of rRNAs isolated from ribosomes which had been treated with increasing concentrations of RNase and precipitated thereafter with magnesium ions. The quantities of both *28S* and *18S* RNAs decrease while the amount of *4—8S* material increases. While the quantity of the remaining *28S* RNA can be estimated from the gradient profile, changes in the quantity of *18S* RNA cannot be evaluated, since the *18S* material present in the gradient may be contaminated with the breakdown products of *28S* RNA. Fig. 2 shows that after treatment with RNase and precipitation with magnesium ions the decrease of the amount of *28S* RNA is not proportional to the concentration of RNase. As it was shown earlier (Hüvös et al., 1970) the peak of *28S* RNA shifted to a zone of a lower sedimentation constant upon treatment with RNase. In the calculations of the amounts of the remaining *28S* RNA this shift was not taken into consideration. This calculation, therefore, gives the maximum values for intact, remaining *28S* RNA.

Effect of RNase on polysomes. RNase sensitivity of rRNA and mRNA

The rather sensitive response of rRNA to mild RNase treatment made it interesting to compare the sensitivities of rRNA and mRNA towards RNase. This was carried out in a semi-quantitative manner, by comparing the effect of low RNase concentrations on the polysome profile and the rRNA pattern of the same ribosome preparation.

When our standard technique was used to remove RNase, the lowest amounts of RNase applied, 0.05 μg RNase/mg rRNA, did not cause detectable changes

in *18S* or *28S* RNA content, nor could we observe significant changes in the polysome profile. As, however, after magnesium precipitation ribosomes may tend to aggregate and thus sediment like polysomes, a slight loss in polysomes may not have been detected. These experiments were therefore carried out omitting precipitation with magnesium acetate in the following way:

Aliquots of ribosome suspensions enriched in polysomes were incubated with or without RNase, layered on a 10—30 per cent linear sucrose gradient and

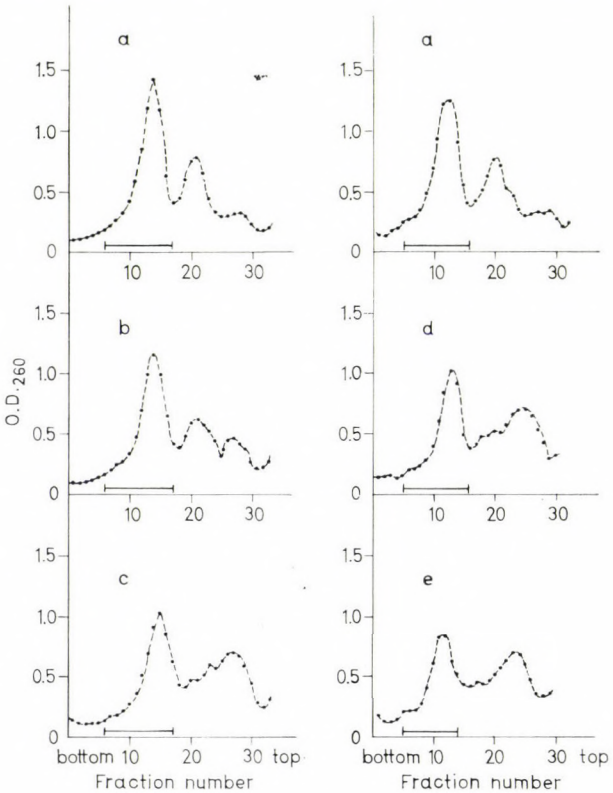

Fig. 1. Sedimentation profiles of RNAs from RNase treated ribosomes. Ribosome suspensions corresponding to 8 mg rRNA/ml were treated with *a)* 0; *b)* 0.05; *c)* 0.4; *d)* 0.7; *e)* 1.0 μg RNase/mg RNA for 10 min at 0 °C, and precipitated with magnesium acetate to remove the bulk of RNase. Samples containing 0.7 mg RNA were treated with DEP to destroy the residual RNase activity. Preparation for sedimentation analysis and centrifugation of the samples were performed as described in Methods

centrifuged to investigate the sedimentation profile of polysomes. 20 minutes after starting centrifugation of these samples DEP was added to other aliquots of these suspensions taken for RNA analysis, to destroy the action of RNase. 20 minutes was estimated as the time required for *80S* ribosomes to leave the initial zone containing RNase. In this way the duration of RNase action was roughly

comparable in the samples taken for polysome and rRNA analysis, respectively, although somewhat higher losses of polysome content may be observed by this technique probably because some RNase molecules move with the particles and continue acting on them during centrifugation.

The changes in polysome and rRNA profiles were examined, using different concentrations of RNase.

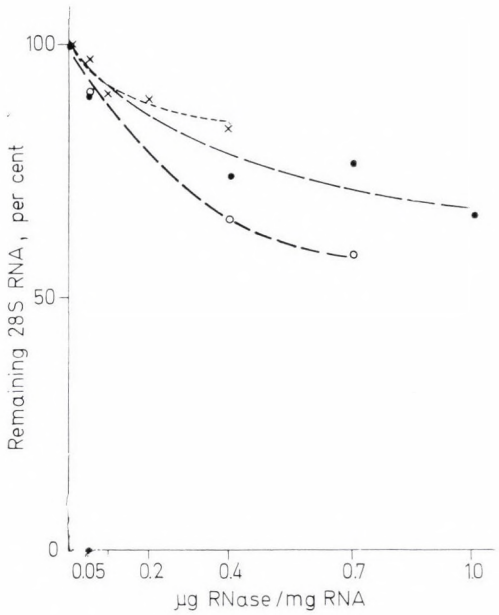

Fig. 2. Effect of RNase concentration on the degradation of *28S* RNA. Ribosomes were incubated with the indicated amounts of RNase, precipitated with magnesium acetate and treated with DEP. The quantity of intact *28S* RNA remaining after RNase treatment was calculated from the corresponding gradient profiles shown in Fig. 1 (underlined area) in terms of total OD_{260} units recovered. The amount of *28S* RNA in the control sample was taken as 100 per cent. The three curves of this figure represent three independent experiments

As can be seen in Fig. 3A, treatment of ribosomes with 0.19 μg RNase/ml (0.05 μg RNase/mg rRNA) results in small changes in the polysome profile, whereas no significant changes are found in the sedimentation profile of RNA.

2.1 μg RNase/ml (0.5 μg RNase/mg rRNA) causes a larger but not total destruction of polysomes, and significant changes can also be detected in the RNA profiles (Fig. 3B). Quantitative evaluation of the gradient profiles shows that the amount of polysomes containing more than two ribosomes decreased by 55 per cent and the amount of monosomes increased by 150 per cent. At the same time the amount of intact *28S* RNA decreased by 20 per cent. It can be concluded that the sensitivity of messenger and ribosomal RNA to nucleolytic digestion does not differ significantly.

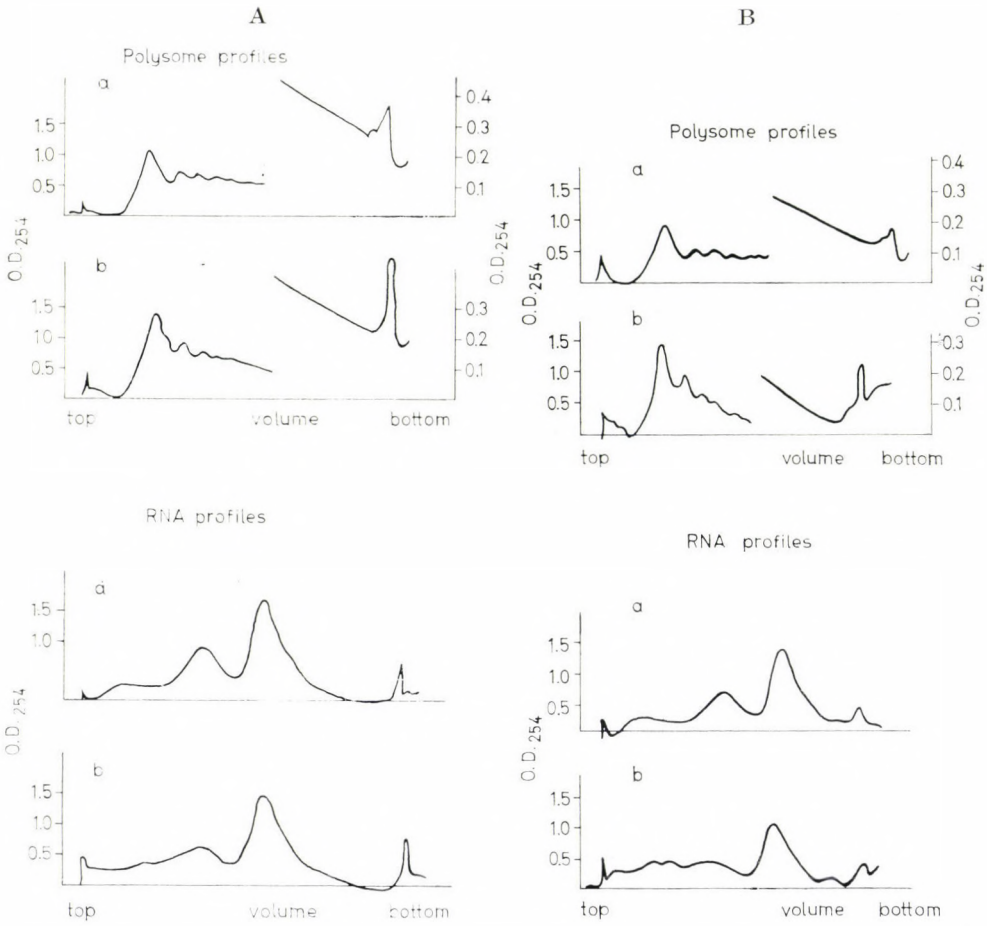

Fig. 3. Effect of RNase on polysome content and rRNA profile. A) Ribosome suspensions were incubated at 0 °C *a)* without RNase; *b)* with 0.19 μg RNase/ml (0.05 μg RNase/mg RNA). Five minutes after the addition of RNase, aliquots were layered on a 10–30 per cent (w/w) linear sucrose density gradient. Centrifugation was started 20 minutes after addition of RNase. 20 minutes after starting the centrifugation of the polysome preparation, aliquots were taken from the original reaction mixtures and DEP was added to them. These suspensions were prepared for RNA analysis and centrifuged as described in Methods. Gradients were analysed in an ISCO model density gradient analyzer. B) Ribosomes were incubated at 0 °C *a)* without RNase; *b)* with 2.1 μg RNase/ml (0.5 μg RNase/mg RNA). Gradient analysis as in Fig. 3A. In order to estimate the amount of heavy polysomes the sensitivity of the analyzer was raised fivefold, indicated by the break in curves

Effect of RNase treatment on amino acid incorporation

Ribosomes were treated with RNase under the same conditions as before. For subsequent assay of amino acid incorporation DEP as RNase inhibitor could not be applied. Therefore RNase was removed by precipitating the particles with magnesium acetate. As shown in the previous paper (Hüvös et al., 1970), this treat-

ment removes the bulk of RNase but leaves behind some traces of enzyme bound to the particles. During incubation at 37 °C for 10 minutes this bound RNase did not exhibit any activity towards RNA substrate in the assay of Schucher and Hokin (1954), and only very little activity towards RNA in the particles themselves, as checked by density gradient analysis (Fig. 4). Its activity was observed

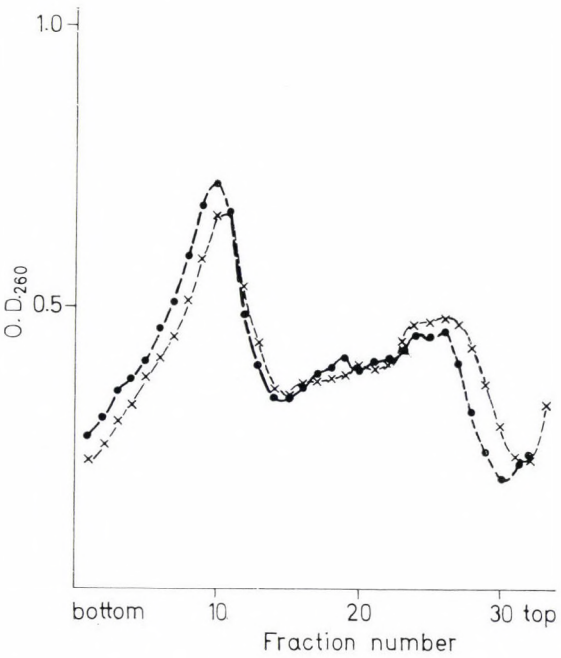

Fig. 4. Effect of incubation at 38 °C on the integrity of rRNAs of RNase-treated ribosomes. Ribosome suspensions, corresponding to 8 mg rRNA/ml were treated with RNase in a final concentration of 3.2 μg/ml for 10 min at 0 °C, then precipitated with magnesium acetate and washed as described in Methods. Aliquots corresponding to 0.7 mg rRNA were incubated: × — ×, for 10 minutes at 38 °C; ● — ●, for 10 minutes at 0 °C. After incubation DEP was added to both samples, followed by the addition of EDTA and SDS. Further treatment and centrifugation were performed as described in Methods

only when in the presence of SDS the nucleoproteid structure was destroyed (Hüvös et al., 1970). It seems therefore that the enzyme may bind to ribosomes in an inactive form (like the "latent" RNase of E. coli) and has to be liberated for activation. If this is the case, amino acid incorporation of RNase-treated ribosomes can be measured after removal of RNase without interference by traces of bound RNase present. The time curve of amino acid incorporation by these ribosomes was indeed linear for the period of the assay, confirming that probably no further damage occurred to the ribosomes while testing their activity.

A decrease of amino acid incorporation was found by this technique with RNase-treated ribosomes. The loss of activity increased with increasing RNase concentration, no complete inactivation has been reached, however, in the concentration range 0.4—8.0 μg RNase/ml (0.05—1.0 μg RNase/mg rRNA). In Fig. 5 amino acid incorporation is plotted against RNase concentration used for the

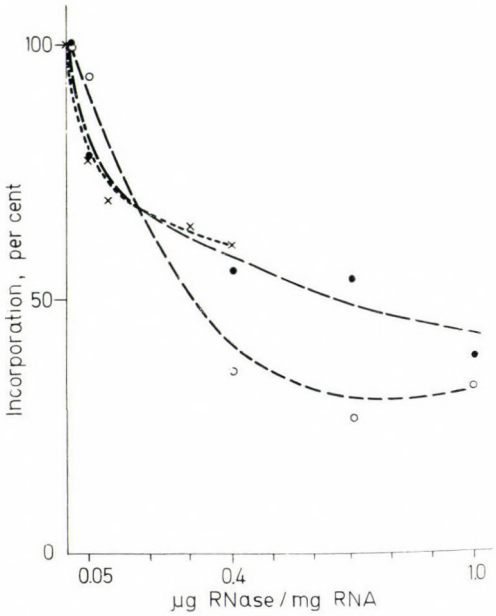

Fig. 5. Effect of RNase on the incorporating activity of ribosomes. Ribosomes were incubated with the indicated amounts of RNase for 10 min at 0 °C, and after the removal of RNase by precipitating ribosomes with magnesium acetate, their incorporating ability was tested as described in Methods. The values are corrected for samples incubated in energy free system. Results of three independent experiments are illustrated

pretreatment of ribosomes. As can be seen, a rapid decrease is followed by little further change. The curve is very similar to that obtained when the *28S* RNA content of RNase-treated ribosomes is plotted against RNase concentration. In Fig. 6 the *28S* RNA content of ribosome preparations treated with different amounts of RNase is plotted against the amino acid incorporating activity of the same ribosome preparations. It can be seen that there is a linear relationship between *28S* RNA content and amino acid incorporating activity of the ribosomes, reduction of amino acid incorporation being always somewhat greater than loss of *28S* RNA on a percentage basis.

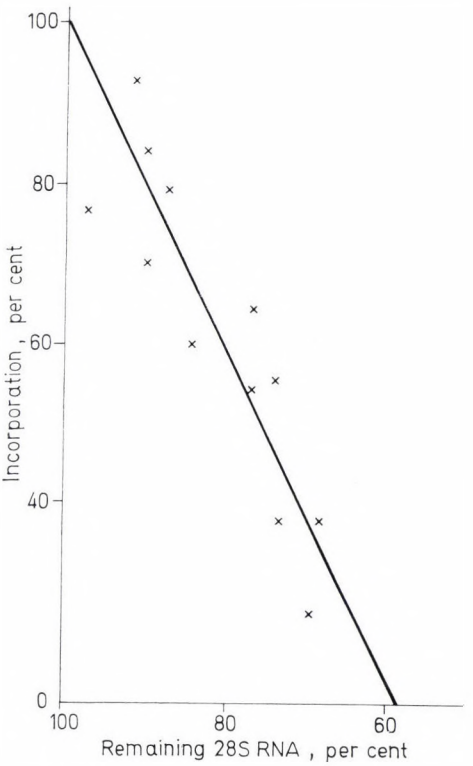

Fig. 6. Correlation between the *28S* RNA content and amino acid incorporating ability of ribosomes treated with RNase and precipitated with magnesium acetate. The data presented here are obtained from several independent experiments. For details see text

Discussion

Ribosomal RNA is usually considered a rather stable component of the particles, relatively resistant to nucleases. In contrast to that, high sensitivity towards RNase of messenger RNA has been observed in different cells (Chantrenne et al., 1967). The present results show, however, that even mild treatment of pigeon liver ribosomes with pancreatic RNase is sufficient to induce some breaks in both *28S* and *18S* RNA, thereby reducing the size of the RNA molecules. The same treatment causes partial breakdown also of mRNA. The lowest amounts of RNase applied, 0.05 μg RNase/mg rRNA, caused no change in rRNA and only a very slight loss of polysomes. Thus, with respect to their response to mild RNase treatment, rRNA and mRNA do not show great differences. Similar results were obtained by Fenwick (1968) on HeLa cell and E. coli ribosomes.

These data are not in disagreement with the possibility of protection of RNA by ribosomal proteins. The nucleoproteid structure may prevent total degradation of RNA. Strong digestion of ribosomes was shown to lead to partial

breakdown of RNA (Petrovic, Brkic, 1969; Cox, 1969) and to a release of nucleotides (Santer, Smith, 1966). These observations together with our findings suggest that in the nucleoprotein particles many areas of RNA may be protected, but there are some sites in the ribosome (possibly on the surface) where RNA is easily accessible to RNase. These results are in accordance with the recently proposed model of reticulocyte ribosomes (Cox and Bonanou, 1969). The similarity of RNase-sensitivity of rRNA and mRNA points to the possibility that some areas of mRNA are also protected against RNase action. The observation that mRNA may be associated with proteins (Hoagland, Askonas, 1963; Samarina et al., 1966; Perry, Kelly, 1968) supports this idea.

We also followed the changes in amino acid incorporation, ensuing RNase treatment, of the ribosomes. A decrease was found in the activity of ribosomes. This decrease varied with RNase concentration in the same way as did the loss of *28S* RNA. Expressed in percentages of the control value, amino acid incorporation was always somewhat lower than *28S* RNA content. As at the same time there is also a loss of polysomes, it is uncertain to what extent the breakdown of rRNA and mRNA participates in the deterioration of biological activity. The decrease of the amino acid incorporating activity of ribosomes following RNase treatment and precipitation with magnesium acetate may be connected also with the observed increase in the protein/RNA ratio (Nomura, Traub, 1968; Brentani, et al., 1968). The parallelism between *28S* RNA content and amino acid incorporation is, however, striking. The amount of *28S* RNA remaining after RNase treatment is the maximal measure of intact ribosomes present. A possible interpretation of the above correlation is therefore that the intact structure of ribosomal particles (or of their RNA) may be necessary for amino acid incorporation. This parallelism is a further indirect proof of the conclusion that the sensitivity of mRNA and of rRNA towards pancreatic RNase does not differ significantly.

The authors are indebted to Prof. F. B. Straub for his interest in the work, and for his valuable discussions. The skillful technical assistance of Mrs J. Jeszenői is gratefully acknowledged.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (2), pp. 203—213 (1970)

Characterization of the Less-reactive SH Groups of D-glyceraldehyde-3-phosphate Dehydrogenase

I. Kinetic Analysis of Mercaptide Formation

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(Received February 20, 1970)

The overall reaction of the SH groups of D-glyceraldehyde-3-phosphate dehydrogenase with p-mercuribenzoate can be divided into three phases: 1) the mercaptide formation of one reactive (nucleophilic) SH group per subunit; 2) the reaction of one of the three less-reactive SH groups per subunit (SH_{II}); 3) the reaction of the buried SH_{III} and SH_{IV} groups. The reaction of SH_{II} follows apparently first order kinetics with respect to the enzyme, but the rate constant varies with the concentration of PMB. The reactivity of this SH_{II} can be interpreted on the basis of fluctuation of the structure of the protein molecule. The first order rate constant for the reaction of the buried SH_{III} and SH_{IV} groups increases as the enzyme concentration is lowered, probably because of the faster denaturation of the dissociated subunits.

Introduction

Muscle D-glyceraldehyde-3-phosphate-dehydrogenase (GAPD) consists of four identical subunits and contains four cysteinyl residues per subunit (Harris, Perham, 1965). One SH group per subunit is extremely reactive (nucleophilic SH group, SH_I, Racker, Krimsky, 1958; Pihl, Lange, 1962; Boross, Cseke, 1967) and plays a direct role in the enzymic process (Krimsky, Racker, 1955; Koeppe et al., 1956; Harris et al., 1963). Two SH groups per subunit react rapidly with p-mercuribenzoate (PMB), which means that the enzyme contains, in addition to the nucleophilic SH group, another easily accessible SH group (SH_{II}) (Szabolcsi et al., 1960). The other two SH groups per subunit (SH_{III} and SH_{IV}) react with PMB only after prolonged incubation; at pH 8.5 and 5 °C this reaction follows first order kinetics. These latter residues are probably buried in the interior of the enzyme molecule and become exposed owing to a conformational change (Friedrich, Szabolcsi, 1967).

The question arises whether SH_{II} reacts with PMB in a way similar to SH_{III} and SH_{IV}, i.e. the exposure of SH_{II} results in some structural change of the protein molecule after the modification of the nucleophilic thiol group. As reported in a preliminary paper (Boross et al., 1969) the mercaptide formation of SH_{II} follows an apparently first order kinetic with respect to the enzyme. This first order rate

constant, however, depends on the PMB concentration, which indicates a more complex mechanism of the reaction. We suggest that the unusual kinetic behaviour of SH_{II} can be explained on the basis of a fluctuating protein structure.

Materials and Methods

GAPD was isolated from swine muscle according to the method of Elődi and Szörényi (1956) and was recrystallized four times. The molecular weight was taken to be 140 000 (Elődi, 1958). Enzyme solutions were prepared freshly: the crystals were dissolved in tris buffer, pH 7, ionic strength 0.05 and the solution was gel-filtered on a Sephadex G-100 column equilibrated with the same buffer. The removal of ammonium sulphate was checked by conductivity measurements.

All experiments described in this paper were carried out with NAD-free enzyme. Firmly bound NAD was removed by charcoal-treatment (Velick, 1953). Enzyme concentration was determined spectrophotometrically at 280 nm. The molar extinction coefficients for the GAPD–NAD complex and for NAD-free GAPD were 1.4×10^5 and 1.27×10^5, respectively.

PMB was a commercial preparation of British Drug Houses. The concentration of PMB solutions was determined spectrophotometrically ($\varepsilon_{234\,nm} = 17\,400$; Boyer, 1954).

The molar extinction coefficient of the mercaptide bond between protein SH groups and PMB was determined by titration with PMB ($\Delta\varepsilon_{255\,nm} = 6\,000$). In the kinetic experiments the number of mercaptide bonds formed was calculated accordingly by Boyer's method (Boyer, 1954).

Carboxymethylation of protein was carried out as previously described (Boross, Cseke, 1967). Iodoacetic acid was recrystallized four times from carbon tetrachloride.

The measurements were carried out in Unicam SP 500 and SP 700 spectrophotometers, with thermostated cell compartment at 5 °C.

Results

1. Kinetics of mercaptide formation. Dependence upon PMB concentration

To the enzyme dissolved in tris buffer, pH 7, ionic strength 0.05, 2, 3, 4, and 8 moles of PMB were added per subunit and the formation of mercaptide bonds was measured (Fig. 1). The overall process can be divided into three phases. The initial unmeasurably quick change in extinction means the reaction of the nucleophilic SH group with PMB. The next phase, which also corresponds to one thiol group per subunit (SH_{II}), is very rapid but still measurable by conventional methods. The third phase, which corresponds to the mercaptide formation of residues SH_{III} and SH_{IV}, is clearly distinguishable from the second one. In the last phase the overall reaction can be described by simple first order kinetics, since reaction velocity is independent of PMB concentration. The reactions of these two SH groups with PMB cannot be distinguished from each other. This finding

is in good agreement with earlier experiments of Friedrich and Szabolcsi (1967). The authors explained first order kinetics by a rate-limiting structural change required for the exposure of buried SH groups, induced by the blocking of SH_I and SH_{II}.

Contrary to the reaction of SH_{III} and SH_{IV}, the rate of reaction between SH_{II} and PMB is dependent upon PMB concentration (Fig. 1).

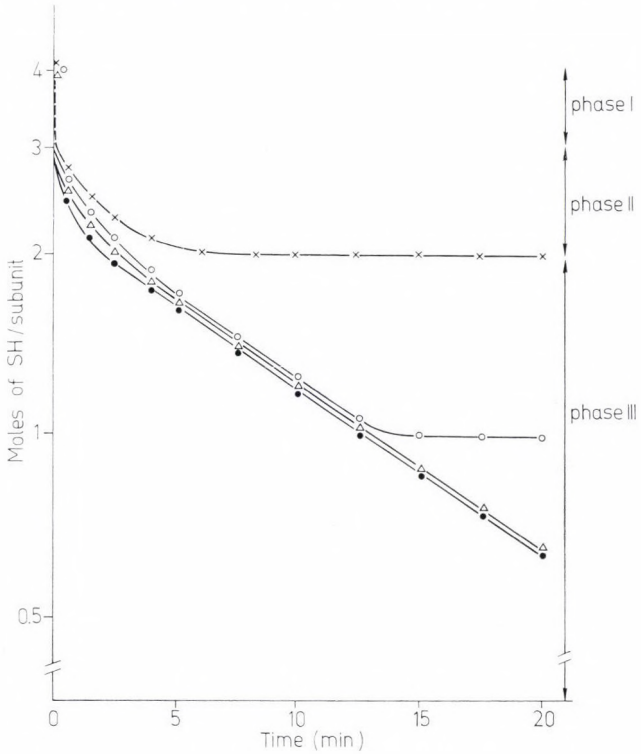

Fig. 1. Semilogarithmic plot of the reaction of SH groups with PMB. The reaction of NAD-free GAPD (1.25×10^{-6} M) with 2 (× — ×), 3 (○ — ○), 4 (△ — △) and 8 (● — ●) moles of PMB per subunit in 0.05 ionic strength buffer, tris pH 7, at 5 °C

Our data suggest that the mercaptide formation of buried SH groups commences in a subunit of the enzyme molecule only if the SH_{II} has already reacted. Furthermore, if it is taken into account that the reaction of buried SH groups (phase III) is very slow compared to the reaction of SH_{II} (phase II), it can be conceived that the change in extinction at the beginning of phase II represents, with good approximation, the modification of SH_{II}. In fact, the semilogarithmic plot of values obtained in the first minute of the reaction is a straight line (Fig. 2).

However, the reaction is only apparently of first order. Fig. 3 shows the dependence of the first order rate constant calculated from the initial phase of the

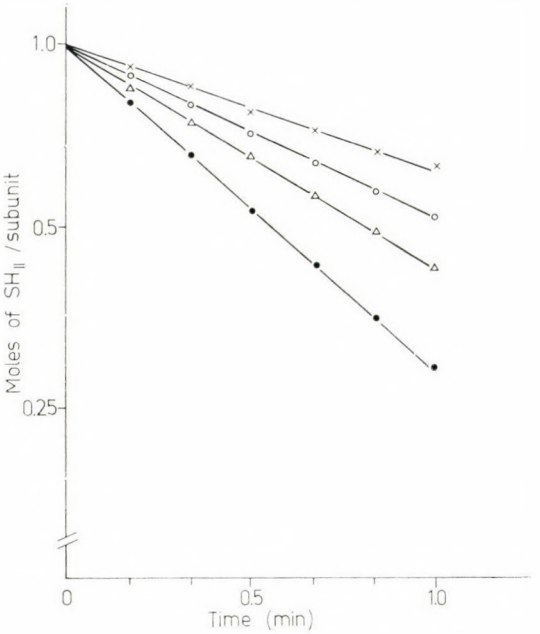

Fig. 2. Effect of PMB concentration on the mercaptide formation of SH_{II}. A more detailed analysis of phase II in Fig. 1. Symbols and reaction conditions are the same as in Fig. 1

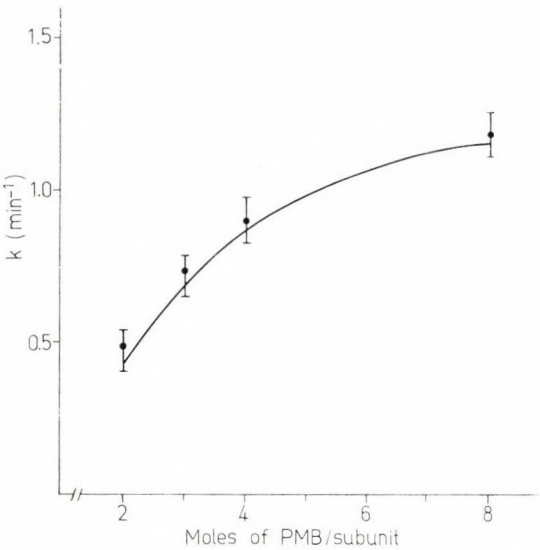

Fig. 3. Dependence of the apparent first order rate constant on PMB concentration as calculated from the initial part of the mercaptide formation of SH_{II}. The points represent the mean of several experiments. Calculations were made from the first minute of mercaptide formation. The continuous line was calculated from Eq. (8) (Cf. Discussion)

reaction on PMB concentration. Since the relationship is not linear, the reaction of SH_{II} with PMB cannot be regarded as a pure second order reaction either.

If under the above experimental conditions PMB is added to mercaptoethanol, an unmeasurably rapid, instantaneous reaction is observed. The relatively slow reaction of SH_{II} with PMB therefore indicates that this thiol group cannot be regarded as a free aliphatic mercaptane. A possible explanation is that the exposure of SH_{II} is brought about by a change of enzyme structure that may be induced by the blocking of SH_I. In this case, however, simple first order kinetics would be

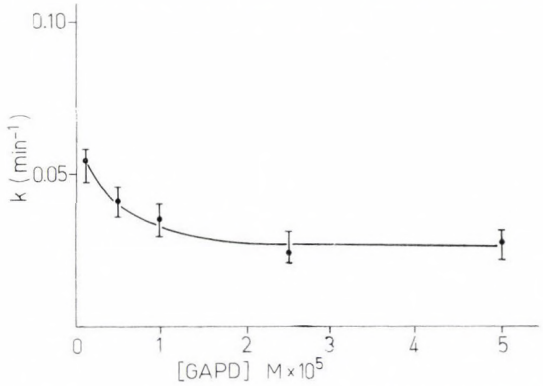

Fig. 4. Dependence of the first order rate constant of mercaptide formation of SH_{III} and SH_{IV} upon GAPD concentration. The reactions were carried out in 0.05 ionic strength tris buffer, pH 7, at 5 °C with 8 moles of PMB per subunit

expected also for SH_{II}, just as with SH_{III} and SH_{IV}. This criterion is not fulfilled in the above experiment, and the following experiments also rule out this assumption.

a) One mole of PMB was added to the enzyme per subunit and the mixture was incubated. At various times samples were withdrawn and 1 mole of PMB per subunit was added to them. No instantaneously reacting, i.e. fully exposed SH_{II} could be detected even after about half an hour of incubation, although in the presence of 2 moles of PMB per subunit SH_{II} forms mercaptide within a few minutes.

b) We found that in tris buffer the carboxymethylation of SH_I did not affect the kinetics of the reaction between SH_{II} and PMB. Moreover, the period of several hours that elapsed between the completion of carboxymethylation and the addition of PMB failed to influence the rate of mercaptide formation.

Consequently, the possibility that the modification (mercaptidation or carboxymethylation) of SH_I triggers the exposure of SH_{II} can be excluded. The reaction of SH_{II} is limited by a structural change different in character from that operating in the case of buried SH groups.

2. Effect of enzyme concentration on mercaptide formation

The reaction of SH_{II} and that of the buried SH groups with PMB was studied in solutions of different enzyme concentrations.

The rate constant for SH_{II} did not change when the concentration of the enzyme was varied and PMB concentration was kept constant, i.e. the reaction was of first order with respect to the enzyme.

The first order rate constant of the mercaptide formation of buried SH_{III} and SH_{IV} increased towards low enzyme concentrations (Fig. 4). An analogous phenomenon was observed in the case of another denaturation process, the spontaneous heat inactivation of GAPD. At 10 °C the rate of activity loss markedly increased at protein concentrations below 1×10^{-5} M (Vas, Boross, 1970).

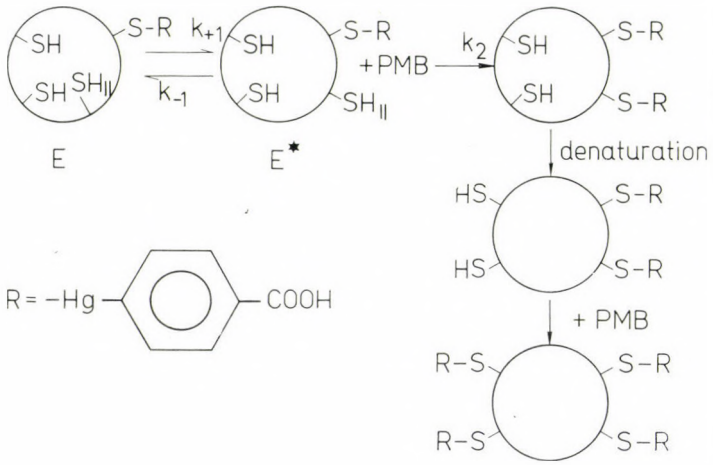

Fig. 5. Scheme for the reactions of SH groups of GAPD with PMB. The circles represent one subunit of GAPD

Discussion

The above experiments convincingly prove that SH_{II} of GAPD can be regarded as a "less-reactive" thiol group, i.e. its reaction with PMB is not instantaneous in contrast with the mercaptide formation of aliphatic mercaptanes or of the nucleophilic SH group of the enzyme. This reduced reactivity is most probably due to the fact that SH_{II} is in an unfavourable state with regard to the reaction with PMB. This state may be the participation in a complex (e.g. to serve as a ligand of Zn ion bound to the enzyme) or a sterically hindered position due to another functional group.

Our experiments indicate that the availability of SH_{II} does not depend on the structural change that follows the modification of SH_I. Therefore we assume that SH_{II} is exposed owing to the structural fluctuation of the enzyme molecule. In other words, GAPD molecules occur with certain probability in conformations in which SH_{II} is accessible to PMB. The probability of occurrence of this form must be very small, as the amount of SH_{II} that reacts instantaneously with PMB is too low to be detected.

On this basis the reaction of SH_{II} with PMB may be described by the following simplified scheme (cf. also Fig. 5):

$$E \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E^* + PMB \xrightarrow{k_2} E - PMB \longrightarrow \text{denaturation} \tag{1}$$

E and E* are the two possible states of the enzyme molecule, and in the absence of PMB there is a fluctuational equilibrium between them. The probability of occurrence of state E*, in which SH_{II} is accessible to PMB, is very small, i.e. $k_{+1} \ll k_{-1}$. Since the reaction of free SH groups with PMB is very fast, one can assume that the formation of E* is the rate limiting step of the overall process, i.e. $k_{+1} \ll k_2$. In such a system the concentration of E* will tend to zero, therefore the change of its concentration will also be negligible, that is, a steady state is maintained over a large part of the reaction (Benson, 1960):

$$\frac{d[E^*]}{dt} \approx 0 \tag{2}$$

The steady-state equation for [E*] is

$$\frac{d[E^*]}{dt} = k_{+1}[E] - k_{-1}[E^*] - k_2[PMB][E^*] \approx 0 \tag{3}$$

The rate of mercaptide formation is

$$v = k_2[E^*][PMB] \tag{4}$$

From Eqs (3) and (4)

$$v = \frac{k_2 k_{+1}[PMB]}{k_{-1} + k_2[PMB]} \cdot [E] \tag{5}$$

Equation (5) can be written in two forms:

$$\frac{1}{v} = \frac{k_{-1}}{k_2 k_{+1}[E]} \frac{1}{[PMB]} + \frac{1}{k_{+1}[E]} \tag{6}$$

$$\frac{1}{v} = \left(\frac{k_{-1}}{k_2 k_{+1}[PMB]} + \frac{1}{k_{+1}}\right) \cdot \frac{1}{[E]} \tag{7}$$

That is, if $\frac{1}{v}$ is plotted against $\frac{1}{[PMB]}$ a straight line is obtained with an intercept on the ordinate $\frac{1}{k_{+1}[E]}$ and slope $\frac{k_{-1}}{k_2 k_{+1}[E]}$. On the other hand, if $\frac{1}{v}$ is plotted against $\frac{1}{[E]}$, a straight line starting from the origin is obtained. As

shown in Fig. 6 the experimental data can be described by these equations if the initial rates and initial enzyme and PMB concentrations are used for the calculations.

From the intercept on the ordinate in Fig. 6a the value 1.67 min^{-1} is obtained for k_{+1}. Using this value, from the slope of the line we can calculate $\frac{k_{-1}}{k_2}$

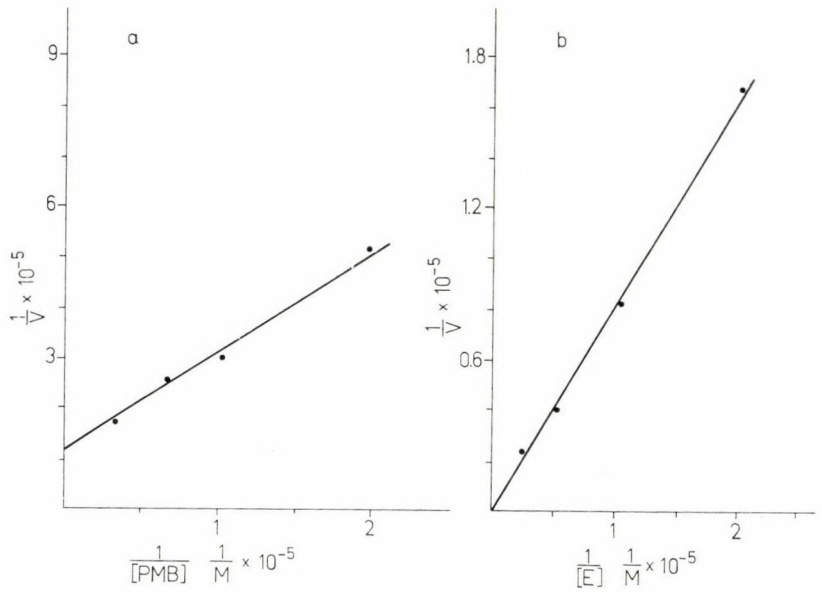

Fig. 6. Double reciprocal plot of the effect of PMB (a) and enzyme (b) concentration on the initial rate of mercaptide formation of SH_{II}. The initial rate was calculated from the first 15 sec of the reactions and is expressed as the concentration of mercaptides, in moles per litre, per minute. In Fig. 6a the enzyme concentration was 1.25×10^{-6} M, whereas in Fig. 6b the PMB concentration was 4×10^{-5} M, and the [E] values mean the subunit concentration. [PMB] represents the actual concentration of PMB remaining after the reaction of the nucleophilic SH group

which is 1.61×10^{-5} M. Since k_{-1} is a first order rate constant and k_2 is a second order rate constant, and $[E] = 0.5 \times 10^{-5}$ M, the half times of the two reactions fall into the same order of magnitude.

If we calculate the slope of the straight line described by Eq. (7), using the values of k_{+1} and $\frac{k_{-1}}{k_2}$ thus obtained, the calculated value agrees within the limits of error with the one determined experimentally as shown in Fig. 6b. This is a strong support for the validity of the proposed fluctuational scheme.

In this case the reaction is indeed of first order with respect to the enzyme, and the first order rate constant from Eq. (5) is

$$k' = \frac{k_2 k_{+1} [PMB]}{k_{-1} + k_2 [PMB]} \tag{8}$$

It can be seen that k′ varies with PMB concentration. The continuous line in Fig. 3 is the theoretical curve of Eq. (8). The apparently first order rate constants calculated from the initial phase of the reaction agree well with Eq. (8).

However, the dependence of the rate constant on PMB concentration also means that in the course of the reaction, as PMB is being consumed, the rate constant must also change. The time course of the reaction of SH_{II} with PMB can be calculated from Eq. (5).

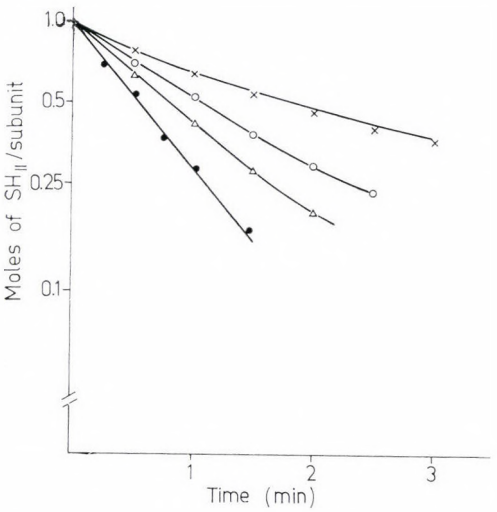

Fig. 7. Time course of the mercaptide formation of SH_{II} at various PMB concentrations. Reaction conditions and symbols are the same as in Fig. 1. The continuous lines were calculated according to Eqs (9) and (10), and the points were obtained by subtracting the reaction of SH_{III} and SH_{IV} from the experimental data

The solution of this equation is

$$\frac{k_{-1}}{k_2}\frac{1}{[PMB]_0-[E]_0}\ln\frac{[E]_0([PMB]_0-x)}{[PMB]_0([E]_0-x)}+\ln\frac{[E]_0}{[E]_0-x}=k_{+1}t \qquad (9)$$

where x = concentration of mercaptide bonds formed in time t, $[E]_0$ and $[PMB]_0$ are the initial concentrations of SH_{II} and the reagent, respectively. If $[E]_0 = [PMB]_0$, the integrated form of Eq. (5) is

$$\frac{k_{-1}}{k_2}\frac{x}{[E]_0([E]_0-x)}+\ln\frac{[E]_0}{[E]_0-x}=k_{+1}t \qquad (10)$$

The continuous lines in Fig. 7 represent the theoretical curves calculated from Eqs (9) and (10). In the calculations the moles of PMB added were always reduced by one because of the instantaneous reaction of SH_I. It is clear

from the figure that at low PMB concentrations the apparent first order rate constant decreases during the reaction, whereas at high reagent concentrations the change is negligible.

The reaction of SH_{II} cannot be measured separately and followed up to completion, since the reaction of buried SH groups, initiated by the blocking of SH_{II}, also becomes detectable. Knowing the rate constant of mercaptidation of these SH groups, approximative calculations were made for this secondary reaction. The equation for consecutive first order reactions was used, taking into account that the apparent first order rate constant of mercaptide formation of SH_{II} decreases during the reaction. The calculated values were subtracted from the number of mercaptide bonds determined experimentally, thus the number of mercaptide bonds formed by SH_{II} were obtained. The points in Fig. 7 represent these values, which fit well the theoretical curves obtained from Eqs (9) and (10).

At high PMB concentrations (whether excess PMB was given to dilute enzyme solution or an amount of PMB equivalent to SH_{II} was given to the concentrated protein), the rate constant calculated on the basis of Eq. (8) hardly changed during the reaction. At the same time, in this case the reaction of SH_{II} is much faster than that of SH_{III} and SH_{IV}, from which it follows that the probability of the buried SH groups to react to any appreciable extent before the completion of the reaction of SH_{II} is much smaller. Thus, here the reactions of the two kinds of SH groups can be clearly distinguished and measured separately. The value of k' gradually approaches k_{+1} towards high PMB concentrations, i.e. when PMB $\rightarrow \infty$, $k' \rightarrow k_{+1}$. In other words, at high PMB concentrations mercaptide formation becomes so fast that E* cannot be re-formed into the original enzyme, E. In this case the rate of the overall reaction is indeed limited only by k_{+1}. The experimental data shown in Fig. 3 fulfil these expectations.

Finally a few remarks should be made about the reaction of SH_{III} and SH_{IV}. On the one hand, the apparently first order rate constant for these two buried SH groups is independent of PMB concentration (Friedrich, Szabolcsi, 1967), which suggests that here we deal with a unidirectional denaturing alteration.

On the other hand, the first order rate constant of mercaptidation of buried SH groups depends upon the protein concentration (Fig. 4). It may be assumed that this phenomenon is related to the dissociation of GAPD molecules into subunits, inasmuch as the tetrameric, dimeric, or monomeric molecular forms have different reactivities. Hoagland and Teller (1969) reported that the GAPD tetramer dissociated into dimers at concentrations lower than 1 mg/ml ($K_d = 2 \times 10^{-6}$ M). This finding is in accordance with our results. From the similar dependence upon protein concentration of the rate of heat denaturation it follows that dissociation into subunits influences also this denaturation process.

The authors wish to express their thanks to Prof. F. B. Straub for helpful discussion.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (2), pp. 215—223 (1970)

Characterization of the Less-reactive SH Groups of D-glyceraldehyde-3-phosphate Dehydrogenase

II. Effect of Coenzyme, Anions and pH on the Reactivity

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(Received February 20, 1970)

The kinetics of reaction of the three less-reactive SH groups per subunit of D-glyceraldehyde-3-phosphate dehydrogenase with p-mercuribenzoate were studied under various experimental conditions. It has been found that mercaptide formation of SH_{II} is slowed down by the coenzyme as well as certain anions (first of all phosphate and arsenate), and is accelerated by chloride. The reaction rate has a minimum value in neutral solution. Since the reactivity of SH_{II} has been interpreted on the basis of fluctuation of the protein structure (Vas, Boross, 1970), these results refer to the influence of bound NAD or some ions on the fluctuation of the enzyme molecule in the neighbourhood of SH_{II}. The pattern of inhibition by NAD indicates the existence of cooperative interactions between the subunits of the protein molecule. The reactivity of SH_{III} and SH_{IV} towards PMB is decreased by increasing concentrations of some anions, whereas it is unaffected by the coenzyme. The rate of the mercaptide formation of these buried thiol groups is practically constant in a wide pH range. Since the rate of this reaction is limited by the denaturation of the protein the experimental findings yield informations on the structural stability of the whole enzyme molecule.

Introduction

Muscle D-glyceraldehyde-3-phosphate-dehydrogenase (GAPD) has four identical subunits and contains four cysteinyl residues per subunit (Harris, Perham, 1965). One SH group per subunit is extremely reactive (nucleophilic SH group, SH_I; Racker, Krimsky, 1958; Pihl, Lange, 1962; Boross, Cseke, 1967). In our preceding paper, the lesser reactivity of the other three thiol groups per subunit of GAPD (SH_{II}, SH_{III} and SH_{IV}) towards p-mercuribenzoate (PMB) has been described (Vas, Boross, 1970). On the basis of kinetic analyses we interpreted the diminished reactivity of SH_{II} by the fluctuation of the protein structure. This mechanism differs in many aspects from that of the reaction of buried thiol groups (SH_{III} and SH_{IV}), i.e. from denaturation changes of the protein molecule (Friedrich, Szabolcsi, 1967).

We have observed during the study of the nucleophilic SH group of the enzyme that various factors, such as the presence of polyvalent anions, the removal of bound coenzyme or its replacement by NADH, affect the reactivity of SH_I (Boross et al., 1969). It is known that SH_I corresponds to the Cys-149 residue in the amino acid sequence, and at a distance of four residues from this group

there is another cysteinyl residue, Cys-153 (Harris, Perham, 1968). SH_{II} is presumably identical with Cys-153, as several authors claim that after the modification of Cys-149 this thiol group reacts earlier than Cys-244 and Cys-281 do (Olson, Park, 1964; Harris, Perham, 1964; Davidson et al., 1967; Little, O'Brien, 1969; Parker, Allison, 1969; Wassarman, Major, 1969; Boross, 1969). Although this question is not yet conclusively answered, there is undoubtedly an SH group in or near the active centre of the enzyme beside the nucleophilic SH_I that directly participates in catalysis.

The question arises, how the factors that so profoundly influence the reactivity of SH_I affect the neighbourhood of the active center. It seemed right to assume that by studying the kinetics of the modification of SH_{II} and of the buried SH_{III} and SH_{IV} residues with PMB, we could gain information about the structure of the enzyme molecule in different environments.

Materials and Methods

The experiments were carried out with swine muscle GAPD in the same manner as described in our preceding paper (Vas, Boross, 1970).

The pH of the reaction mixtures was adjusted by dilution of the gel-filtered enzyme solution with an appropriate buffer. In the acidic region acetate buffers, ionic strength 0.05, were used. The pH was always measured at the temperature of the experiments (5 °C) and was checked after the experiment.

The following chemicals were used: PMB (British Drug Houses), NAD (Reanal), NADH (Reanal), NADP (Sigma) were commercial preparations. PGA was prepared from fructose-1-6-diphosphate as described by Szewczuk et al. (1961). The other chemicals were reagent grade preparations.

Results

1. Effect of coenzyme on mercaptide formation

After addition of PMB to the enzyme containing firmly bound NAD, SH_{II} reacted slower than in the case of charcoal-treated enzyme (Table 1). The reaction of the buried SH_{III} and SH_{IV} groups was not affected by NAD. The same effects were observed, when first PMB (equivalent to the amount of nucleophilic SH group) was added to the NAD-free enzyme, then 1 mole of NAD per subunit and then PMB again. The inhibitory effect of NAD could be observed also in this case.

Contrary to the effect of NAD, the addition of NADH or PGA to the NAD-free enzyme did not influence the reaction of either SH_{II} or the buried SH groups with PMB. NADP, which does not bind to the enzyme (Arnon et al., 1954), had no effect on the reaction either.

To characterize the inhibitory effect of NAD, the dependence of the extent of inhibition on the amount of coenzyme added to the NAD-free enzyme was

Table 1

The apparent first order rate constants for the initial part of the reaction of SH_{II}

GAPD concentration 1.25×10^{-6} M, in tris buffer of ionic strength 0.05, at 5 °C

pH	Additions	GAPD + 2 moles PMB/subunit	GAPD–NAD + 2 moles PMB/subunit
		min^{-1}	
7.0	—	0.52	0.30
	1 mole NADP/subunit	0.52	—
	1 mole NADH/subunit	0.52	—
	50 moles PGA/subunit	0.52	—
	0.3 M sulfate	0.17	0.06
	0.3 M phosphate	0.09	0.05
	1 M chloride	1.90	0.78
8.5	—	0.96	0.60
	0.3 M sulfate	0.24	0.15
	0.3 M phosphate	0.14	0.09
	1 M chloride	2.20	0.95

studied. The rate constants measured at various NAD concentrations are shown in Fig. 1. The high light absorption of NAD did not allow us to further increase the concentration of the coenzyme.

In our previous experiment with NAD-free enzyme we have established that the apparent first order rate constant of mercaptidation of SH_{II} is independent of protein concentration (Vas, Boross, 1970). In the presence of NAD this kinetic constant decreased with increasing enzyme concentration, gradually approaching a minimum value (Fig. 2). It should be noted that this experiment was carried out in the presence of phosphate to slow down the otherwise rapid reaction (see Section 2) and to increase in this way the accuracy of the measurement.

2. Effect of anions on mercaptide formation

The presence of various anions in the reaction mixture substantially influenced the rate of reaction with PMB of both SH_{II} and buried SH groups. It did not affect either reaction whether K^+, Na^+ or NH_4^+ was used as cation. Figs 3 and 4 show the change of rate constants as a function of anion concentration.

Mercaptide formation with SH_{II} was slowed down by sulfate, phosphate, and arsenate, whereas it was increased by chloride. Phosphate and arsenate ions produced maximum inhibition already in very small concentrations, i.e. their effect on the reaction of SH_{II} was much more specific than that of sulfate or chloride (Fig. 3). The anions affected the reaction of SH_{II} also in the presence of NAD; their effects were practically additive (Table 1).

As seen in Fig. 4, the reaction of buried SH groups was not so specifically influenced by phosphate ion as that of SH_{II}; the inhibition was about the same as with sulfate ion. The mercaptide formation of SH_{III} and SH_{IV}, in contrast with SH_{II} was slightly slowed down by chloride ion.

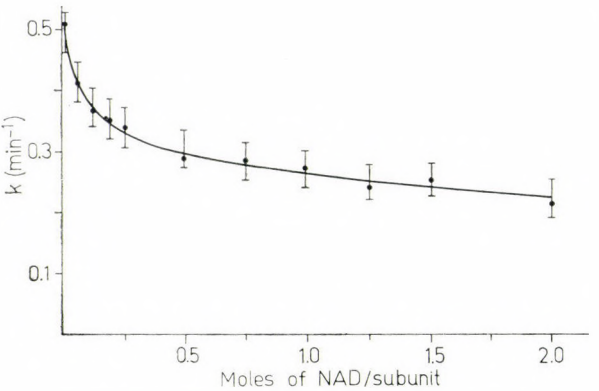

Fig. 1. Effect of NAD concentration on the apparent first order rate constants of the initial part of the reaction of SH_{II} with PMB. Different amounts of NAD were added to $1.25 \times \times 10^{-6}$ M NAD-free GAPD in 0.05 ionic strength tris buffer, pH 7, at 5 °C. The reaction was started by the addition of 2 moles of PMB per subunit. The continuous line is a hyperbola corresponding to the straight line in the double reciprocal plot of $\frac{1}{\Delta k}$ vs. $\frac{1}{[NAD]}$ (cf. the text). The symbols are the mean values of several experiments

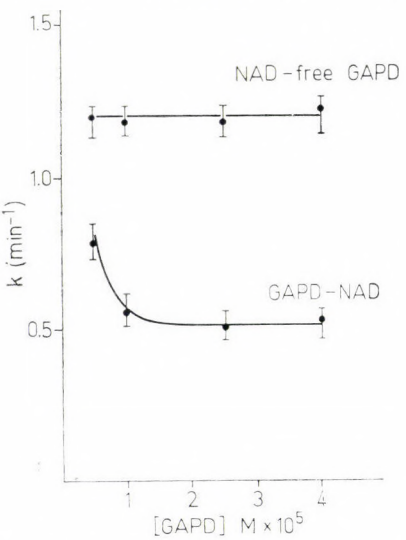

Fig. 2. Effect of protein concentration on the apparent first order rate constants of the initial part of the reaction of SH_{II} with PMB. To GAPD solutions of various concentrations PMB was added in amounts to give a constant final concentration of 1.6×10^{-4} M after the reaction of SH_I. The experiment was carried out in the presence and absence, respectively, of firmly bound NAD in tris buffer, pH 7, containing 0.16 M phosphate, at 5 °C

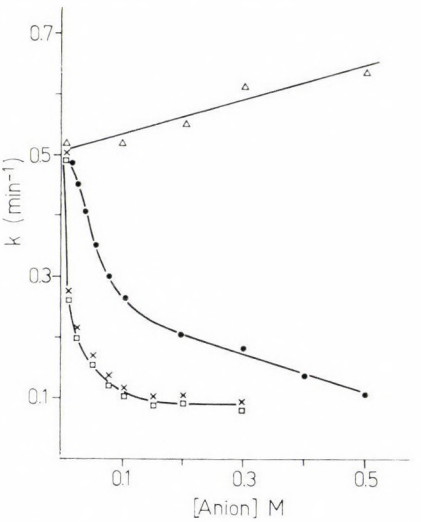

Fig. 3. Dependence on anion concentration of the apparent first order rate constants of the initial part of the reaction of SH_{II} with PMB. 1.25×10^{-6} M GAPD was treated with 2 moles of PMB per subunit in 0.05 ionic strength tris buffer, pH 7, in the presence of sulfate (● — ●), chloride (△ — △), phosphate (× — ×), and arsenate (□ — □) at 5 °C

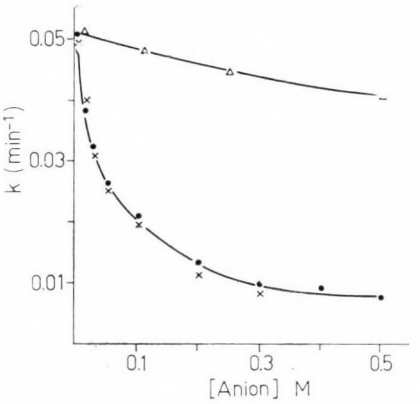

Fig. 4. Dependence on anion concentration of the first order rate constants of the reaction of buried SH groups with PMB. 1.25×10^{-6} M GAPD was treated with 4 moles of PMB per subunit in 0.05 ionic strength tris buffer, pH 7, in the presence of sulfate (●—●,) chloride (△—△) and phosphate (×—×)

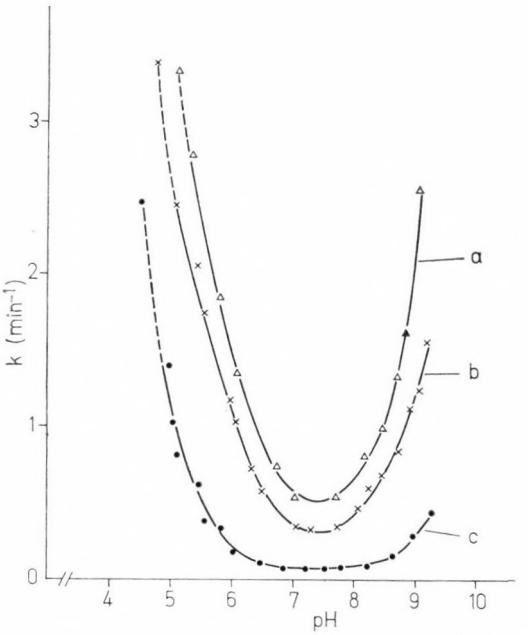

Fig. 5. pH-dependence of mercaptide formation of the SH groups of GAPD. 1.25×10^{-6} M GAPD was treated with 2 moles (curves *a* and *b*) and 4 moles (curve *c*) of PMB per subunit at 5 °C in 0.05 ionic strength tris and acetate buffers in the alkaline and acidic pH-ranges, respectively. Curves *a* and *b* refer to the initial phase of the reaction of SH_{II} in the absence and presence of NAD, respectively. Curve *c* shows the change of the rate constant of SH_{III} and SH_{IV}

3. Effect of pH on mercaptide formation

The rate constants of mercaptidation of SH_{II} and of the buried SH groups changed with pH according to a minimum-curve (Fig. 5). While for SH_{II} the curve attained its minimum at pH 7.0 to 7.5, for the buried SH groups the rate constant scarcely changed over a wide range of pH.

The inhibitory effect of NAD on the reaction of SH_{II} prevailed in a wide pH-region. The minimum of the curve was at the same pH-value as for NAD-free enzyme.

Discussion

In a previous paper (Vas, Boross, 1970) we have pointed out that the kinetic analysis of the mercaptide formation with SH_{II} supported the following mechanism:

$$[E] \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [E^*] + [PMB] \xrightarrow{k_2} [E] - [PMB] \qquad (1)$$

where [E] and [E*] are two conformational states of the protein. This suggested mechanism can be used for the interpretation of the results presented in this paper. It seems reasonable to assume that the coenzyme affects fluctuation of the protein by altering k_{+1}. This does not necessarily involve the displacement of fluctuational equilibrium, since k_{-1} may also change simultaneously. To examine the effect of coenzyme, the kinetic analysis was carried out for the reaction of SH_{II} in the presence of NAD. Fig. 6 shows that $\frac{1}{v}$ is a linear function of $\frac{1}{[PMB]}$ also in this latter case. This supports the validity of Eq. (1) in the presence of NAD, too. On the other hand, it can be calculated from the intercept and the slope of the curve that the value of $\frac{k_{-1}}{k_2}$ is the same for the free enzyme and for the enzyme containing NAD. As k_2 is characteristic of the reaction between free SH groups and PMB, k_{-1} could not change either in the presence of NAD. On the other hand, k_{+1} falls in the presence of NAD to about half of its value found in the absence of NAD (9.8 min^{-1} and 1.67 min^{-1}, respectively), i.e. NAD displaces the fluctuational equilibrium towards the form that contains SH_{II} in a masked position.

It should be emphasized in connection with the inhibitory action of NAD that the coenzyme is able to form a complex with the enzyme even after blocking the nucleophilic SH group with PMB. This finding is at variance with earlier experimental data on rabbit muscle GAPD (Velick et al., 1953; Velick, 1958).

The binding of NAD may also explain the dependence upon protein concentration of the rate of mercaptide formation with SH_{II} in the case of enzyme containing NAD (Fig. 2). Namely, by increasing the concentration of enzyme the concentration of NAD also increases, the dissociation of the enzyme–NAD complex becomes repressed and thus the inhibition is made more prominent.

The inhibitory effect of NAD is visualized in more detail in Fig. 1, which shows the variation of rate constants with NAD concentration at a given protein concentration. The following conclusions can be drawn from these experiments:

a) If the extreme case were assumed that the SH_{II} groups of GAPD–PMB–NAD complex react with PMB infinitely slowly, i.e. k tends to zero towards high NAD concentrations, even then it follows from the experimental curve that binding of NAD to a single subunit per molecule protects the whole molecule

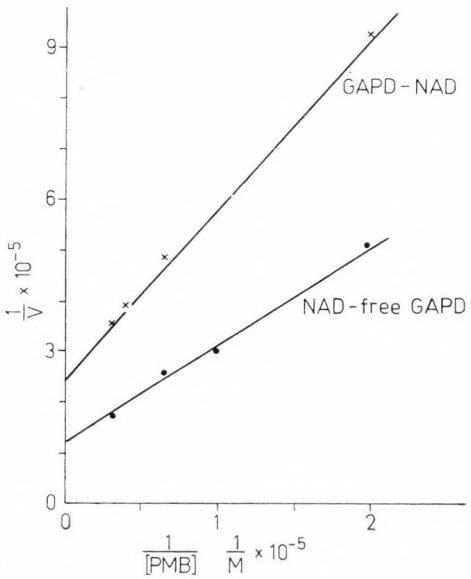

Fig. 6. Double reciprocal plot of the effect of PMB concentration on the initial rate of mercaptide formation of SH_{II}. The initial rate was calculated from the change of optical density at 255 nm in the first 15 sec of the reactions and is expressed as the concentration of mercaptides, in moles per litre per minute. Enzyme concentration was 1.25×10^{-6} M for both GAPD–NAD and NAD-free GAPD. $\frac{1}{v}$ is plotted against $\frac{1}{[PMB]}$ according to the equation (cf. Vas, Boross, 1970):

$$\frac{1}{v} = \frac{k_{-1}}{k_2 k_{+1}[E]} \cdot \frac{1}{[PMB]} + \frac{1}{k_{+1}[E]}$$

against PMB: in the presence of 0.1 mole of NAD per subunit the k-values decrease by 25 per cent as compared with the NAD-free enzyme. This conclusion is all the more justifiable, if the dissociable character of the complex is taken into account, which means that a part of the amount of NAD added to the reaction mixture is in the free state and obviously cannot exert any inhibitory effect. Furthermore, at a high protein concentration (Fig. 2) the curve does not tend to zero but rather to a finite limit with increasing NAD concentrations. It follows that the binding of 0.1 mole of NAD per subunit effects an inhibition of more

than 25 per cent. Thus the inhibitory effect of NAD appears as if multiplied by the cooperative interaction between subunits, which has also been postulated by Kirschner et al. (1966) and Conway and Koshland (1968). Our results agree with those of Listowsky, who found that one mole of NAD per enzyme molecule (tetramer) was sufficient to bring about almost all the changes in optical rotatory dispersion which could be effected by NAD (Listowsky et al., 1965).

b) The curve in Fig. 1 can be described within the limits of error by a hyperbola. This can be demonstrated by Südi's method (1970): the reciprocals of the differences between rate constants measured with NAD-free protein and with enzyme containing different amounts of NAD are plotted as a function of the reciprocals of NAD concentrations. A linear relationship is obtained, from which it follows that there is a hyperbolic relationship between the values of rate constant and of NAD concentration. The continuous line in Fig. 1 is a calculated hyperbola with the parameters derived from the double reciprocal plot. It can be seen that the theoretical curve satisfactorily fits the experimental data.

Taking into account the aforementioned and the fact that the dissociation constant of NAD is also different for the four subunits of the native enzyme (Conway, Koshland, 1968; De Vijlder, Slater, 1968), we suppose that the binding of a single NAD molecule per enzyme molecule causes the observed inhibition and the hyperbola describes the dissociation of this NAD molecule. The dissociation constant calculated from the linear double reciprocal plot is 1×10^{-6} M. Presumably this is the dissociation constant of the NAD molecule that binds most strongly in the protein containing 1 mole of PMB per subunit.

Our experiments also support the idea that SH_{II} is located in the neighbourhood of the active center. This may be the explanation of the fact that NAD influences the reactivity of only SH_{II} and that phosphate and arsenate ions affect the environment of SH_{II} much more specifically than that of SH_{III} and SH_{IV}. As phosphate is one of the substrates of the enzyme, the phosphate-binding site is presumably near SH_{II}. On the other hand, the effect of anions on buried SH groups can be interpreted in terms of an environmental influence on the steric structure of the whole protein molecule.

The pH-dependence of mercaptide formation (Fig. 5) is in good agreement with the fluctuation and denaturation models of the two kinds of "less-reactive" SH groups. The more pronounced dependence of the reaction of SH_{II} upon pH reflects the structural alteration of a small part of the protein molecule. On the other hand, the fact that the rate of mercaptide formation of buried SH groups hardly changes in a wide pH-range indicates that gross structural changes of the protein molecule are required for these thiol groups to react, and it has been shown (Elődi, 1960; Bolotina et al., 1967) that the physico-chemical parameters of the enzyme remain also unchanged in a wide range of pH.

The authors wish to express their thanks to Prof. F. B. Straub for helpful discussion

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Structural Investigations on Pancreatic α-Amylase

II. Determination of the Molecular Weight by Sedimentation and Light Scattering

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(Received February 16, 1970)

The molecular weights of hog pancreatic amylase and of the two fractions obtained from the recrystallized enzyme by DEAE-Sephadex chromatography (amylase I and II) were measured by three independent methods: sedimentation velocity and diffusion, sedimentation equilibrium, and light scattering. All three preparations sedimented as single sharp peaks indicating homogeneity in molecular size. The weight average molecular weights for amylase, amylase I and II were 52 600 ± 2500, 51 800 ± 2500, and 52 500 ± 2500, respectively. Other molecular parameters, i.e. sedimentation constant (4.5 ± 0.16 *S*) and diffusion constant (7.6 ± 0.2 × 10^{-7} cm^2 sec^{-1}), coincided within the limits of error for the three samples studied. The B value, the second virial coefficient, was 2.3 × 10^{-4} as calculated from light scattering data. These data suggest that the three amylase preparations are identical with respect to their molecular parameters.

The molecular weight of pancreatic α-amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) has been determined in a number of laboratories but the values reported fell in a wide range from 40 000 up to 61 000 (Caldwell et al., 1954; Loyter, Schramm, 1962; Danielsson, 1947; Kranz et al., 1965; Kluh, Sorm, 1968; Malacinski, Rutter, 1969). It is not clear whether this variation in molecular weight found with amylase is to be attributed to the different techniques applied in various laboratories or to the different procedures used for the isolation of the enzyme. Obviously, the large amount of proteolytic enzymes present in amylase preparations in the early stages of the purification procedure may influence the integrity of amylase. Proteolytic enzymes can be inhibited by specific inhibitors, e.g. diisopropylfluorophosphate.

Recrystallized pancreatic amylase can be resolved by DEAE-Sephadex chromatography into two or four fractions of the same specific activity (Szabó, Straub, 1966; Juhász, Szabó, 1967; Marchis-Mouren, Pasero, 1967), similarly to amylases from other sources (see e.g. Malacinski, Rutter, 1969). The molecular basis of this fractionation is not yet clear.

If the molecular weights of amylase I and II were different the variations in molecular weight obtained in different laboratories could be due to different amylase I and II ratios in the examined preparations.

In the present work we measured the molecular weight of hog pancreatic amylase and of its fractions obtained by DEAE-Sephadex chromatography (amylase I and II) by three independent methods: sedimentation velocity and diffusion, sedimentation equilibrium, and light scattering.

Materials and Methods

Hog pancreatic amylase was prepared according to Hatfaludi et al. (1966), and amylase I and II were kindly supplied by Dr T. M. Szabó. The protein was twice recrystallized and stored as a suspension in 0.01 M tris buffer, pH 7.4, containing 0.001 M calcium chloride and 0.001 M diisopropylfluorophosphate. The specific activity of the preparations was about 4000 amylase units as measured according to Smith and Roe (1949). Amylase I and II were separated by DEAE-Sephadex chromatography (Szabó, Straub, 1966).

Protein concentration was determined spectrophotometrically in 0.01 M tris buffer pH 7.4, at 280 mμ and was calculated on the basis of an extinction coefficient of $E^{1\%}_{1cm} = 24$ (Hsiu et al., 1964). For all measurements 5×10^{-3} M tris buffer, pH 7.4, which contained 5×10^{-3} M calcium chloride and 2×10^{-4} M β-mercaptoethanol, was used.

The determination of sedimentation and diffusion constants and the sedimentation equilibrium runs were carried out in a MOM G 120 type analytical ultracentrifuge equipped with both interference and Philpot—Svensson optics. The temperature stability was within ± 0.10 °C and the accuracy of rotor speed was ± 0.1 per cent. Sapphire cell windows were used when interference optics were employed.

Sedimentation velocity was determined in standard double-sector cells at a rotor speed of 50 000 rpm with Philpot—Svensson optics. Protein concentration varied from 0.2 to 0.5 per cent. The temperature was kept at 20 °C. The data were extrapolated to 0 protein concentration and were corrected for water and temperature.

Diffusion constant was measured in a capillary type, double sector, synthetic boundary cell. The protein solutions were thoroughly dialyzed against the buffer. Care was taken to form a sharp boundary free of turbulence. Low rotor speed (1000 to 3000 rpm) was used to reduce the sedimentation transport. Protein concentration varied between 0.1 to 0.5 per cent. Diffusion constants were calculated by the area method and the data were corrected for 0 protein concentration and 20 °C.

Sedimentation equilibrium measurements were carried out both by the high speed equilibrium method of Yphantis (1964) and by the low speed equilibrium procedure. The height of the liquid column in the high speed equilibrium measurements was about 2 to 3 mm. Initial protein concentration was 0.5 mg/ml and a 28 000 rpm rotor speed was chosen. The low speed measurements were performed at 8000 rpm rotor speed with a 2 mm high column of protein solution of 1 mg/ml concentration. The temperature in both cases was kept at 20 °C.

The *partial specific* volume of amylase was determined in 12 parallel experiments in pyknometers of 5.0 ml volume at 20 ± 0.05 °C temperature. The same value was used for the calculation of the molecular weights of amylase I and II, too.

Light scattering was measured in a Brice–Phoenix S-2000 type light scattering photometer at 436 and 546 mμ wavelengths. Solutions were purified by centrifugation in a preparative ultracentrifuge at 20 000 rpm for 2 to 3 hours and by filtering through a G 5 sintered glass filter under pressure directly into the care-

fully cleaned photometer cell. The intensity of scattered light was measured at 90° with protein solutions of 0.5 to 6.0 mg/ml concentration. The dn/dc increment of the refractive index was determined with a Brice–Phoenix differential refractometer at 436 and 546 mμ wavelengths and was found to be 0.168 and 0.160 at the two wavelengths, respectively. The molecular weights were calculated from the intercept of the plot Hc/τ *vs.* c (Stacey, 1956), and the second virial coefficient B was obtained from the slope of the straight line. The error of determinations was 8 per cent.

Results and Discussion

Amylase, amylase I and II sedimented as single sharp peaks, and the schlieren curves recorded at different enzyme concentrations were symmetrical. Thus all three preparations may be regarded as homogeneous.

The sedimentation and diffusion constants of the three amylases coincided within the limits of error (Table 1). The sedimentation constant exhibited a very

Table 1

Molecular parameters of amylase

Parameter	Method		Amylase	Amylase I	Amylase II
$S^0_{20,w}$, S			4.46 ±0.16	4.42±0.16	4.61±0.16
$D^0_{20,w} \times 10^{-7}$ cm²/sec			7.62 ±0.20	7.51±0.20	7.71±0.20
$\bar{V}$	pycnometry		0.728±0.03		
$\bar{M}_{sD}$			52 400±3000	52 700±3000	53 400±3000
$\bar{M}_n$			50 000±2500	50 500±2500	49 800±2500
$\bar{M}_w$	high speed equilibrium		51 500±2500	51 500±2500	52 400±2500
$\bar{M}_z$			53 200±2500	53 500±2500	54 000±2500
$\bar{M}_w$	low speed equilibrium		53 000±2000	52 200±2000	52 500±2000
	light scattering	436 mμ	52 700±4000		
$\bar{M}_w$		546 mμ	53 500±4000		
		436 mμ	2.3×10^{-4}		
		546 mμ	2.35×10^{-4}		

slight concentration dependence (Fig. 1), which suggests the absence of strong interactions between molecules under the experimental conditions applied.

The partial specific volume 0.728 determined by pyknometry was used in all calculations.

The $\bar{M}_{sD}$ average molecular weights calculated from the sedimentation constants, diffusion constants, and partial specific volume for amylase, amylase I, and II were 52 400, 52 700, and 53 400, respectively.

Molecular weights were determined also by sedimentation equilibrium experiments. The function of ln y *vs.* r^2 was plotted from the data of high speed

equilibrium experiments and straight lines were obtained (Fig. 2) characteristic of monodisperse systems. The $\overline{M}_n$, $\overline{M}_w$, and $\overline{M}_z$ average molecular weights are shown in Table 1. The minor deviations of the different average values may be due to the presence of a small amount of aggregates in the solutions.

The molecular weights obtained from low speed equilibrium experiments appear to be somewhat higher than those obtained by the high speed method. These differences can also be attributed to the presence of a small amount of aggre-

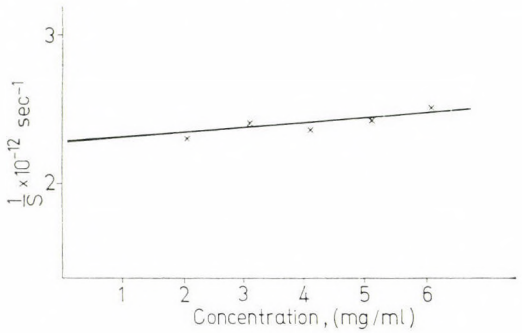

Fig. 1. The concentration dependence of the sedimentation coefficient of amylase

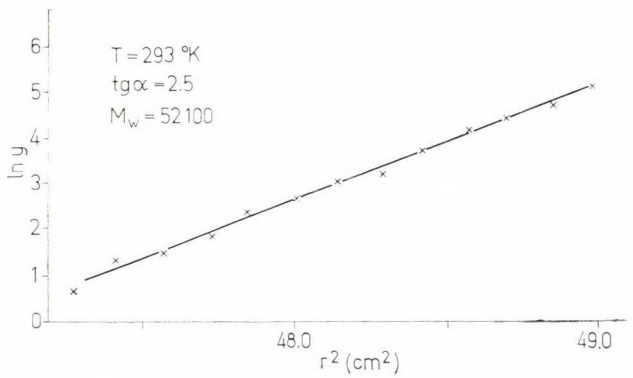

Fig. 2. Plot of ln y (fringe height) *vs.* r^2 in a typical high speed sedimentation experiment. The initial concentration of amylase was 0.5 mg per ml. Solvent: 5×10^{-3} M tris buffer, pH 7.4, containing 5×10^{-3} M calcium chloride, 2×10^{-4} M β-mercaptoethanol and 10^{-3} M diisopropylfluorophosphate. Rotor speed: 28 000 r.p.m.

gates, since the high speed method selectively emphasizes the effect of the lower molecular weight component.

The determination of molecular weight by light scattering was carried out only with amylase. Molecular weights of 52 700 and 53 500 were obtained by measurements at 436 and 546 mμ, respectively. These data coincide with those obtained by hydrodynamic methods (see Table 1). The second virial coefficient B

was 2.3×10^{-4} as calculated from the slope of plot Hc/τ *vs.* c (Fig. 3). This value is characteristic of a globular protein in weak interaction with the surrounding solvent medium.

The molecular weight data as well as the other parameters of amylase, amylase I and II listed in Table 1 indicate that these three samples appear to be

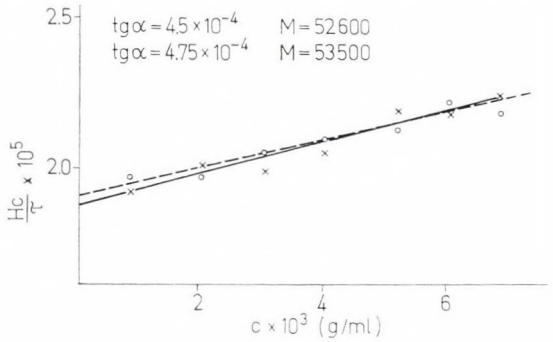

Fig. 3. Plot of $\frac{Hc}{\tau}$ *vs.* amylase concentration in a light scattering experiment. Solvent: 5×10^{-3}M tris buffer, pH 7.5, containing 5×10^{-3} M calcium chloride, 2×10^{-4} M β-mercaptoethanol and 10^{-3} M diisopropylfluorophosphate. Temperature: 22 °C. Wavelengths: o—o 546 mμ, ×—× 436 mμ

practically identical as far as the size and hydrodynamic properties are concerned. Thus, it is still to be unraveled whether the separation of amylase I and II on DEAE-Sephadex is due to conformational differences or to slight variations in amino acid composition.

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Identification of 1-Dimethylamino-naphthalene-5-sulfonyl Amino Acids by Thin Layer Electrophoresis

(Short Communication)

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(Received February 18, 1970)

A highly sensitive method for the determination of the N-terminal amino acid residues of peptides was introduced by Gray and Hartley (1963; Gray, 1967). They applied 1-dimethylamino-naphthalene-5-sulfonic chloride* to label the N-terminal amino group. After acid hydrolysis the strongly fluorescent DNS-derivative of the N-terminal residue could be identified either by thin layer chromatography (cf. Pataki, 1966; Woods, Wang, 1967) or by electrophoresis on paper (Gray, Hartley, 1963; Gray, 1967) or on cellulose thin layer (Arnott, Ward, 1967).

A sensitive and fast method of separation by thin layer chromatography was described by Woods and Wang (1967). The only disadvantage of this method has been that it is based on a two-dimensional separation. Therefore a whole plate is required for each sample.

Identification by high voltage electrophoresis on paper (Gray, Hartley, 1963; Gray, 1967) by the combination of two buffer systems gave satisfactory results in the separation, but the duration of the identification process was rather long (about 4—5 hours) and the sensitivity of this method was relatively low (1—5 nmoles of DNS-amino acids were required).

Thin layer electrophoresis on cellulose (Arnott, Ward, 1967) gave only a poor resolution and each run took about 3 hours.

Concerning the resolving power, sensitivity and the time required for separation, reasonably good results were obtained by using silica gel thin layer plates (MN-Polygram, SIL-NHR, Macherey, Nagel & Co., Düren) for electrophoresis.

The acid hydrolyzate of the DNS-peptide containing 0.1—1 nmole of the DNS-amino acid is dried down, redissolved in 5 μl 0.05 M ammonium hydroxide and applied to a pre-coated thin layer plate (10 cm × 20 cm). The position of the start is seen in Fig. 1. The size of the spot should be kept small, not exceeding 2 mm in diameter. To avoid overspreading, continuous drying in warm air is necessary during the spotting.

After applying the sample the plate is carefully wetted with buffer (pyridine–acetic acid–water 8 : 12 : 980 v/v/v, pH 4.35), the excess of the buffer is blotted away and the wet plate is placed on the electrophoresis plate. If an equipment not

* Abbreviation: 1-dimethylamino-naphthalene-5-sulfonyl = DNS.

specially designed for thin layer electrophoresis is used, the cooling plate is much longer than the thin layer plate itself. Therefore, to assure the electric contact, a wet paper wick should be laid from the edge of the thin layer plate to the electrode vessels. The cover is then replaced and the pressure adjusted to about 1.1—1.4 atm.

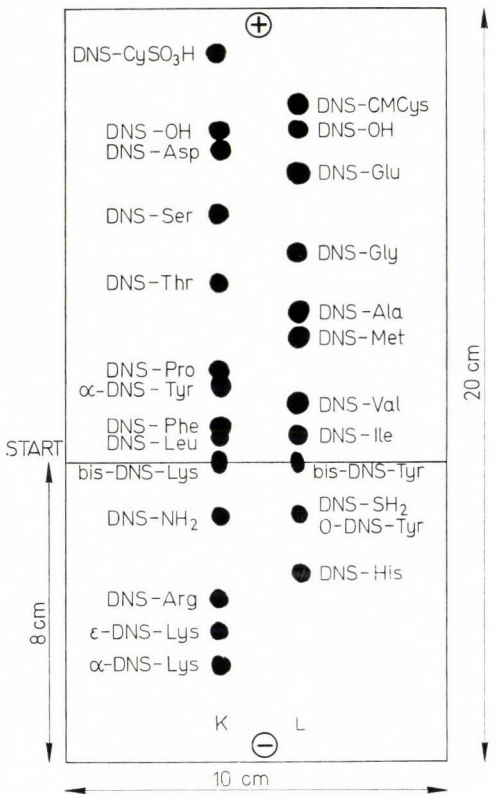

Fig. 1. Electrophoretic separation of the DNS-amino acids by thin layer electrophoresis, Pyridine-acetic acid–water 8 : 12 : 980 v/v/v, pH 4.35. 90 V/cm length, 8 mA/cm width. 25 min

Depending on the effectivity of cooling and the pressure applied, at 90 V/cm (in the case of a Shandon high voltage equipment this corresponds to 4000 V) the intensity of current is 6—8 mA/cm width. After 20—25 min run the plate is removed, dried in warm air and the DNS-derivatives are identified under UV light. Both short (254 mμ) and long wave (364 mμ) lights are suitable, although a prolonged exposition of the plate to 254 mμ light results in a yellow discoloration.

As illustrated in Fig. 1, nearly all of the DNS-amino acids (applied in two marker mixtures, K and L, respectively) are well separated. Unsatisfactory is the

separation of DNS-Ile, DNS-Leu and DNS-Phe, which overlap; neither bis-DNS-Tyr nor bis-DNS-Lys move off the origin and this makes their identification difficult.

These uncertainties can be cleared up by an additional chromatography following the electrophoresis. In this case the sample should be spotted about 2 cm apart from the longer edge of the plate. The distance of the origin from the cathode side should be the same as with the one-dimensional run. After the electrophoretic run the plate is dried and put in a chromatographic vessel for ascending chromatography perpendicular to the direction of the electrophoresis, in a chloroform–tert.-amylalcohol–acetic acid 70 : 30 : 0.5 v/v/v system. The differences in the R_f values of the amino acids mentioned above are great enough to get a good separation (DNS-Ile: 0.89, DNS-Leu: 0.63, DNS-Phe: 0.42, bis-DNS-Tyr: 0.23, bis-DNS-Lys: 0.33; Morse and Horecker, 1966).

An additional aid to the identification of DNS-His and DNS-Arg can be the application of the Pauly and Sakaguchi reactions. This is of special interest when the amount of ε-DNS-Lys is high.

The greatest advantage of this identification method is the rather short duration of the procedure. In most cases the DNS-method is combined with the Edman-degradation (Gray, Hartley, 1963a; Gray, 1967a). Since the time required for the Edman-degradation and for the preparation of the sample to the determination of the N-terminal residue is about 5—6 hours, it means a great help that to obtain the result of the previous Edman-degradation does not take more than one hour. In this way it is easily possible to keep the "one residue per day" sequencing speed.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (2), pp. 235—238 (1970)

Effect of Diethyl Pyrocarbonate on the Biological Activity of Intact TMV and TMV-RNA*

(Preliminary Communication)

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(Received March 4, 1970)

Recently, a new method based on the use of DEP* as a nuclease inhibitor has been developed for the extraction of undegraded nucleic acids from plant tissues (Solymosy et al., 1968; Lázár et al., 1969), human placenta (Abadom, Elson, 1970) and *E. coli* cells (Summers, 1970). Also, DEP has been shown to have a protective effect on polyribosome structure (Weeks, Marcus, 1969) and the integrity of ribosomal RNA (Hüvös et al., 1970) during the isolation of ribosomes. The successful application of DEP as a nuclease inhibitor warranted investigations on its mode of action. Whereas progress has been made in the study of chemical reactions of DEP with amino acids (Mühlrád et al., 1967) and amino acid residues in proteins (Ovádi et al., 1967; Hegyi, Mühlrád, 1968; Mühlrád et al., 1969; Ovádi, Keleti, 1969; Wolf et al., 1970) less attention has been devoted to the possibility of a reaction between DEP and nucleic acids. Although Fedorcsák et al. (1969) reported on the template activity and Abadom and Elson (1970) on the amino acid acceptor activity of RNA extracted by the DEP-method, the possibility of *some* (limited) reaction between DEP and nucleic acids has not been ruled out. The biological activity of viral RNA is known to be altered or lost upon a change of most probably only *one nucleotide residue* in the polynucleotide chain (Schuster, Schramm, 1958). Therefore, as the most sensitive test, the effect of DEP on infectious TMV-RNA and intact TMV, in which the nucleic acid is embedded in a protein shell, was investigated.

TMV was purified from infected Samsun tobacco plants, and infectious RNA was isolated from purified TMV as described by Fraenkel-Conrat (1966).

Treatment of intact TMV with DEP was carried out as follows: 1 ml samples containing 200 μg, 20 μg and 2 μg TMV, respectively, in 0.1 M phosphate buffer, pH 7.0, were thoroughly mixed with DEP at a rate of 25 μl DEP per 1 ml sample (a fivefold excess over saturation level). The mixtures were kept in an ice bath for 30 min and then used for infectivity assays. TMV preparations treated as above except the addition of DEP served as control.

* Abbreviations used: DEP, diethyl pyrocarbonate; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; TMV, tobacco mosaic virus.

Treatment of TMV-RNA with DEP was carried out as follows: 0.5 mg of TMV-RNA in 1.6 ml 0.1 M phosphate buffer, pH 7.0, was saturated and thoroughly mixed with DEP at a rate of 25 μl DEP per 1 ml sample. The mixture was kept for 30 min in an ice bath. TMV-RNA was then precipitated with 3 volumes of ice cold 96 per cent ethanol in the presence of one tenth volume of 20 per cent sodium acetate, pH 5.5. The precipitate was kept at —20 °C for 45 min and washed twice with 70 per cent ethanol to remove DEP. The washed TMV-RNA precipitate was taken up in 3 ml of 0.1 M phosphate buffer, pH 7.0, and the clear solution was used for inoculation. TMV-RNA preparations treated as above except the addition of DEP served as control.

Infectivity was tested with both intact TMV and TMV-RNA by the local lesion assay (cf. Roberts, 1964) on half leaves of *Nicotiana tabacum* L. cv. Xanthi.

Table 1

Effect of treatment with DEP on the infectivity of intact TMV

Inoculum	Number of local lesions*	
	Experiment No. 1	Experiment No. 2
Untreated TMV		
200 μg	261	485
20 μg	123	155
2 μg	36	23
DEP-treated TMV		
200 μg	140	194
20 μg	64	74
2 μg	24	19

* Average of six replicates.

Table 2

Effect of treatment with DEP on the infectivity of TMV-RNA

Inoculum	Number of local lesions*
Untreated TMV-RNA	
Experiment No. 1	78
2	115
3	132
DEP-treated TMV-RNA	
Experiment No. 1	0
2	0
3	0

* Average of six replicates.

It was found that the infectivity of the intact virus was reduced upon treatment with DEP (Table 1), and that of TMV-RNA was completely abolished by DEP treatment (Table 2). The deleterious effect of DEP treatment on the infectivity of intact TMV may be due to the reaction of DEP with the protein moiety of the virus, possibly inhibiting the attachment of the virus to proper cell receptors or inhibiting the release of viral RNA in vivo. This latter possibility is supported by the observation that so far no or little TMV-RNA could be extracted from tobacco mosaic virus by conventional methods in the presence of DEP (Bagi et al., 1970).

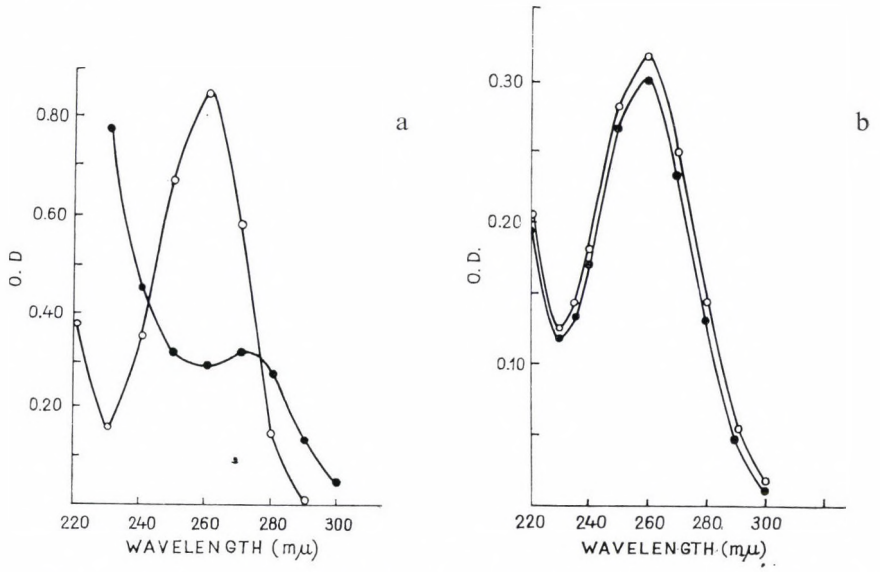

Fig. 1. Effect of treatment with DEP on the UV spectra of adenosine-5' phosphate (a) and TMV-RNA (b). ○—○, untreated; ●—●, treated with DEP

As to the mode of action of DEP on nucleic acids the carbethoxylation of the amino groups of nucleic acid bases seems to be the most likely explanation. This hypothesis is supported by the finding that DEP does not inhibit the transforming activity of bacterial DNA (Fedorcsák, Turtóczky, 1966). Bacterial DNA is a double stranded structure in which the bases are shielded owing to their steric position. The observation that transfer RNA possessing biological activity can be isolated by the DEP method from E. coli (cf. Fedorcsák et al., 1969) and human placenta (Abadom, Elson, 1970) is also in line with the hypothesis that fully or partially double stranded molecules react with DEP only to a limited extent, if at all. It should be stressed, however, that even the single stranded TMV-RNA appears to have a fairly limited reaction with DEP. This is shown by the following observations. If tested individually, all four bases, nucleosides and nucleotides of RNA seem to react with DEP. These reactions are reflected

in changes in infrared and ultraviolet spectra as well as electrophoretic behaviour (Kapovits et al., 1970). As a representative example the change in the ultraviolet spectrum of adenylic acid after DEP treatment is shown in Fig. 1a. The reaction apparently depends on steric factors since, in contrast to the bases and nucleotides, no spectral changes were detected in DEP-treated TMV-RNA (Fig. 1b). This suggests that DEP only reacts with some of the nucleotide residues in TMV-RNA. Such a limited reaction does not result in a spectral change of TMV-RNA, but apparently suffices to inactivate the molecule.

All these results indicate that DEP reacts with the building blocks of nucleic acids as well as with *some* nucleic acid species. The reaction with nucleic acids appears to depend on the secondary structure of nucleic acids and offers a new tool for studying the differential reactivity of various portions of nucleic acids possessing a secondary structure. Detailed results on the chemical reactions of DEP with bases, nucleosides, nucleotides and nucleic acids will be published elsewhere.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (2), pp. 239–248 (1970)

On the Dynamics of Accommodation for the Muscle-response of Anodonta Larva and the Electric Response of Anodonta Nerve

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The accommodation of the response of larval adductor muscle and that of the cerebrovisceral connectivum (CVc) of adult *Anodonta cygnea* L. has been investigated by impulses increasing exponentially. The excitation was indicated by an action potential of a given size and by a contraction, respectively. Under identical conditions the accommodation constant of CVc is 69 ± 17, and that of the glochidia is 57 ± 15 msec (mean value $\pm$ S.D.). The accommodation "constant" (λ) depends on the steepness of the stimulus-pulse and on the duration of the pulse, i.e. it is not constant. There exists an accommodation going together with threshold decrease, if $\lambda > T \times [\exp (d/T) - 1]$. Such cases were found with both experimental objects at $d = 100$ msec. A low accommodation constant corresponds to a jump to a new fixed threshold value which is d/λ. There is a complete transition between the two extremes. The accommodation and the anti-accommodation can have a role in the rhythmic activity of the systems. The dynamics of the accommodation is different depending on the degree of activation of CVc and the direction of the activation of glochidia, a fact indicating the presence of separate excitable structures. An approximation of strength–duration relationship on the basis of Hill's hypothesis is not of sufficient accuracy.

Introduction

One of the early efforts to describe the threshold increase of excitable tissues under the influence of direct current is the two-parameter linear hypothesis of the subthreshold excitation (Monnier, 1930, 1952; Rashevsky, 1933, 1936; Hill, 1936). The phenomenon was characterized by Solandt (1936) and Hill (1936) by the λ accommodation constant in a rather simplified way. Later on, some authors, for example Valbo (1964) stressed the extremely complicated nature of the processes reflected by λ. Guttmann and Barnhill (1968) propose the modification of the non-linear axon model of Hodgkin and Huxley (1952) on the basis of the temperature dependence of λ. Ernst (1969) thinks such an application of the mathematical methods concerning the continuous quantities to be generally defective. On the other hand Noble (1966), in agreement with several other authors, prefers the old — much more simplified — not accommodating, one-parameter model for short pulses.

On the basis of the background outlined above we think that the analysis of the accommodation remains one of the important questions of the hypotheses

wanting to describe the dynamics of the excitation and that of the threshold in particular. Therefore we have analyzed the apparent accommodation of two differently indicated responses (contraction and action potential) on composed objects.

Methods

The isolated cerebrovisceral connectivum of *Anodonta cygnea* L. (CVc) was kept in Marczynsky (1969) solution until recording; during the experiment it was lifted into paraffin oil, and the action potentials evoked through Ag electrodes were recorded by a DISA 14CCO2 symmetrical a.c. amplifier. A DISA Multistim stimulator was applied for stimulation. The stimulation was performed by means of pulses of the form $W_T = W_0 [1 - \exp(-d/T)]$ integrated by a simple RC-circuit of capacity output, where d is the duration of the pulse, $T = RC$ the time constant varying between 10 μsec and 2000 msec and W_0 is the output voltage of the stimulator.

The other object was the Anodonta larva, glochidium (Herbers, 1914), which was stimulated through a liquid (Lábos, 1964, 1967). The stimulus was indicated by the phasic contraction of its adductor muscle.

The λ accommodation constant was determined by the method of Solandt (1936) on the basis of the time constant dependence of the apparent threshold (V_T):

$$\lambda = TV_0/(V_T - V_0), \text{ where } V_0 = \lim V_T, \text{ if } T \to 0.$$

The experiments were performed at a temperature of 25—28 °C. A total of about 50 nerves and 100 glochidia were used for the measurements.

Results

1. The apparent and real increase of threshold voltage

When the time-constant dependence of the threshold is measured at a given arrangement of electrodes (Lábos, 1967, 1968) and by a pulse width of $d = 100$ msec, as shown in Figs 1A and 1B, then, if the time constant $T = 1000$ msec, a 15—20 times higher apparent, but only a 1.5—2 times higher real threshold is obtained.

According to Solandt (1936) the V_T-dependence is strictly linear; it is though not linear in Hill's theory (1936) but the deviation is very slight. With CVc, λ is really independent of T in certain cases, but with glochidia and, often with CVc too, we generally find the logarithmic connection $\lambda_T = \alpha \lg T + \beta$ (Fig. 2). Such an approximation can be only a partial one whatever high be the accuracy. Undoubtedly the threshold does not change near $T=0$, there is no accommodation, i.e. $\lambda \to \infty$, therefore, the steeply changing case of Fig. 2 must also be considered as real. So the λ_T relation has a steeply falling, and a settled interval, or perhaps one increasing again slowly.

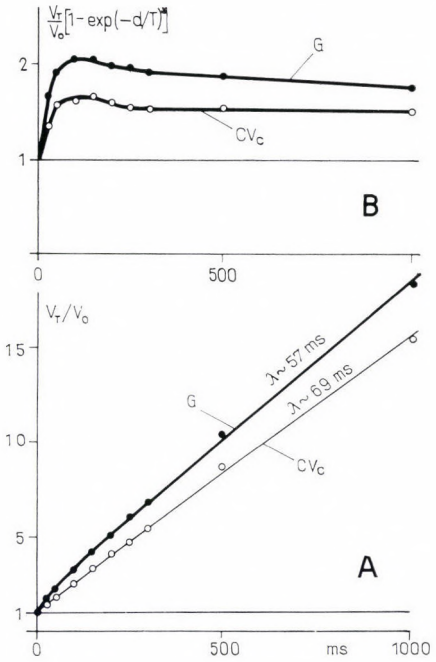

Fig. 1. The dependence of the relative apparent (A) and the relative real (B) stimulus threshold on the time-constant T of the exponentially rising stimulus pulse. G – the average of 10 measurements performed on glochidia; the space between electrodes is 4 mm, $d = 100$ msec. The average of the variation coefficients is 24.4 per cent. CVc – 12 measurements; the distances between the 4 electrodes are: 1–1–10 mm. The length of the earth plate was 10 mm

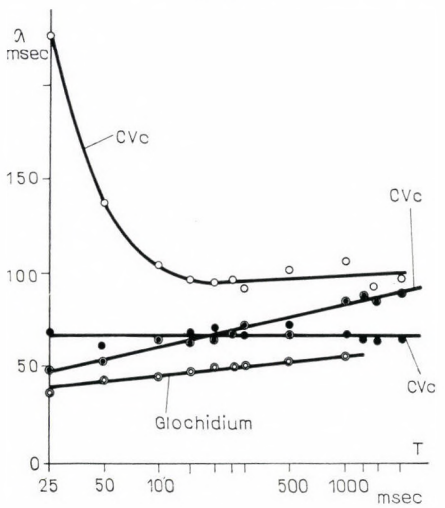

Fig. 2. The different T-dependence of the λ accommodation coefficient in some cases if $d = 100$ msec. In the linear cases $\lambda_T \sim$ const. and $\lambda_T \sim \alpha \lg T + \beta$, respectively

For the real relative threshold increase V'_T/V_0

$$\frac{V'_T}{V_0} = \left(\frac{T}{\lambda_T} + 1\right) \cdot [1 + \exp(-d/T)] \tag{1}$$

is obtained.

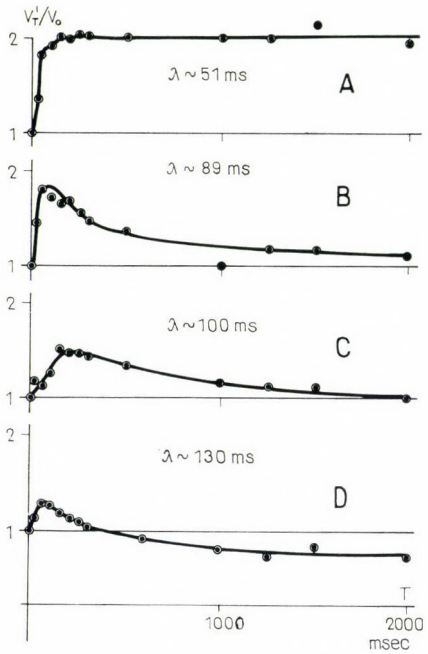

Fig. 3. The dependence of the real, relative threshold increase on the time constant T. 4 CVc of different accommodation constants. Case D represents an anti-accommodation. In each case $d = 100$ msec

The curve of the real threshold increase has interesting characteristics because of the shortness of the $d = 100$ msec pulse already in the case of λ, independent of the time constant. If T and d are given, there exists a limit-accommodation constant, λ_0. If $\lambda < \lambda_0$, the measured threshold is increased, if $\lambda > \lambda_0$ the threshold is decreased.

Its value is

$$\lambda_0 = T\,(e^{d/T} - 1) \tag{2}$$

and $\lim \lambda_0 = d$, if $T \to \infty$.

So with $d = 100$ msec the T and λ_0 values belonging together are:

T	50	100	250	500	1000	2000	∞	msec
λ_0	321	171	123	110	105	102	$d = 100$	

Out of the cases of Fig. 3, D shows such an anti-accommodation phenomenon, which cannot be found in Hill's hypothesis. Transitions can be found between the

real and the excitational accommodation. In the second extreme case the threshold settles to a new stable value (Fig. 3A). This new stable value is approximately d/λ, which corresponds well to the limiting value of V'_T/V_0 in the case of $T \to \infty$.

2. Transient and steady-state accommodation

The accommodation constant depends also on the duration of the pulse. If d increases, also λ usually increases. Fig. 4 shows that with an increasing duration of the direct current the magnitude of the threshold increase of CVc is smaller, approaching a constant in a non-linear way. The standard deviation of the λ values is high, the variation coefficient reaches even 50 per cent. The increase is significant. We met a similar regularity for the accommodation of glochidia.

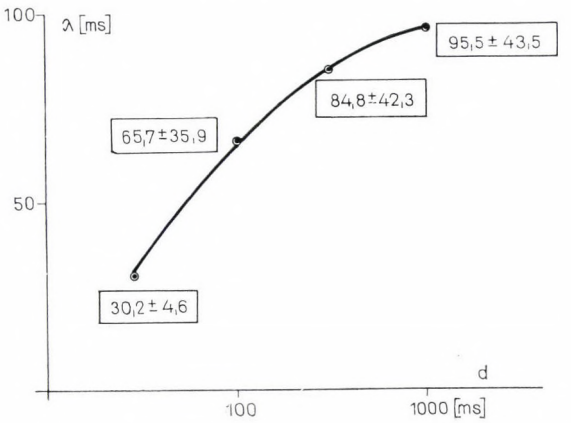

Fig. 4. The pulse-width dependence of the accommodation constant of CVc. Average of 22 cases ± S.D.; $T = 1000$ msec. The curve can be well approximated as follows: $\lambda(d) \sim \lambda_\infty/(1 + 129\ d^{-1.167})$ where $\lambda_\infty \sim 100$ msec; d in msec

3. The activation dependence of the λ value of CVc

Considering that CVc is a compound nerve (Zsukov, 1946; Lábos et al., 1963) and the measurement of the threshold has been carried out at a given activation (Lábos, 1967), at a given proportion of the maximal action potential, the activation dependence of λ can reflect the fibre composition. Fig. 5 shows that λ is a parameter sensitive enough for differentiating different groups of fibre, though, if the activation is 40—80 per cent, it is constant in many cases, independently of the size of activation. At the bottom of Fig. 5 we indicated the Blair-constant (Blair, 1932; Lábos, 1967) calculated on the basis of the strength–duration curve measured for the same case. It can be seen that this quantity $k = d^{-1} \cdot \lg [V.(V - R)^{-1}]$ depends differently on d in the differently accommodating cases. The steepness of this dependence can inform us even about the fact whether the accommodation is anomal, i.e. it goes together with a threshold decrease; with a 38 per cent activation in the given case $\lambda > \lambda_0$.

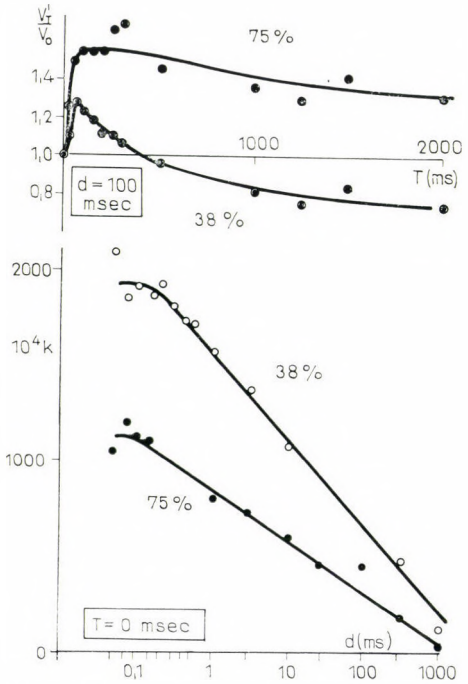

Fig. 5. The threshold necessary for producing the action potentials of 750 μV and 1.5 mV of CVc was measured (38 and 75 per cent activations, respectively). Above: the T dependence of the real relative threshold increase; below: the d-dependence of the Blair-constant k

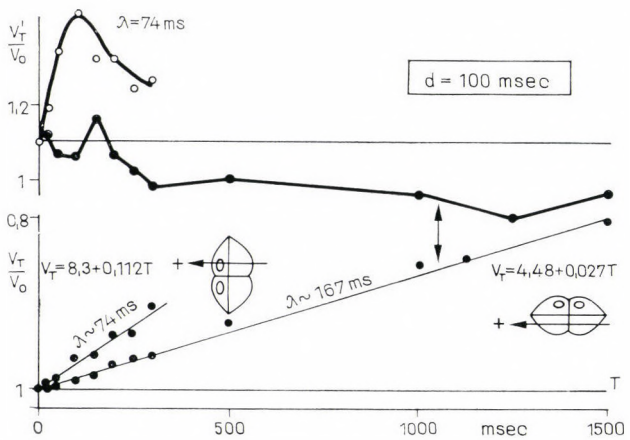

Fig. 6. The relative apparent and real threshold increase. Response of glochidia to transversal and longitudinal stimuli

4. The accommodation of the motor response of the glochidium is direction-dependent

It has been pointed out previously that the excitability of the adductor of the glochidium is anisotropic, which can be explained by the complicated nature of the response system (Lábos, 1968). Fig. 6 shows that the accommodation of the longitudinal and that of the transversal excitability of the animal also differ from each other. In the given case a threshold decrease belongs to the longitudinal accommodation, because with the $d = 100$ msec used here $\lambda = 167$ msec corresponds to the $\lambda > \lambda_0$ case from about $T > 100$ msec. Thus a longitudinal threshold decrease may be accompanied by a transversal threshold increase.

Discussion

After comparing the accommodation of different vertebrate and invertebrate preparations (Bullock, Horridge, 1965) with that of the glochidia and the CVc we are of the opinion that λ has not a value making much difference. This fact is expressed in the opinion of several authors that accommodation is partly of connective tissue origin (Frankenhauser, 1952; Tasaki, 1950; Diecke, 1954; Taylor, 1965). It is well known that, on the other hand, there are also definitely accommodating and adapting unicellular preparations (Araki, Otani, 1959; Bradley, Somjen, 1961; Arvanitaki et al., 1964, 1965; Arvanitaki, Romey, 1966). Therefore the integrating quality of the connective tissue can only hide the real threshold dynamics, but it is not the reason for the accommodation.

On the basis of the experiments several simple arguments can be advanced against the popular opinion that the λ constant alone can give an account of the dynamics of the accommodating threshold. The change of the accommodation depends on d, T, the activity and the direction, and we can state only concerning the last two factors that the compound nature of the response system of CVc or the glochidium is responsible for them.

We consider the existence of the limit-accommodation to be an important fact. While, on the one hand, it is true that autoactive nerve cells are accommodated rhythmically under the influence of a stimulus of permanent intensity (Granit, Skoglund, 1943; Arvanitaki et al., 1964, 1965; Arvanitaki, Romey, 1966), it is also true, on the other hand, that the accommodation does not require an unambiguous change of the threshold: the threshold can increase and decrease. And this phenomenon can be the basis of a rhythmic excitation. It is interesting to mention here that Cooley and Dodge (1968) with the analogous modelling of the modified Hodgkin—Huxley equations (1952) experienced a slow threshold-oscillation while applying a slowly increasing intensity of stimulus. The close correlation existing between the oscillations and accommodation of adaptive regulators is also known from technics (Chestnut, Mayer, 1955). Therefore the accommodation, as a process hindering rhythmicity, contains an information concerning the conditions of the rhythmic response.

Table 1

The Hill-approximation

d	Experimental	Theoretical make	Theoretical break	Deviation from the experimental, per cent
0.05	41.70	40.71	>1000	− 2.4
0.07	31.40	29.05		− 7.5
0.10	22.00	20.82		− 5.4
0.15	15.40	14.00		− 9.1
0.20	11.70	10.66		− 8.9
0.30	8.40	7.28		−13.3
0.50	5.45	4.59		−15.8
0.70	4.13	3.43	1000	−17.0
1	3.25	2.58	100	−20.7
3	1.63	1.31	50	−19.7
10	1.09	1.10	12.5	+ 0.9
30	1.00	1.28	4.5	+28.0
100	1.00	2.18	1.89	+89.0
300	1.04	10	1.12	+ 7.6
1000	1.06	202	1.00	− 5.7
				$\delta \sim 27.5$

$\lambda_1 = 131$ msec, $\tau_1 = 2$ msec, $h_1 = 1.0138$, $g_1 = 1.0155$

With the aid of Hill's (1936) theory the strength–duration relation (SDR) can be reconstructed if the time constant τ proportional to Blair's (1932) $1/k$ constant and the λ accommodation constant are known. They can be calculated from the experimental data of Fig. 5. If $z = V_d/V_\infty$ and the value of d is small, then

$$\tau \sim -\frac{d}{\ln\left(1-\frac{1}{z}\right)} \tag{3}$$

From the experimental data of Fig. 5 we obtained the following values: $\lambda_1 = 131$ and $\tau_1 = 2$ msec, $\lambda_2 = 75$ and $\tau_2 = 3.3$ msec.

If $\lambda/\tau = f$, the equation of the making (M) and breaking (B) SDR by Hill (1936) is

$$Z_M = \frac{h}{e^{-d/\lambda} - e^{-d/\tau}}$$

and

$$Z_B = \frac{1}{(1-e^{-d/\lambda})^g}$$

where $h = [f/(f-1)]\, f^{-1/(f-1)}$ and $g = f/(f-1)$; if calculated: $h_1 = 1.0138$, $g_1 = 1.0155$, $h_2 = 0.9928$, $g_2 = 1.0460$.

In Table 1 the experimental and theoretical making and breaking thresholds and the deviation are indicated for two cases of Fig. 5. It can be seen that the

oj SDR

Experimental	Theoretical make	Theoretical break	Deviation from the experimental, per cent
80.65	66.63	10^6	−17.4
51.71	47.50		− 8.2
36.84	32.13		−12.8
26.40	22.06		−16.4
21.10	18.65		−21.6
15.14	11.28	1000	−26.5
10.32	7	200	−32.2
8.32	5.23	100	−37.2
6.38	4	77	−26.3
2.95	1.78	25	−39.7
1.53	1.20	8.2	−21.6
1.05	1.48	3	+41.0
1.02	3.75	1.38	+35.0
1.00	54.25	1.02	+ 2.0
1.03		1.00	− 3.0
			$\delta \sim 26.7$

$\lambda_2 = 75$ msec, $\tau_2 = 3.3$ msec, $h_2 = 0.9928$, $g_2 = 1.0460$

Table 2

Test of the validity of the one-time-constant model (Blair, 1932) for Cooley and Dodge's (1968) data and for Anodonta CVc

d (msec)	Giant axon model (Cooley, Dodge, 1968)		CVc, 25–28 °C	
	τ (6 °C) ms	τ (18 °C) ms	τ (75 per cent) ms	τ (38 per cent) ms
0.05	2.08	0.84	2.07	4.10
0.10	2.09	0.82	2.28	3.85
0.20	2.07	0.83	2.24	4.16
0.50	2.01	0.81	2.50	4.91
1	1.96	0.74	2.72	5.87
2	1.83	0.54	Ø	Ø
3	Ø	Ø	3.14	7.26
10	Ø	Ø	3.97	9.46

deficiency of Hill's hypothesis is 25—27 per cent, and its multiplying by a factor does not help. Furthermore the anomaly is very great with long pulses where the make and brake responses are separated. On the basis of Fig. 4 it is justified to join at least a *d*-dependent resistance-coupling between Hill's (1936) *RC*-block equivalents of the subthreshold excitation and the excitation passing on. The theory gives a qualitative account of the anomalies that can be experienced really

at great pulse-times, but it separates the threshold dynamics from the dynamics of the excitation. Only non-linear models can eliminate this deficiency. But while analysing e.g. the features of the calculated and experimental SDR of Cooley and Dodge (1968) which were obtained with the Hodgkin—Huxley model, it was observed that the Blair-constant does not show the decrease characteristic of the accommodation, which refers to a difference of this kind between the giant axon and the present objects, and/or to the limits of the Hodgkin—Huxley approximation (Table 2).

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (2), pp. 249—257 (1970)

Effect of Heavy Water on the Amino Acid Incorporation of Subcellular Liver Particles

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(Received November 21, 1969)

The intensity of *in vitro* protein synthesis in guinea-pig liver microsomes increases 1.5 to 3-fold at a high concentration of heavy water. This is valid both for endogenous and exogenous (synthetic) messenger directed amino acid incorporation. The increase in endogenous amino acid incorporation should be attributed mainly to the heavier particles. Out of the various steps of protein synthesis, the intensities of aminoacyl-tRNA transfer and peptide-bond synthesis increase upon the effect of *in vitro* deuterization. Neither ribosomes obtained by deoxycholate treatment from microsomes, nor isolated mitochondria and nuclei displayed increased amino acid incorporation in a medium with D_2O. The increasing effect of heavy water on the *in vitro* protein synthesis is a feature characteristic not exclusively of microsomes from guinea-pigs. An increase can be observed also in microsomes from rat and chicken liver. The results suggest that heavy water influences some translation modulation mechanism operating at the microsomal level.

Introduction

It is well known that hydrogen bonds play an important role both in the structure and function of various biopolymers. The exchange of hydrogen by deuterium alters the hydrogen bonds, consequently, their role in the function.

Only very few data are available on the effect heavy water might exert on the protein synthesizing activity in subcellular liver particles. In previous experiments reported from this laboratory it was demonstrated that, at a high D_2O concentration, the guinea-pig liver microsomes incorporated appreciably more amino acids. On the contrary, the biosynthetic activity in ribosomes, obtained by deoxycholate treatment from the same fraction, remained the very same both in H_2O and D_2O media (Holland, Antoni, 1968). The present paper deals with the detailed results of experiments performed in order to elucidate the heavy water induced increase in protein synthesis.

Abbreviations used: ATP — adenosine triphosphate; GTP — guanosine triphosphate; poly-U — polyuridylic acid; TGA — trichloroacetic acid; PGA — perchloric acid.

Materials and Methods

Animals

Male and female guinea-pigs ranging from 350 to 440 g in weight, white rats weighing 150—200 g and one week old Leghorn chickens were used. Prior to the experiments the animals were given food and drinking water *ad libitum*.

Cell fractionation

The animals were sacrificed, their livers removed, cut into small pieces with scissors and washed with chilled physiological NaCl-solution. All subsequent operations were performed at 0—4 °C. The tissue fragments were homogenized in a 2.5 volume of Medium A (0.02 M tris-HCl, pH 7.6, 0.025 M KCl, 0.005 M $MgCl_2$, 0.006 M 2-mercaptoethanol, 0.25 M sucrose) in a glass homogenizer fitted with a teflon pestle. The homogenate was centrifuged at 600 *g* for 10 minutes. The purified nuclear fraction suitable for amino acid incorporation was isolated from the precipitate according to Blobel and Potter (1966). The various mitochondrial and microsomal fractions were precipitated from the nuclear supernatant by subsequent differential centrifugation: the precipitate of higher *g* value was always obtained from the previous supernatant. The fractions obtained were washed with Medium A and next suspended in Media B (0.02 M tris-HCl, pH–pD 7.6, 0.075 M KCl, 0.01 M $MgCl_2$, 0.006 M 2-mercaptoethanol, 0.25 M sucrose), with H_2O and D_2O, respectively.

The microsomal and ribosomal fractions were isolated as described by Korner (1961) and similarly suspended in Media B with H_2O and D_2O, respectively. The pH-5 fraction was prepared from the microsomal supernatant according to Hoagland et al. (1958).

Protein synthesizing systems

The amino acid incorporation system contained the following components: 20 mM tris-HCl (pH 7.6), 10 mM $MgCl_2$, 75 mM KCl, 6 mM 2-mercaptoethanol, 1 mM ATP, 0.1 mM GTP, 5 mM creatine phosphate, 20 μCi/ml creatine phosphokinase, ^{14}C-amino acid, 2 μCi/ml; cold amino acid mixture, 0.05 mM per each amino acid, save the one given in labelled form; no cold amino acid mixture was added to the ^{14}C-protein hydrolysate. Depending on the type of the experiment, microsomes, ribosomes, mitochondria or cell nuclei were added in concentrations of 1.5 to 2 g per ml incubation mixture, calculated according to the protein content; pH-5 fraction (1.5 mg protein per ml) was administered only in the case of microsomal or ribosomal amino acid incorporation.

Incubation was always performed by shaking at 37 °C. The incorporation rate was always calculated from the mean values of two samples. The reaction was stopped by chilled TCA (final concentration 5 per cent). The labelled protein fraction was isolated according to Siekevitz (1952). pH-5 fraction, dissolved in water and in heavy water, respectively, containing about 0.2 mg tRNA and 1.5 mg protein, was used to study the functioning of the adaptor system. Since it was established that the majority of tRNA are in a free, uncharged state in the

pH-5 fraction obtained from liver hyaloplasma, no preincubation was required for decharging. The reaction was stopped by cold TCA (10 per cent v/v). The precipitate was washed three times with cold TCA, extracted with hot PCA (0.5 N) and finally used for the determination of radioactivity and RNA-content.

The aminoacyl-tRNA transfer reaction was measured according to the method developed by Nirenberg and Leder (1964). A tRNA fraction (2.36×10^5 cpm/mg; 0.45 mg/ml) that had been charged by a mixture of ^{14}C-amino acids, was added to the microsomes (2 mg protein/ml) in the presence of the hyaloplasma containing the transfer factors (1.7 mg protein/ml). After incubation for a sufficient period of time, the reaction was stopped by chilled Medium B. The aminoacyl-tRNA–microsome complex was adsorbed onto a Millipore filter, washed with Medium B and the radioactivity of the filter determined. The intensity of the synthesis of the peptide bond was determined in an aliquot of the incubation mixture, by measuring radioactivity in the fraction insoluble in hot TCA.

Chemical assays

The protein content was determined according to Lowry et al. (1951) with bovine albumin as standard. RNA was determined by the orcinol colour reaction on the basis of the ribose content (Mejbaum, 1939).

Determination of radioactivity

Dissolved in 85 per cent formic acid, the protein precipitate was plated on aluminium planchets; the RNA nucleotide fraction was extracted with hot PCA, rendered ion-free by KOH and transferred on aluminium planchets. The samples were dried and their radioactivity determined in a Frieseke—Hoepfner gas flow counter. The radioactivity of the Millipore filters was determined under the same conditions.

Materials

The following chemicals and uniformly labelled amino acids were used: ATP, GTP, creatine phosphate, polyuridylic acid (Reanal, Budapest); creatine phosphokinase (Calbiochem, Luzern). Amino acids: ^{14}C-lysine, 112 mCi/mM (UVVVR, Prague); ^{14}C-valine, 160 mCi/mM, ^{14}C-phenylalanine, 244 mCi/mM, ^{14}C-protein hydrolysate of Chlorellae, 54 mCi/mAtom (Radiochemical Centre, Amersham).

The available labelled alga protein hydrolysates are known to contain appreciable acid-insoluble activity yielding high blank values in *in vitro* amino acid incorporation systems. Accordingly, this acid-insoluble activity was removed by PCA from the amino acid preparations used in the experiments.

Results

The *in vitro* endogenous amino acid incorporation by ribosomes from guinea-pig liver increases 1.5 to 3-fold in media with 70 to 90 per cent of heavy water (Holland, Antoni, 1968). The increase occurs also when amino acid incor-

poration is induced by some synthetic messenger (Table 1). As it clearly appears from Table 1 both the endogenous and the poly-U induced ^{14}C-phenylalanine incorporation appreciably increase in the incubation mixture containing 80 per cent D_2O.

The pD and pH optima of endogenous amino acid incorporation agree in both media (Fig. 1). However, it should be noted that the dissociation constant

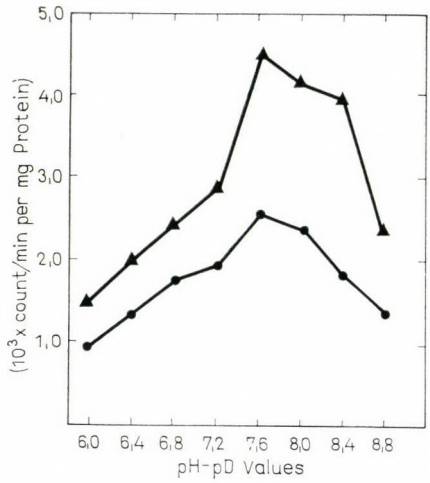


Fig. 1. Effect of pH—pD on the microsomal amino acid incorporation. Incorporation of ^{14}C-lysine was performed for 45 minutes in H_2O (●) and in 75 per cent D_2O (▲), respectively

Table 1

Poly-U directed ^{14}C-phenylalanine incorporation by guinea-pig liver microsomes; effect of high concentration of heavy water

Incubation was carried out for 60 minutes

System	^{14}C-phenylalanine c.p.m. /mg protein	
	in H_2O	in 80 per cent D_2O
Complete system — poly-U	298	1036
Complete system + poly-U (200 μg/ml)	2660	6558
Poly-U directed increase	2362	5522

of heavy water being different, the pD values measured by the electrical pH-meter were corrected according to Glasoe and Long (1960), by adding 0.4.

The microsomal fraction is known to be rather heterogeneous. Therefore, it was advisable to study to what extent did the heavy water induced increase in amino acid incorporation affects the various microsomal fractions. Following ^{14}C-valine incorporation performed in media with H_2O and D_2O, respectively,

the distribution of radioactivity between the various subfractions of the microsomal fraction was analysed by sucrose density gradient centrifugation (Fig. 2). As it clearly appears from Fig. 2, the D_2O induced increase in amino acid incorporation was mainly found in the more rapidly sedimenting fractions.

The purpose of the experiments to be described below was to elucidate which phases of the protein synthesis are affected by the increased activity induced by heavy water. As compared to the medium with H_2O, the medium with D_2O does not induce any increase in the isolated aminoacyl-tRNA system (Table 2). Since heavy water is known to have no effect on the functioning of the complete

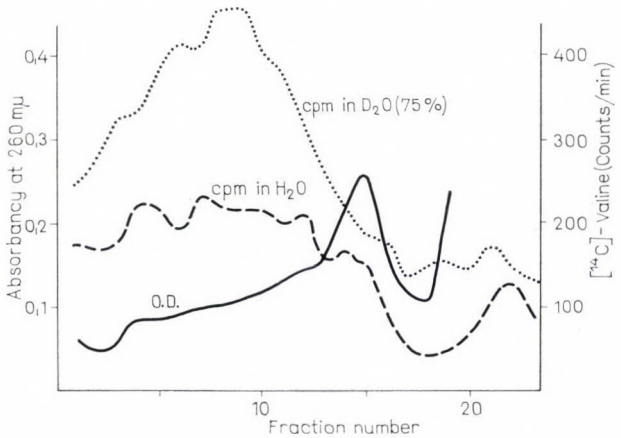


Fig. 2. Distribution of radioactivity between the various microsomal fractions after ^{14}C-valine incorporation in H_2O and D_2O (75 per cent) for 45 minutes; linear sucrose gradient (30—50 per cent). 0.5 ml of the incubation mixture was layered on the gradient (4.5 ml) and centrifuged for 110 minutes with 44 000 r.p.m. in the SW50 Spinco rotor. After running the bottom of each tube was punctured and fractions of three drops were collected and appropriately diluted. After measuring extinctions at 260 mμ, the fractions were precipitated with cold TCA (5 per cent v/v) in the presence of 1 mg bovine albumin as carrier. Incorporated radioactivity was determined as described in Materials and Methods

Table 2

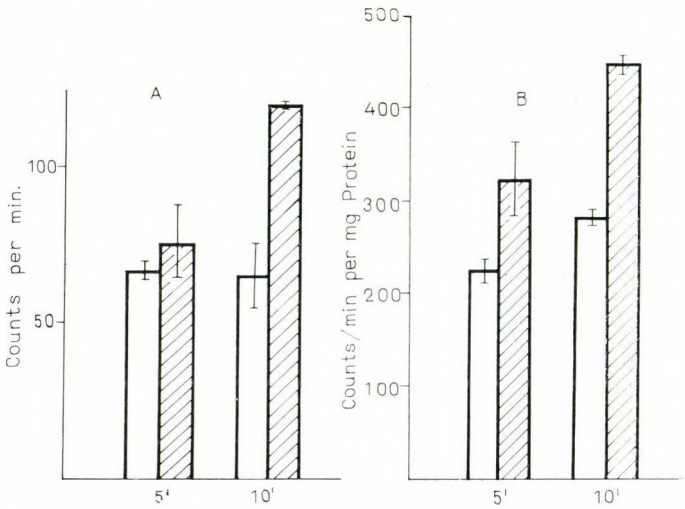
Effect of heavy water on the amino acid charging of tRNA both in the absence and in the presence of microsomes

In both cases (pH-5 fraction alone and pH-5 fraction in the presence of microsomes) incubation lasted for 30 minutes. The composition of the systems is given in Materials and Methods

System	Aminoacyl-tRNA synthesis (^{14}C-valine c.p.m./mg RNA)		Amino acid incorporation (^{14}C-valine c.p.m./mg protein)	
	in H_2O	in 75 per cent D_2O	in H_2O	in 75 per cent D_2O
pH-5 fraction	108 838	107 450	—	—
pH-5 fraction + microsomes	40 050	44 480	3850	7112

ribosomal system, this result was not surprising (Holland, Antoni, 1968). The problem that still requires elucidation is whether the same is valid for microsomes, too. When measuring aminoacyl-tRNA synthesis in the presence of microsomes, only a slight, 10—20 per cent, increase can be detected in the medium with D_2O (Table 2) that *per se* by no means explains the considerable increase seen in the whole protein synthesis.

The analysis of the further steps of protein synthesis reveals that the more intensive amino acid incorporation found in the presence of heavy water is a consequence of an increased aminoacyl-tRNA transfer and increased peptide bond synthesis (Figs 3A—B).



Figs 3A—B. Effect of heavy water on the aminoacyl-tRNA-transfer reaction (A) and peptide-bond synthesis (B). For details see Materials and Methods. Radioactivity values of transfer reaction are given for 0.5 ml incubation mixture. ☐ in H_2O; ▨ in D_2O (80 per cent)

It is well known that, in addition to cytoplasmic microsomes, intensive protein synthesis is proceeding both in the mitochondria and in the nucleus, which, however, differs from the microsomal protein synthesis in many respects (tRNA and ribosomal structure, sensitivity to antibiotics, etc.). On this basis it was indicated to study the intensities of amino acid incorporation in the mitochondria and in the nucleus in media with H_2O and D_2O, respectively, and to compare the results with those obtained for microsomes. As it clearly appears from Fig. 4, while the amino acid incorporation increasing effect of the D_2O medium is easily detectable in the fraction sedimented at 60 000 *g*, consisting mainly of microsomes, there is no significant difference in the two different media between the functional activities of the fractions sedimented at 6500 and 26 000 *g*.

Similarly, no appreciable difference is found between amino acid incorporation by nuclei suspended in water and in heavy water, respectively (Table 3).

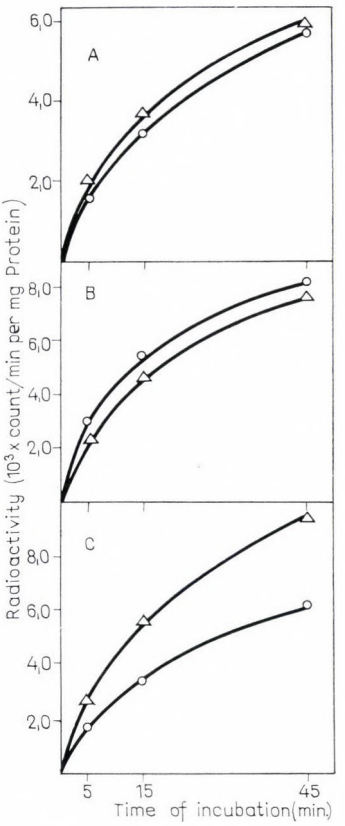


Fig. 4. Amino acid incorporation by various cytoplasmic particles in H_2O and D_2O. The incorporation mixtures did not contain pH-5 fraction, save the cytoplasmic particles sedimented at 6500 *g* (A), 26 000 *g* (B) and 60 000 *g* (C) for 10 minutes, respectively. A mixture of labelled amino acids (^{14}C-protein hydrolysate) was used: incubation was carried out in H_2O (○) and 80 per cent D_2O (△)

Table 3

Amino acid incorporation by isolated liver cell nuclei in H_2O and D_2O. Guinea-pig liver cell nuclei were isolated according to Blobel and Potter

For incubation mixture see Materials and Methods. ^{14}C-protein hydrolysate was used as radioactive precursor

Incubation time	Radioactivity (c.p.m./mg protein)	
	in H_2O	in 80 per cent D_2O
15 min	2035	2063
30 min	2755	2719

Accordingly, a high concentration of heavy water exclusively influences the microsomal protein synthesis *in vitro*.

The experiments reported on hitherto were performed with particles isolated from guinea-pig liver. However, the changed reaction of liver microsomes to heavy water is not characteristic exclusively of this species. A similar, increased amino acid incorporation upon the effect of deuterium was found *in vitro* also in the case of microsomes isolated from rat or chicken liver.

Table 4

Effect of heavy water on amino acid incorporation by rat and chicken liver microsomes

^{14}C-protein hydrolysate was used in the rat liver microsomal system and ^{14}C-valine for chicken liver microsomes. In both experiments incubation lasted for 50 minutes

Liver microsomes	^{14}C-amino acid c.p.m./mg protein	
	in H_2O	in 80 per cent D_2O
rat	12 461	23 710
chicken	388	1 414

Discussion

The ribosomes represent the protein manufacturing particles both in microorganisms and in higher-developed organisms. Within the cells of higher organisms the majority of ribosomes are functionally related to the endoplasmic reticulum. It is strongly suggested and supported also by a number of observations that the endoplasmic reticulum has some function that is more than a mere structural one. For instance, it is known that the *in vitro* biosynthesis of more complex proteins, consisting of several subunits (as antibodies, thyreoglobulin, glycoproteins, cellulase, etc.) has been accomplished in a microsomal system only (Nezlin, 1968; Wagner, Cynkin, 1969; Davies, Maclachlan, 1969). In this connection one should recall the experiments performed by Jondorf. Following pretreatment of rats with emetin and streptovitacin-A *in vivo*, he demonstrated an increase in the protein synthesis at the microsomal level, *in vitro* (Jondorf, 1968; Jondorf, Szapary, 1968).

The increase in amino acid incorporation found in the presence of a high concentration of heavy water is also one of the phenomena that occur at the microsomal level of protein synthesis. The fact that neither the mitochondrial nor the nuclear protein syntheses show any change under similar conditions also suggests that heavy water influences some such modulation mechanism at the translation level that operates in microsomes only and is presumably in some connection with the endoplasmic reticulum.

As demonstrated earlier by Henderson et al. (1967), certain control mechanisms are sensitive to the isotope effect of the deuteron. Thus, e.g. E. coli β-galactosidase synthesis may be inhibited by heavy water. Furthermore, any medium with D_2O alters protein synthesis in avian erythroblasts (Henderson et al., 1967a).

Moreover, D_2O is capable of appreciably influencing the allosteric sensitivity of the dehydrogenase enzyme to glutamic acid in isolated calf liver (Henderson, Henderson, 1969). The authors are of the opinion that these effects of heavy water are due in part to the replacement of H bonds by D bonds, and in part to the additional stabilizing effect resulting from the replacement of bound water by D_2O.

In the course of the replacement process some conformational change will occur that becomes stable. It is very likely that the increased activity of microsomes in heavy water is the consequence of some conformational change. Whether this change in the structure affects only some factor localized in the cell membrane or the connection between the membrane and ribosomes, requires further elucidation. Experiments in progress are designed to answer this problem.

The authors thank Prof. V. Várterész for his interest in this work. The skilled technical assistance of Mrs. Hanna Sinay and Mrs. Klára Szilvásy is gratefully acknowledged.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (2), pp. 259—260 (1970)

Freeze-Etching Investigation of the Ultrastructure of Striated Myofibrils

(Preliminary Report)

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(Received February 19, 1970)

The discussed problems of striated myofibrils require to adopt newer and newer experimental methods (e.g. Ernst, 1963).

As it was previously pointed out (Achátz, 1967, 1968) optic artefacts could be produced by superposition of layers forming an ultrathin section used for studying the muscle with electron microscope. Artefacts can be brought about by procedures of preparation needed for electron microscopic investigation (fixing, dehydration, staining) as well; however, very little is known about these structural alterations.

The freeze-etching technique developed by Moor et al. (1961) avoids such preparative operations. The flying muscle of the honey bee *Apis mellifica* has presently been investigated by the freeze-etching method according to the procedure described by Moor and Mühlethaler (1963). The dissected muscle fibers after having been kept in 30 per cent glycerine for one hour were freezed in Freon 22 at a temperature of —150 °C. Then the preparation was set on the precooled freezing table of a Balzers BA 510M cryostat under vacuum. The sectioning was followed by sublimation for one minute, finally a platinum-carbon replica of the sample was made. The replica was investigated by a Philips EM 300 electron microscope.

A part of the cross-section of a myofibril is shown in Fig. 1. The plane of the section was not exactly at right angles to the longitudinal axis of the fibril,* therefore this figure presumably shows the cross-section of the A band and Z-membrane of a contracted fibril. The network surrounded by a hexagonal system of holes is seen in the field of the A-band. Very likely a comparison can be made between this system of holes and the tube-like filaments seen on micrographs of some ultrathin sections (Hodge, 1955, 1956). The holes can represent longitudinal tube-like formations; also a certain transversal structure can be seen (Ernst et al. 1969). The structure of a Z-membrane is very complicated and a number of new experimental data are required to analyse it.

* This could be concluded from the width of the cross-section of the Z-membrane; the real angle might be about 80°.

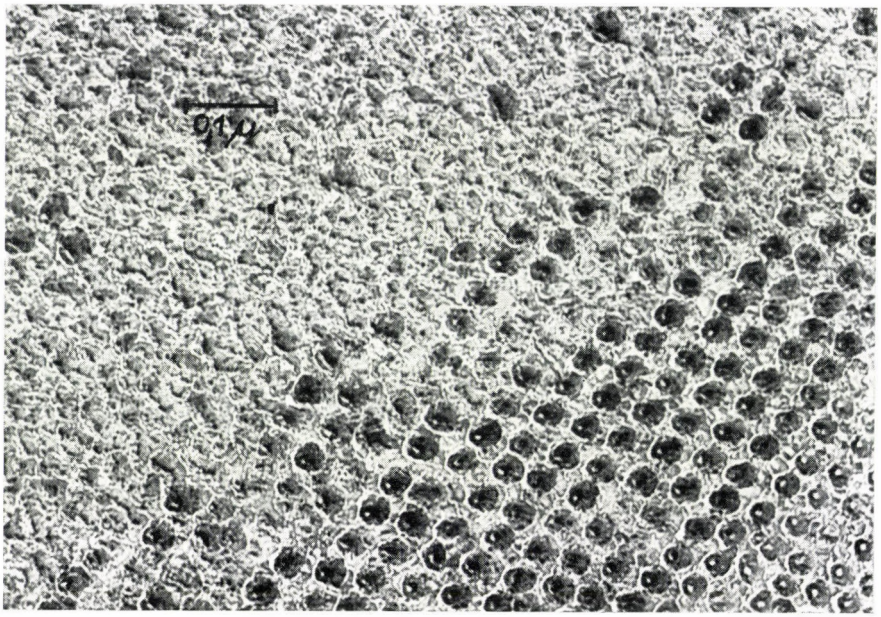

Fig. 1. Cross-section of a fibril of bee flying muscle, × 110 000

According to the data presented above the electron micrographs obtained from replicas of myofibrils prepared by freeze-etching method show such a structure which is hardly compatible with the pictures found in ultrathin muscle sections made by traditional technique. Therefore, in our opinion, it is important to carry out further studies on various kinds of muscle by this method.

These experiments were performed in the Electron Microscope Laboratory of the Measuring Service Department of the Hungarian Academy of Sciences.

The author is indebted to the scientific advisor Tibor Peres for his active participation in the work.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (2), pp. 261—262 (1970

The in vivo Absorption of Chlorophyll-b in Higher Plants

(Short Communication)

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(Received December 2, 1969)

In connection with the *in vivo* absorption of chlorophyll-b only the position of the red band at 650 nm was known earlier (Bogorad, 1965). Recently Yentsch and Guillard (1969) measured the complete absorption spectrum of chlorophyll-b *in vivo* in *Stichococcus mirabilis* and *S. cylindricus*. No similar information on higher plants is so far known.

Our investigation was carried out on normal peas (Deep Yellow Viktoria) and on their chlorophyll-b free mutants (No. 1206 A) induced by X-ray irradiation. The chlorophyll content of the mutant plants equals 40—50 per cent of that of the normal ones, while the carotenoid content is 70—100 per cent (Müller, 1964). The plants were grown under controlled conditions for a period of three weeks. The intensity of the illumination was 8000 lux and this was supplied by white fluorescent lamps (Daylight) in 12 hr light and 12 hr dark cycles at 30 °C and 25 °C, respectively.

The difference of the *in vivo* absorption of the normal and mutant leaves was measured with a Unicam SP 800 spectrophotometer in the subsidiary sample place mounted close to the detector. The normal leaves (containing both chlorophyll-a and b) were placed to sample beam, while the mutant ones (containing chlorophyll-a only) were used as reference. The mutant leaves have a lower absorption due to the lower chlorophyll content. Considering this fact the mutant leaves were used in several layers in order to receive a zero absorption difference at 680 nm. Since the chlorophyll-a content of the sample and of the reference was equal under these conditions, we received the absorption spectrum of chlorophyll-b *in vivo* directly as the difference of the absorption of the leaves. Furthermore the usual *in vivo* absorption measurement was carried out on the leaves and derivative absorption spectra were calculated at 2 nm intervals.

The comparison of the derivative spectra (Fig. 1) clearly shows the lack of chlorophyll-b in the mutant plants. The *in vivo* forms of chlorophyll-a seemed to be identical in both substances with the only difference that the chlorophyll-a 670 (?) presented itself at 672 nm in the case of the normal, and at 668 nm in the case of the mutant leaves.

The *in vivo* absorption spectrum of chlorophyll-b is shown in Fig. 2. For comparison the absorption in acetone is also demonstrated. It may be seen th a

the blue peak is shifted from 454 to 471 nm, while the red one from 645 to 650 nm. The *in vivo* red maximum agrees with the data given by others and the blue one differs in 1 nm from the location measured in algae (470 nm). We assume that the difference of the carotenoid content, if any, does not cause any interference in the development of the blue peak. The absorption maxima of β-carotene in the lamellas occur at 468 and 493 nm (Tae et al., 1968). Our curve does not show any shoulder around 493 nm; thus, presumably, the position of the blue peak in the *in vivo* absorption of chlorophyll-b was not interfered by the shorter carotene peak either.

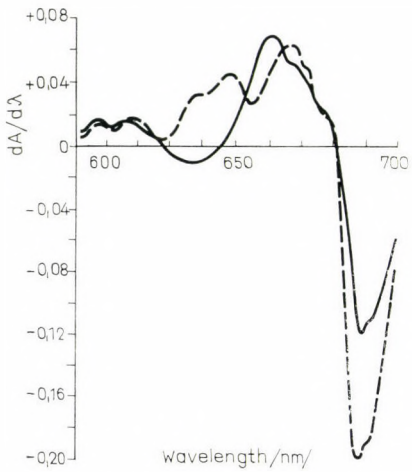

Fig. 1. Derivative absorption spectra of normal (– – –) and mutant (———) leaves

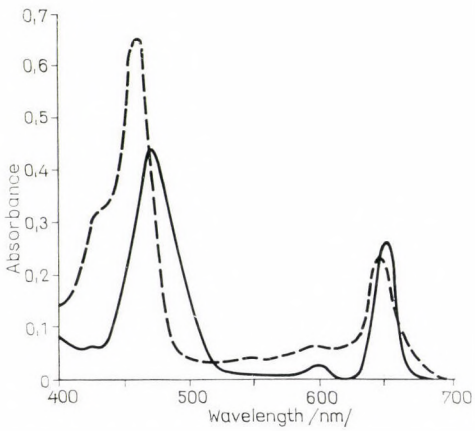

Fig. 2. Comparison of *in vivo* (———) and acetone (– – –) spectra of chlorophyll-b

A further difference between the *in vivo* and acetone spectra is the change in the ratio of the blue/red peaks: in an extract this value is 2.94 while *in vivo* it is 1.66. In algae a ratio of about 1.35 was recorded (Yentsch, Guillard, 1969). It may be interesting to note that in the case of chlorophyll-a, unlike in chlorophyll-b, a slight increase was measured in favour of the *in vivo* conditions (Kahn, Bannister, 1965).

We are indebted to Professor F. Müller, Hohenheim, for his kind supply of the seeds. The assistance of Sz. Eszter Barsi is appreciated.

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Book Review

Fortschritte der experimentellen und theoretischen Biophysik. Heft 11. Biophysikalische Untersuchungen von Zellen und Geweben durch passive elektrische Verhalten. Edited by F. Pliquett. VEB Georg Thieme Verlag, Leipzig, 1969, 106 pages. Price: 30,90 MDN.

In the first part of the paper the author defines generally the passive electric parameters; the dielectric constant, the conductivity, the time of relaxation and its distribution. He gives a brief survey of the characteristic frequency dependence of the electric parameters of the biological materials, and of the connection between the structural elements of different orders of magnitude and the electric characteristics that can be measured in every single frequency band. In the further chapters he treats in detail the methodological and theoretical problems of measurements at low and extremely low (10^{-3} Hz) frequencies. He gives an account of several original methods of measurement with which the low-frequency dielectric data of a single cell and tissues, respectively, can be determined. According to his measurements performed at unicells the physical and chemical changes following the growth, division and perishing of the cell lead to characteristic changes of the dielectric data. He observed similar changes also during the autolysis of the heart muscle. The author supposes that, at extremely low frequency, a dispersion caused by macromolecular complexes, by larger structural elements, appears, and therefore the measurements of low frequency show the macromolecular changes sensitively. The literary enumeration and experimental data of the paper introduce the reader first of all into the biophysical problematics of low-frequency measurements.

G. Masszi

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

A kézirat nyomdába érkezett: 1970. III. 26. — Terjedelem: 12,75 (A/5) ív, 78 ábra

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (3), pp. 265–272 (1970)

Some Aspects of Oxidative-Reductive Changes in Chloroplast Suspensions During the Process of Ageing

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The changes in the oxidation-reduction potential (redox potential, RP) due to periodic illumination of chloroplast suspensions of primary bean leaves shows characteristic differences, depending on the age of the leaf. These changes seem to be in relation with the chlorophyll concentration in the chloroplasts and with some of the photochemical activities, e.g. Hill-reaction, measured by the reduction of DCIP. The RP of samples of different ages starts from nearly the same value ($E_h = 385$ mV $\pm$ 10 mV), increases during the period of illumination and decreases in the dark. These changes are less regular in the case of chloroplasts of young leaves. The overall trend of the curves is characterized by a decrease of RP with successive illumination periods. This may be attributed to the exhaustion of the redox poising capacity of the system, which is more obvious in young leaves than in old ones.

Introduction

The process of ageing as well as the juvenility and senescence phenomena have attracted the interest of investigators in recent years. In plants, changes in the structure, chlorophyll content and photosynthetic effectiveness of chloroplasts during ageing have been reported by several authors (e.g. Ljubesic, 1967; Sironval, Englert-Dujardin, 1963; Clendenning, Gorham, 1950; Miller, 1960; Godnev et al., 1967). On the other hand, no explanation of the mechanism of oxidation-reduction changes during the process of ageing has been given. Energy transfer in photosynthesis is known to consist of a series of redox reactions. Thus, with the aid of RP measurements we can obtain a clear picture of the functional and dynamic changes of plant organisms during the process of ageing. The aim of the present investigations was to study the oxidative-reductive changes in chloroplast suspensions of primary bean leaves with their age. To compare our results with literary data, special attention was given to the photochemical activity (Hill reaction) of chloroplasts as well as to their chlorophyll content. It will be shown that in chloroplast suspensions it is possible to measure a characteristic RP value, though not giving a direct information on the biochemical conditions in the chloroplast themselves, is proportional to the resultant RP due to the concurrent changes in

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the chloroplasts. Namely, owing to the photochemical reactions, products of different normal potential and different oxidative and reductive capacity will be dissolved and diffused from the chloroplasts in the suspension, thus producing a resultant RP value.

It has to be remembered that both types of metabolic processes which take place simultaneously in green plants and autotrophic organisms, i.e. photosynthesis and respiration are opposite from the point of view of RP. Chloroplast suspension, in our case, can be considered as nearly free from respiratory effects which can generally be attributed to mitochondria localted in other parts of the cell. Thus, the process of photosynthesis with its light and dark reactions predominates in clean chloroplast suspensions. The dark reactions are exergonic. They take place without external energy transfer and are directed towards the equilibrium state. Consequently the course of their change can be followed by RP measurements (Dechev, Moscona, 1964).

The light reactions are endergonic and the final state which can be reached in the process is the stationary state. However, a system reaching stationary state will be far away from a thermodynamical equilibrium because the resulting products of light reactions would not persist in the equilibrium state. Thus, though the mixed potentials produced are generally not accessible for mathematical treatment (Spiro et al., 1961) so far, the results of RP measurements can give information about the direction and the time course of the process. Summing up, measurements of RP do not depend only on the composition of the system and on the reduced and oxidized state of the components, but also on the degree of approximation to the thermodynamic equilibrium the system has reached.

Materials and Methods

Plant material. Bean plants *(Phaseolus vulgaris* L.) cv. Surecrop were grown in pots in a temperature and light controlled growing chamber at 25—30°C and 2 000 lux. A sand culture technique was applied using Prianishnikov nutrient solution. The plants were grown as described by Szalai and Frenyó (1962).

Representative samples were taken at random from the primary leaves when the plants were 8 (very young), 13, 20 (mature), 27 (senescent) and 32 days old (very old). The experiment was repeated using a series of successive sowing dates so as to be able to perform the measurements on the above mentioned stages at the same time.

Chloroplast isolation. Chloroplasts were isolated according to Nobel (1967) with the following modifications:

1. To avoid contact with metal, the leaves were torn into small pieces by hand instead of using scissors (Arnon, 1949).

2. The grinding in the nylon bag lasted 1 min instead of 10 sec.

3. Every 5 g of plant material were ground in 20 ml of isolation medium (0.2 M sucrose buffered with 0.02 M Tris-hydroxy-methylaminomethane, HCl; pH 7.9).

4. An additonal wash was used with the same amount of buffer solution.

5. Resuspension was made in 10 ml buffer solution.

6. The whole process of isolation was made at a temperature not exceeding 2°C and under dim green light or in the dark.

Light-microscope studies showed that the chloroplast preparations contained only a few per cent of whole cells and that isolated chloroplasts resembled the structures seen in the intact cells.

Redox potential measurements. Redox potential of the chloroplast suspensions was measured by a "Biological pH Meter" (Radelkisz, Budapest) using a smooth platinum electrode with 200 mm^2 area against a saturated calomel electrode. Measurements were made at room temperature every 15—30 sec during successive illumination and dark periods of 5 min each. The sample cuvette (20 mm thick, containing 5 ml of chloroplast suspension) was illuminated by a high pressure stabilized mercury lamp with 40 000 lux intensity. This intensity was considerably higher than that necessary for light saturation of the sample, as shown by RP measurements performed with different ranges of variable light intensity.

Test of photochemical activity. The activity of chloroplast suspensions was investigated by measurement of the Hill reaction, according to the indophenol reduction method of Holt et al. (1951). The mixture contained 1—6 ml chloroplast suspension in Tris buffer and 10 ml 2-6-Dichlorophenolindophenol solution. The reaction rates were determined as the difference in absorbance at 540 nm of the dye for both illuminated and dark control samples (Δ A), divided by the chlorophyll content of the suspension (C_{chl}). The photometric measurements were carried out with a spectrophotometer "Spectromom 360" (Magyar Optikai Művek, Budapest). The illuminated samples were exposed to the light of a mercury lamp for 3 minutes after being kept in the dark for 5 minutes.

Determination of chlorophyll content. This was made by the method of Arnon (1949) using 80 % acetone for extraction. The suspension in acetone was centrifuged twice after removing the buffer. The chlorophyll content of the final extract was measured spectrophotometrically using the same spectrophotometer as above.

Results and Discussion

In discussing the changes in RP during illumination of the active chloroplast suspension, it seems advisable to treat the features characteristic of the different phases of ageing first (Fig. 1).

Redox curves for the isolated chloroplasts kept in the dark showed that the RP rate was stabilized in a few minutes at a potential depending on the isolation process, chiefly on the washing procedure. The stability of this starting potential depends mainly on impurities such as respiratory enzymes present in the cell-residues of the suspension. Chloroplast suspensions obtained with the above method gave stabilized RP values. The starting potential measured 5 min after

placing the electrode into the sample kept in the dark was $+385 \pm 10$ mV, irrespective of the age of the plant used. This value, as well as the other potential values measured, are within the overlapping region of redox reaction for pigment systems. The fact that the starting potential was identical shows that in the state of rest of the chloroplasts the RP value is not affected by the age of the plants. On illumination, however, the endergonic reactions of photosynthesis mentioned above

Fig. 1. Changes in RP of chloroplast suspensions treated in different ways and of a cell homogenate, on intermittent illumination with a period of 5 minutes. 1. Homogenate. 2. Chloroplast suspension washed with 5 ml suspending medium. 3. Washed twice with 5 ml liquid each time. 4. Washed twice with 10 ml liquid each time

begin and the potential increases (Fig. 2). The rate and the degree of this RP change depends also on the illumination and temperature used. Under the conditions of measurement employed, the stationary condition, when the RP does not change any longer, can be generally reached in two minutes. The increase in RP due to illumination can be ascribed to photoreduction of the chlorophyll with a simultaneous oxidation of the redox couples present in the system. After switching off the light the stationary conditions cease and the system turns to tend to the equilibrium through an exergonic reaction chain, and so the RP decreases.

The rate of this decrease is considerably lower than that of the potential increase caused by photoreduction and is stabilized more slowly, in several cases only in 10 minutes. All these changes can be repeated, i.e. on repeating the process of illumination described, the changes take place in the same directions as before.

However, by comparing the quantitative features of these periodical changes as a function of the age of the plants, we have a possibility to investigate the ageing process and the changes it involves. The variations observed in the different

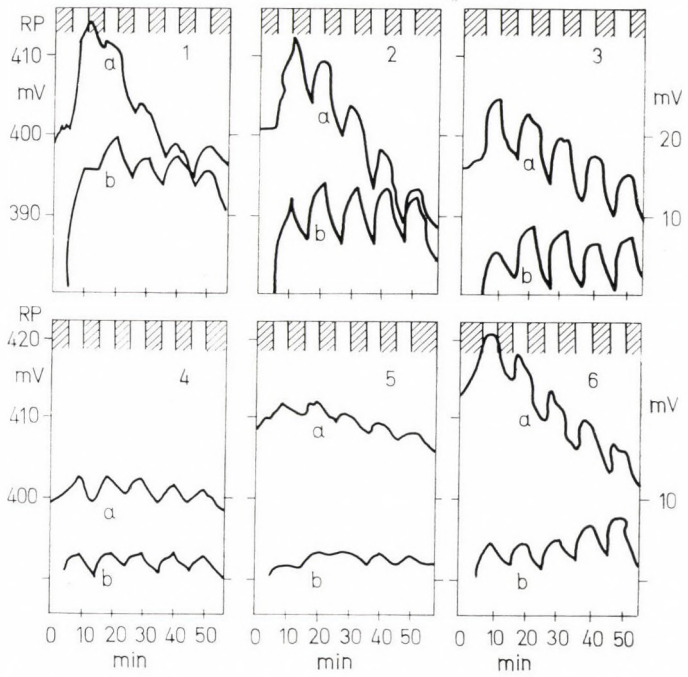

Fig. 2. Changes in RP due to periodical illumination of chloroplast suspensions from primary leaves of plants of different ages. a) Measured curve; b) Corrected curve after eliminating the general decrease of RP by calculation. 1—5. Primary leaves of plants of 6, 13, 20, 27, 32 days, respectively. 6. Mixed leaves of a mature plant

parameters studied show in some cases a monotonous change with the age, while in other cases they exhibit a maximum at the most active leaf stage (Fig. 4). A comparison of the RP changes during the first illumination period in leaves of different ages shows, that the rise of the RP value (height of the potential jumps) during this period gradually decreases with advancing age. The differences between the first and successive potential jumps are relatively high in chloroplast suspensions isolated from young plants and decrease with ageing of the plant till they nearly disappear in the last, senescent leaf stage. Besides the periodical change of the RP and its relatively constant amplitude, a continuous lowering tendency

throughout the whole time of measurement can be observed in the RP curves. The degree of this lowering tendency — which can be expressed as the potential difference per unit time — also decreases with the age of the plants, showing a very small or zero value in the senescent stage. This decrease in the slope of the RP curves with age can be attributed, in addition to the decrease in photosynthetic activity, to certain respiratory processes, associated with mitochondrial contaminations mentioned above or resulting from photorespiration. In accordance

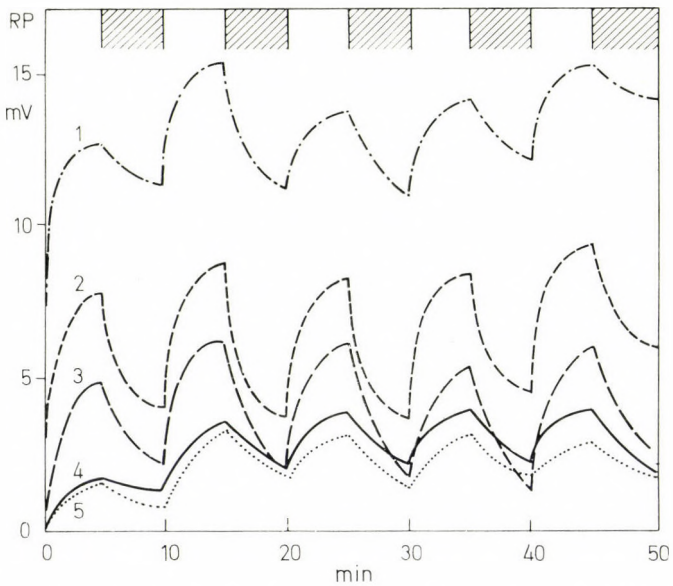

Fig. 3. Corrected curves of the mean values for chloroplast suspensions prepared from plant of different ages. The starting RP values were taken as zero and corrected for the general decrease in RP. Each curve represents the mean of 3 measurements

with literary data the changes in the activity of the Hill reactions used as a control in our measurements also show a decreasing tendency with the age of the plants.

In analyzing the RP curves of chloroplast suspensions isolated from plants of different ages, variations exhibiting a maximum with advancing age can also be found. The amplitudes of the RP curves produced by alternating dark and illuminated periods, as well as the respective mean values, have their maximum in mature plants. This change goes parallel with the total chlorophyll concentration. Namely, according to our measurements, the maximum chlorophyll content of isolated chloroplasts was attained in the 13-day-old sample (fully expanded leaves) when it was nearly the double of that of young expanding leaves; then followed a nearly linear drop to give the minimum value of chlorophyll content in the last, senescent stage (32-day-old plants).

In addition to the facts mentioned above it should be pointed out that the sudden rise of RP during the first illumination period and the tendency of quick lowering of the RP value thereafter, as well as the more irregular course of the curves in the very young plants can lead to the conclusion that the value of RP is relatively less consequent at this age, i.e. the stability of the system is rather low. In systems of such type the redox capacity — defined by the first derivative of the concentration measured chemically or electrochemically against the redox poten-

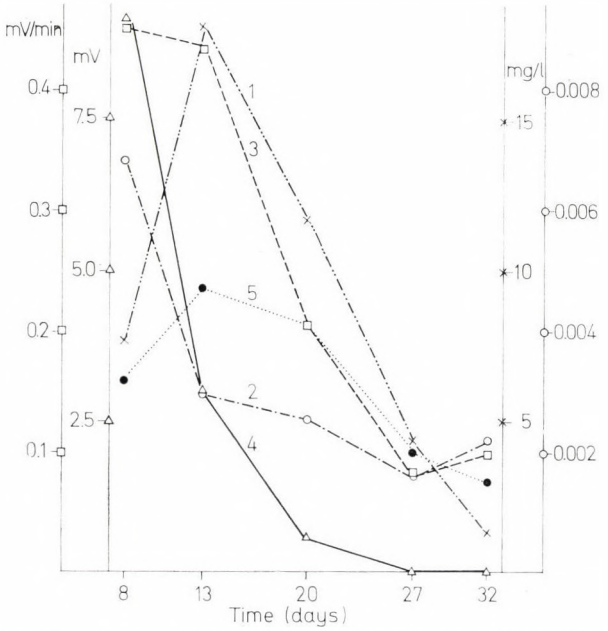

Fig. 4. Changes in different parameters with age of the plants. 1. Total chlorophyll content. 2. Photochemical activity as the reduction rate of DCPIP, given by the difference in absorbance of an illuminated and a dark control sample (Δ A), divided by the chlorophyll content (C_{chl}). 3. Rate of the general decrease of RP in mV/min expressed by the slope of the curves. 4. Difference (in mV) between the RP of the first light reaction and the mean value of those of the subsequent reactions. 5. Amplitudes (in mV) of the RP curves

tial (Nightingale, 1958; Marek, Stur, 1965; Marek, 1967; Marek et al., 1967) — is considerably lower compared to that of systems of older chloroplasts which show a higher stability.

In conclusion, we may state that our RP measurements, which were only of preliminary character, not only show a close relationship with other parameters used for evaluating energetical processes of photosynthesis, but — after further measurements necessary for a more detailed analysis — seem also to be of value in clearing up the mechanisms of this process. More comprehensive studies about the problem of ageing with the use of RP measurements are planned.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (3), pp. 273—277 (1970)

The Effect of X-Rays on the Salt Stimulation of DNA-Dependent RNA Synthesis In Vitro

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(Received January 23, 1970)

The effect of X-rays on DNA-dependent RNA synthesis in vitro has been investigated. The irradiation caused a minor change in the enzyme activity, but significant decrease was observed in the salt stimulation of RNA-polymerase. 25 kR X-ray irradiation stopped the increased RNA synthesis also at high Mg^{++} concentration. The significance of the above findings is discussed.

Introduction

DNA-dependent RNA-polymerase (EC 2.7.7.6.) catalyzes the transcription of the genetic information from DNA to RNA. The activity of RNA-polymerase isolated from E. coli appears to be influenced by salts. The rate of RNA synthesis is stimulated by divalent and monovalent cations as well (Fuchs et al., 1967).

At low salt concentration the polymerase has a typical plateau kinetics i.e. the incorporation of nucleoside triphosphates into synthetized RNA terminates as the incubation time progresses. An increase in the salt concentration of the reaction mixture results in a break of the plateau and in a striking stimulation of both rate and extent of RNA synthesis. At high ionic strength the polymerase continues the transcription along the template and the synthetized RNA contains also the regions of DNA, which are not transcribed at low salt concentration (Qasba, Zillig, 1968). The optimum ionic strength for enzyme activity is near the value which has been found under normal growth conditions in the bacterial cell (Luria, 1960). According to Fuchs et al. (1967), if this mechanism is involved in RNA synthesis in vivo, salts could regulate the rate and extent of RNA synthesis

According to earlier investigations the regulatory sites of aspartate transcarbamylase and phosphorylase *b* were more sensitive to X-rays than the catalytic site, however, phosphofructokinase and fructose-1,6-diphosphatase showed a higher radiosensitivity of their catalytic sites (Kleppe, Spearen, 1967; Damjanovich et al., 1967; Chapman et al., 1968; Little et al., 1968).

This communication presents a short analysis of the radiation sensitivity of salt stimulation of the DNA-dependent RNA-polymerase system from E. coli

Methods

E. coli B was grown at 37°C with vigorous aeration in 25 liter batches of glucose-mineral salt medium containing in 1 000 ml 5 g beef extract; 5 g pepton; 10 g $Na_2HPO_4 \cdot 7\ H_2O$; 10 g glucose and 1 g KH_2PO_4. The cells were harvested when their optical density reached $OD_{540}^{1cm} = 1.8$, i.e. at the end of logarithmic growth phase. The bacterial cells were collected in an "MSE 18" continuous-flow centrifuge. The bacterial sediment was washed twice with ice-cold TMA buffer (0.01 M Tris-acetate, pH 7.5; 0.022 M NH_4Cl; 0.01 M magnesium acetate) and centrifuged for 30 min at 15 000 × *g*. The bacterial sediment was stored in a deep freezer at —20°C until used.

Preparation of RNA polymerase aggregate enzyme. Sixty grams of cell-paste were ground with 120 grams of washed alumina for 30 min and mixed with 80 ml of TMA buffer. The bulk of alumina was removed by centrifugation for 30 min at 5 000 × *g*. The crude extract was centrifuged for 30 min at 25 000 × *g*. The supernatant fluid was centrifuged for 120 min at 105 000 × *g* to remove the ribosomal fraction. The supernatant was decanted and centrifuged again for 180 min at 200 000 × *g*. This 200 000 × *g* pellet contained the bulk of RNA polymerase activity. The pellet was suspended in TMA buffer. All steps of this procedure were carried out at 0 to 4°C.

Irradiation procedure. Samples containing 1650 μg DNA/ml were irradiated in the presence of air at 0°C with a TUR X-ray machine at a dose rate of about 1 600 R/min. The dose was determined with a Fricke-dosimeter, and the DNA content was estimated by the diphenylamine test (Dische, Schwarz, 1937).

Assay of RNA-polymerase. RNA-polymerase activity was measured by incorporation of C^{14}-labelled ATP into acid insoluble material. The usual reaction mixture contained in a total volume of 0.5 ml 0.03 M Tris-acetate buffer, pH 7.9; 0.13 M NH_4Cl; 0.03 M magnesium acetate; 0.001 M of GTP, CTP, UTP, each; 0.0005 M ATP-8-^{14}C, specific activity 0.3 Ci/mole; 0.01 M phosphoenol pyruvate Na salt; 20 μg/ml pyruvate kinase and 300 μg/ml of E. coli aggregate in terms of DNA content. The reaction was started by addition of enzyme. After 30 min incubation at 37°C the reaction was stopped with 1.0 ml of ice-cold water, containing 250 μg/ml bovine serum albumin and the synthetized RNA was precipitated with 1.5 ml 10 % TCA. For kinetic measurements, tenfold volume of the standard reaction mixture was used, 0.25 ml aliquots were removed after different incubation periods, pipetted into 1.25 ml ice-cold water, containing 250 μg/ml of bovine serum albumin and were precipitated with 1.5 ml of 10 % TCA. The precipitate was washed four times with 2 ml ice-cold 5 % TCA. The final precipitate of each reaction mixture was dissolved in 0.5 ml 3.5 % NH_4OH and plated on aluminium planchets, dried and counted in a window-less Gamma gas-flow counter.

Results

The inactivation by X-rays of RNA-polymerase enzyme aggregate of E. coli is shown in Fig. 1. As can be seen, about 25 kR X-irradiation caused a small decrease in enzyme activity. The low inactivation rate may be attributed to the contaminating proteins and nucleic acids. In spite of the low rate determined at low salt concentration (0.03 M Mg^{++}), 25 kR caused a striking decrease in the Mg^{++} and NH_4^+ ion activation of RNA-polymerase. The dose-inactivation curves at low salt concentration were linear in a semilogarithmic plot, demonstrating that the system is inactivated as an exponential function of the dose. The dose-inactivation curves of the salt stimulated RNA-polymerase were not linear in semilogarithmic plots, suggesting that the effect of X-irradiation in this case is counteracted by another effect.

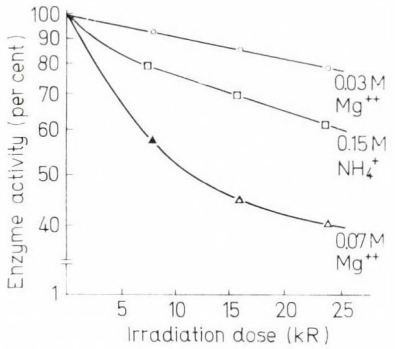

Fig. 1. Inactivation of RNA-polymerase by X-rays. The activity is expressed in per cent of the non-irradiated control. Incubation conditions were as described in Methods. Concentrations of salts were: (○) 0.03 M Mg^{++} and 0.13 M NH_4Cl, (□) 0.03 M Mg^{++} and 0.15 M NH_4^+, (△) 0.07 M Mg^{++} and 0.13 M NH_4^+

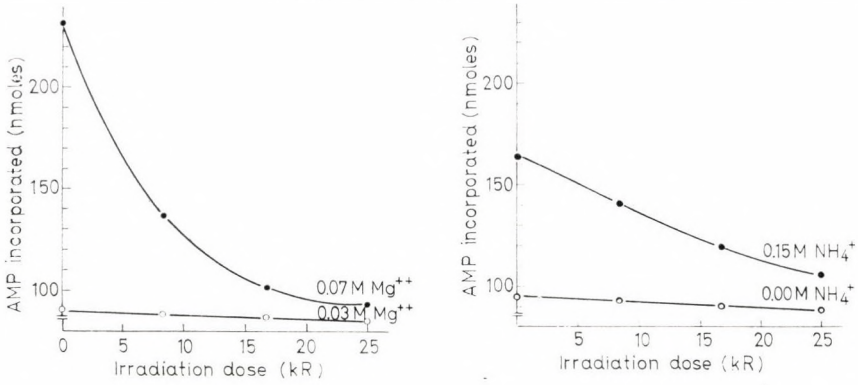

Fig. 2. Changes in the amount of incorporated AMP at low and high salt concentration after X-irradiation of RNA-polymerase. In Fig. 2a concentration of NH_4Cl was 0.13 M; in Fig. 2b concentration of magnesium acetate was 0.03 M

Effects of various Mg^{++} and NH_4^+ concentrations on the activity of non-irradiated and irradiated RNA-polymerase are illustrated in Fig. 2a and 2b. It is apparent that in the presence of catalytic amounts of Mg^{++} with and without 0.15 M NH_4Cl there was no dramatic change in the enzyme activity after a certain dose of X-rays, but the salt stimulation of RNA-polymerase suffered injury upon the same irradiation dose. After 25 kR irradiation we could observe only a slight stimulating effect of high salt concentrations. Fig. 3 shows the time kinetics of

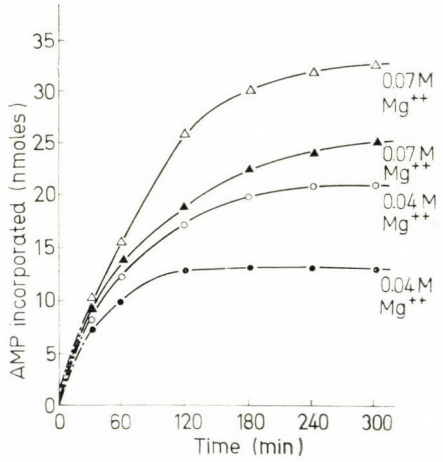

Fig. 3. Effect of different magnesium acetate concentrations on the time kinetics of normal (empty symbols) and irradiated (full symbols) RNA-polymerase. The incubation conditions are described in Methods

DNA-dependent RNA-synthesis in the presence of various Mg^{++} concentrations after 25 kR irradiation. We had chosen those concentrations of Mg^{++} at which the typical plateau form of kinetics did not appear in the case of the non-irradiated enzyme. The irradiated enzyme was unable to continue the RNA synthesis for many hours as the controls and the plateau re-appeared even in the presence of high concentrations of Mg^{++}.

Discussion

The results presented in this paper prove that the ionic stimulation of RNA-polymerase is more radiosensitive than its catalytic function. X-irradiation stopped the increased RNA-polymerase reaction in the presence of high Mg^{++} concentration.

Fuchs et al. (1967) suggested a mechanism for the action of the ionic strength on the polymerase reaction in the presence of double-stranded template. The enzyme has two distinct sites. One is the strand separation site which is required for the separation of the codogenic strand from the non-codogenic strand of DNA,

and the other is the strand exchange site that is necessary for the release of newly synthetized RNA from the template. At low salt concentration the newly synthetized RNA forms a hybrid with the codogenic strand and the polymerase is inactivated by first-order kinetics. This inactivation occurs at both low and high salt concentrations, but in the latter case reactivation takes place. The rate of this reactivation depends on the ionic strength (Fuchs et al., 1967).

We suppose that the most radiosensitive part of the enzyme is the strand exchange site. The indirect proofs are as follows: 1) Reduction of the incorporation of labelled ATP was low, i.e. the damage of the catalytic site was not considerable. 2) If the strand separation site had been more radiosensitive than the strand exchange site, RNA synthesis would not have started or at least the rate of incorporation of labelled ATP would have been retarded.

We cannot disregard completely the damage of the template, but the changes in the salt stimulating effects generally are attributed exclusively to the injury of RNA-polymerase. Experiments with highly purified RNA-polymerase are in progress.

This work was supported by the Hungarian Atomic Energy Committee.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (3), pp. 279–283 (1970)

Studies on the Fluorescence of Glycogen Phosphorylases

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The fluorescence of glycogen phosphorylase *b* and *a* as well as that of amino acid mixtures simulating the tyrosin and tryptophan composition of phosphorylases have been compared. The native enzyme exhibited a significantly stronger fluorescence than the equimolar amino acid mixtures. SDS* and urea, known to split the enzyme into subunits, decreased the fluorescence of phosphorylases. There was a nearly linear relationship between the fluorescence of dimeric and that of tetrameric proteins. Fluorescence intensity proved to be a sensitive tool in studying radiation damage of proteins.

Introduction

The native proteins have fluorescence spectra in the ultraviolet region, due to their aromatic amino acid and particularly to their tryptophan residues (Udenfriend, 1961). The relationship between fluorescence and the degree of molecular organization of macromolecules has been a scarcely investigated problem of molecular biophysics. Glycogen phosphorylases (α-1,4-glucan: ortophosphate glucosyltransferase, EC 2.4.1.1.) are well suited for studying such a question because they are built up from subunits. Phosphorylase *b* most likely consists of two identical subunits and phosphorylase *a* is built up from four subunits. Each subunit of phosphorylase *a* contains also one phosphoseryl residue.

In the present paper we report some experimental data on the correlation between the number of subunits and the fluorescence properties of glycogen phosphorylases.

Materials and Methods

Four times recrystallized phosphorylase *b* was prepared according to Fischer and Krebs (1958). Phosphorylase *a* was prepared from phosphorylase *b* by the use of phosphorylase kinase. Both enzymes were further purified by passing them through a column of Sephadex G-100 (2.8 × 43 cm) equilibrated with 0.05 M

*Abbreviations: AMP: adenosin-5'-monophosphate
G-1-P: glucose-1-phosphate
SDS: sodium dodecylsulphate

Tris-HCl, pH 6.8. The enzyme activity was assayed as described earlier (Damjanovich et al., 1967). The ratio of $OD_{260\ m\mu}/OD_{280\ m\mu}$ was 0.53 showing that the samples were not contaminated with nucleotides.

Urea and SDS were products of Sigma Chemical Co. All the other chemicals were purchased from Reanal Chemical Co.

γ-irradiation (Co^{60}) was carried out by using a cobalt source, with a dose rate of 3.4 kR/min. The dose was measured with Fricke dosimeter.

Fluorescence spectra were determined in a Zeiss PMQ II fluorescence spectrometer and absorption spectra in a Unicam SP-500 spectrophotometer. The experiments were carried out at protein and amino acid concentrations which showed a linear relationship with fluorescence emission.

All experiments were carried out at 25°C.

Results and Discussion

The investigations presented here deal with the fluorescence excitation and emission spectra of phosphorylase *a* and *b* as well as those of amino acid mixtures simulating the tyrosine and tryptophan composition of phosphorylases. The spectra were determined in the presence and absence of detergents — urea and SDS —, and experiments were carried out to determine the effect of G-1-P and AMP on the fluorescence spectra of the enzymes.

As shown in Fig. 1 A the maxima of the fluorescence peaks of phosphorylase swere at 340 mμ, activated at 290 mμ. The fluorescence spectra of tyrosine and tryptophan mixtures, equimolar to their concentrations in phosphorylase *a* and *b*, respectively, were significantly lower and the maxima of the emission peaks appeared at 360 mμ. The relationship between the fluorescence maxima of the samples and the number of subunits is shown in Fig. 1B. The intensities of fluorescence maxima of the proteins were greater than those of equivalent amounts of fluorescent amino acids. The fluorescence intensity of the amino acids was plotted at both 340 mμ and 360 mμ.

Fig. 2 A shows the effect of treatment with urea and SDS on the fluorescence of phosphorylase *a* and *b*. Both agents, known to dissociate phosphorylases into subunits, reduced the fluorescence intensity at all wavelengths (Damjanovich, Kleppe, 1966). SDS decreased the intensity without altering the maxima, whereas urea shifted the fluorescence maxima towards the longer wavelengths, near to the fluorescence maxima of the tryptophan and tyrosine solutions. The concentrations of urea and SDS tested in the experiments did not alter the fluorescence of tryptophan and tyrosine solutions. Fig. 2B shows the effect of urea and SDS on the intensities of fluorescence maxima as a function of molecular organization.

As shown in Figs 1 and 2 fluorescence intensity increased parallel to the number of subunits, i.e. parallel to the degree of organization of the molecules, both before and after treatment with urea or SDS. Since the fluorescence intensity of equimolar concentrations of tryptophan increased slower than that of proteins,

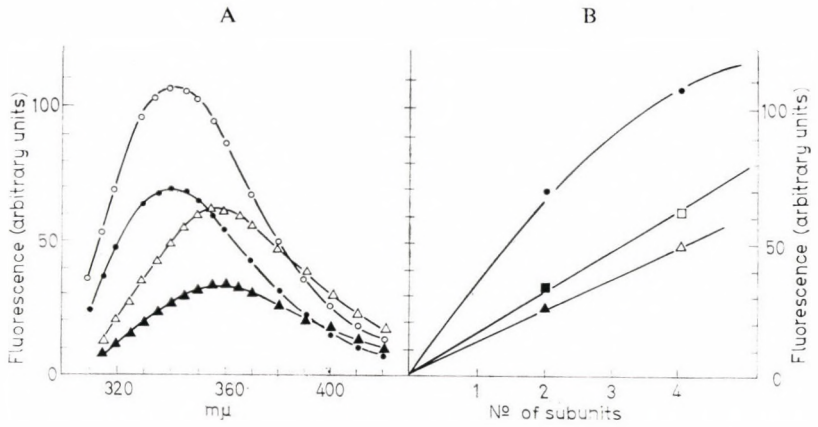

Fig. 1. The fluorescence emission spectra and intensity maxima of phosphorylase *a* and *b* and of suitable tyrosine and tryptophan mixtures. a. Fluorescence emission curves of phosphorylase *a* (○), phosphorylase *b* (●) and of tryptophan and tyrosine solutions, equimolar to the tyrosine and tryptophan content of phosphorylase *a* (△) and *b* (▲). The concentration of phosphorylase *a* and *b* was 10^{-6} M. The amino acid mixtures contained 3×10^{-5} M tryptophan, and 8.8×10^{-5} M tyrosine, or the double of these amounts, according to the amino acid content of phosphorylase *b* and *a*, calculated on the basis of 242 000 and 496 000 molecular weights, respectively (Appleman et al., 1963). All samples were dissolved in 0.05 M Tris-HCl, pH 6.8; 25°C. The excitation wavelength for the emission spectra was 290 mμ; b. Fluorescence maxima of phosphorylases at 340 mμ (●—○) and of tryptophan and tyrosine at 340 mμ (▲—△) and 360 mμ (■—□) plotted against the number of subunits of the phosphorylases. The full symbols stand for phosphorylase *b*, the empty symbols for phosphorylase *a*, and also for the suitable amino acid mixtures, respectively

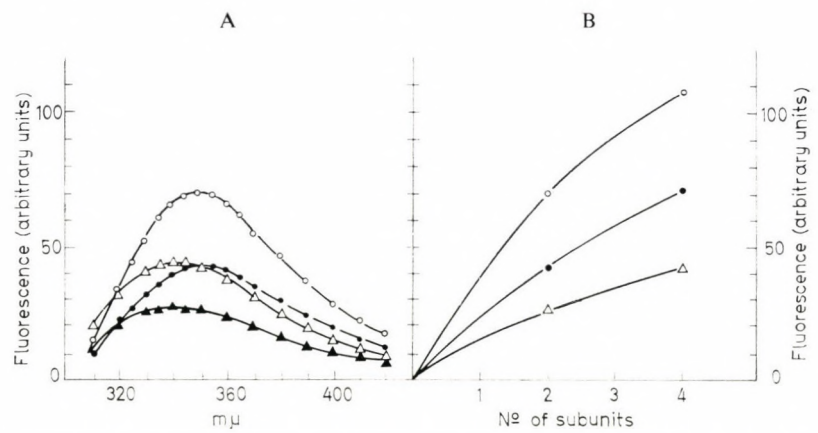

Fig. 2. Effect of urea and SDS on the fluorescence spectra, and intensity maxima of phosphorylases. a. Fluorescence emission curves of phosphorylase *a* (○) and *b* (●) after adding 6 M urea. The effect of 1% SDS on the emission curves of phosphorylase *a* (△) and *b* (▲); b. Effect of urea (●) and SDS (△) on the fluorescence maxima plotted against the number of subunits. The empty circles (○) show the intensity maxima of the untreated enzymes

the existence of intramolecular energy transfer processes along the polypeptide chains towards the tryptophan residues may be suggested. The nearly straight lines of the emission peak values, plotted against the number of subunits, very likely mean that the intramolecular energy transfer processes occur in the subunits or only to a minor extent between the subunits. The mild deviation of the curves may be due to the decreased energy uptake by the interfaces between the subunits.

Urea and SDS split the proteins into subunits. The fluorescence of the subunits did not significantly exceed the fluorescence of the equimolar tryptophan and tyrosine solutions.

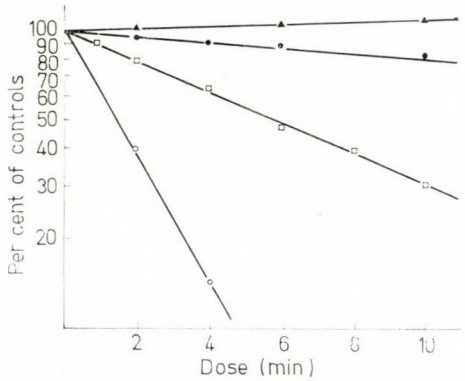

Fig. 3. The effect of γ-irradiation (Co^{60}) on the $OD_{280\ m\mu}$ (△), on the fluorescence intensity at 340 mμ (□), and on the enzymic activity (○) of 10^{-6} M phosphorylase *b*. The effect of irradiation on a 3×10^{-5} M tryptophan solution (●). The enzymic assay was carried out as described earlier (Damjanovich et al., 1967)

It is suggested that this fact is due to the alteration of the right molecular conformation i.e. that of electronic orbitals which depend on the interaction between the subunits.

Experiments were carried out to test whether minor conformational changes could alter the fluorescence of the phosphorylases. The allosteric activator of the phosphorylases, AMP, as well as one of their substrates, G-1-P, are known to change the original molecular conformation of these proteins. Neither 2 mM AMP, nor 32 mM G-1-P per 10^{-7} M protein were able to change the fluorescence of the phosphorylases. Naturally the well known inner filter effect of AMP was taken into account, by comparing the decrease of fluorescence with that of other proteins which were not supposed to bind AMP.

We have concluded that for altering the fluorescence of a protein one has to produce a deep change in the overall conformation. For this reason we irradiated the phosphorylases by γ-rays (Co^{60}) and studied the effect of irradiation on the different characteristic properties of the enzyme (Bacq, Alexander, 1961). Fig. 3 shows the ultraviolet absorbancy, the fluorescence emission maxima and the enzymic activity of phosphorylase *b* plotted against the irradiation doses.

In the investigated dose interval there was no significant change in the qualitative emission spectra. However, irradiation reduced the fluorescence intensity maxima according to an exponential relationship. Fluorescence was reduced by doses which practically did not influence the $OD_{280\ m\mu}$. Also, fluorescence intensity proved to be a sensitive tool in studying the radiation damage of a protein, especially, when the protein had no enzymic activity. The fluorescence intensity of the equimolar tryptophan solution was not as radiosensitive as that of phosphorylase *b*. This observation suggests that irradiation interfered with the suggested energy transfer in the polypeptide chains.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (3), pp. 285–293 (1970)

On the Mechanism of the Degradation of Nucleic Acids by Periodate Oxidation*

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(Received March 4, 1970)

To test the validity of our earlier suggestion concerning the mechanism of elimination, model experiments were carried out with adenosine-5'-phosphate. Upon elimination with cyclohexylamine or lysine, in addition to the free base an intermediate was separated by paper electrophoresis, paper chromatography and DEAE** cellulose chromatography. The occurrence of this intermediate confirms our earlier suggestion that prior to the liberation of the free base the bond adjacent to the oxygen atom of oxidized ribose is cleaved in such a way that some fragment of the oxidized ribose remains temporarily attached both to the phosphate and to the base.

Introduction

As reported earlier (Tankó et al., 1967) considerable work has been done to improve the periodate oxidation method introduced by Whitfeld (1954). The only significant improvement has been achieved by Neu and Heppel (1964). They succeeded by a careful analysis of the experimental conditions to achieve an almost quantitative elimination. Weith and Gilham introduced some ingenious modifications (1967), nevertheless, for a considerable time, the method could not compete with the enzymatic method of Holley et al. (1964). By applying this latter method first Holley et al. (1965) and since then several researchers were able to determine the nucleotide sequence of a number of transfer RNA-s. Quite recently Cory and Marcker (1970) have determined the nucleotide sequence of a methionine t-RNA by enzymatic methods. Khym and Uziel (1968) introduced a fundamental modification by which the periodate method became applicable to the study of polymers. With this procedure the same authors (Uziel, Khym, 1969)

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

** Abbreviations: RNA = ribonucleic acid
DEAE = diethylaminoethyl
CHA = cyclohexylamine
AMP or pA = adenosine-5'-phosphate
Ar = adenosine
A = adenine

were able to determine reliably the sequence of 19 nucleotides in a phenylalanine transfer RNA.

Despite all these important results the exact mechanism of the elimination step is not yet known. Khym (1963) demonstrated the formation of an intermediate of the type of an addition Schiff-base, while Neu and Heppel (1964) attributed the liberation of the base in the presence of large excess of periodate to over-oxidation. When applying DEAE-cellulose chromatography we have found that the liberation of the base proceeds more rapidly than that of the new 3'-P (Tankó et al., 1967). This observation has led us to assume that the first step of the elimination reaction is the cleavage of the bond adjacent to the oxygen atom of oxidized ribose leaving a fragment both on the phosphate and on the base. The present paper reports the separation of one of these intermediates in model experiments with AMP.

Materials and Methods

Adenosine-5'-phosphate was prepared from the Ba salt of ATP by baryta hydrolysis according to the method described by Tankó (1951). The molar extinction coefficient of the product in 0.1 N HCl was $\varepsilon_{257} = 15000\ M^{-1}\,cm^{-1}$ and its phosphorus content was 8.3 %. Periodate oxidation and elimination was carried out with 5 μmoles of AMP as described earlier (Tankó et al., 1967) except that elimination was performed with a 20 fold excess of lysine at pH 6.7—7.0 and at 30°C. In some experiments cyclohexylamine was used. For details see the captions to the Tables and Figures. Paper chromatographic separation was performed on 27×38 cm strips of Schleicher-Schüll 2043/b paper in the solvent system n-butanol-water (86 : 14, by vol.) (Markham, Smith, 1949) by the ascending technique. Paper electrophoresis was carried out in 0.02 M citrate buffer, pH 3.5, with 2.5×50 cm paper strips at 8 V/cm and 0.2—0.3 mA/cm (Tankó, 1959). The components were located on the paper by their UV absorption according to Holiday and Johnson (1949). For DEAE-cellulose chromatography the adsorbent (Whatman DE 50) was treated before use according to Staehelin (1961) and poured into columns of 0.8×15 cm. The column was eluted with NH_4HCO_3, pH 8.6, and fractions of 3 ml were collected at a flow rate of 3 ml per 5—10 minutes by means of an automatic fraction collector "Fractiomat", Labor Factory for Laboratory Equipment, Budapest. The absorption at 260 mμ of the individual fractions was measured in a Beckmann DU spectrophotometer. Phosphorus was determined by a modification (Tankó et al., 1967) of the method of Martland and Robison (1926).

Experimental

To slow down the rate of conversion of the intermediates into the final products, elimination after the oxidation of AMP was performed with a lower amine excess and at lower temperature than optimal. In some experiments CHA, in others lysine was used. The aliquots taken at intervals from the reaction mixture

were separated by paper chromatography using adenine, adenosine and AMP as controls. Inorganic phosphorus and total phosphorus were also determined in the aliquots.

As shown in Fig. 1 the electrophoretic mobility of adenine (A) and adenosine (Ar) at pH 3.5 towards the cathode decreased in the above order. pA remained at the spot of application, most probably owing to endosmotic flow. Iodate rapidly moved towards the anode and did not interfere with the evaluation of

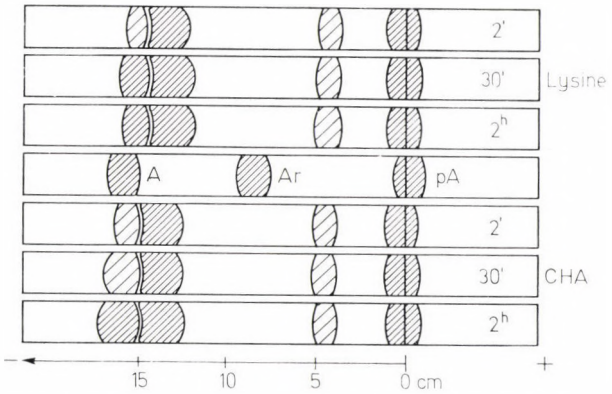

Fig. 1. Paper electrophoretic separation of the products obtained by reacting periodate-oxidized AMP with an amine. Five μmoles of AMP were incubated at 30°C with a 10-fold excess of periodate and a 20-fold excess of CHA or lysine. 1.5 μmole aliquots were taken after a period of 2, 30 and 120 minutes, respectively and reduced with ethyleneglycol. After taking samples for paper chromatography and the determination of phosphorus the pH was adjusted to 3.5 and a mixture containing 0.18 μmole of AMP was applied to each paper strip. Run was performed at pH 3.5 for 3.5 hours at a voltage of 8 V/cm and a current of 0.2—0.3 mA/cm.

the electrophoretograms. When CHA was used a spot which migrated slower than adenine appeared in every case. This might be adenine containing the fragment. After a reaction time of 2 minutes there was almost no free base with CHA. There was a fainter spot ahead of the spot of oxidized AMP. This may represent a complex of oxidized adenosine or AMP with the amine. The results were almost the same when the experiment was performed with lysine. In this case, however, for a reliable detection of the intermediate migrating behind the adenine, elimination reaction had to be performed at pH 6.7—7.0. At pH 5.6—6.0 as applied in our earlier experiments this intermediate was hardly detectable even after a reaction period of 2 minutes, indicating that its decomposition rate was higher under these conditions. The percentage amounts of the reaction products are presented in Table 1.

The data in Table 1 show that the amount of the free base and that of the intermediate located behind it increased with increasing reaction time, but under the limited conditions used the intermediate was still present in a higher proportion

Table 1

Quantitative distribution of products separated by paper electrophoresis after a limited reaction of periodate-oxidized AMP with an amine

Limited reaction was performed at 30°C with 5 μmoles of AMP in the presence of a 10-fold excess of periodate and 20-fold excess of amine. Aliquots taken out after 2, 30 and 120 minutes, respectively, were reduced with ethyleneglycol and after adjusting the pH to 3.5 samples containing 0.18 μmole of AMP were applied to the paper strip. The UV absorption of eluates obtained with 0.02 N HCl was measured at 260 mμ. The data are expressed as μmole and percentage quantities relating to a total of 1.5 μmoles. In the calculations the molar extinction coefficient of adenine + fragment was assumed to be equal to that of adenine ($\varepsilon_{260} = 13\,000\ M^{-1}\,cm^{-1}$)

Elimination		Adenine		Adenine + fragment		Inorganic P	
		μmole	% of total	μmole	% of total	μmole	% of total
CHA	2 minutes	0.11	7	0.39	26	0.41	27
	30 minutes	0.31	21	0.73	49	0.80	53
	120 minutes	0.47	31	0.69	46	0.92	61
Lysine	2 minutes	0.12	8	0.34	23	0.31	20
	30 minutes	0.49	33	0.71	48	0.84	56
	120 minutes	0.57	38	0.68	45	0.86	57

with either CHA or lysine used for elimination. The amount of inorganic phosphorus liberated during the elimination reaction is also shown in Table 1. It can be seen that under the conditions of limited reaction the amount of free adenine was less than that of the liberated inorganic phosphorus, however, together with the intermediate containing the fragment the former was considerably higher than the latter, indicating the formation of a significant proportion of ribose fragments containing phosphorus. Such fragments, however, could not be identified in these experiments.

As shown in Fig. 2 upon the separation of the reaction products by paper chromatography in the n-butanol-water mixture, AMP did not leave the spot of application, while adenine moved considerably faster than adenosine. The intermediate was detected in this way too, although when CHA was used for the elimination reaction, contrary to paper electrophoresis, it ran in front of adenine as a spot with reduced intensity. When lysine was used for elimination at pH 6.7 to 7.0, here too, a separate spot was obtained, but this spot appeared behind adenine as a somewhat elongated but clearly detectable one. Iodate does not interfere with the run and the location of the components, since it is insoluble in this solvent system and so remains together with AMP on the start line. The quantitative ratios of the reaction products separated by paper chromatography are shown in Table 2. The data support the results of paper electrophoresis with the difference that a higher amount of free adenine (but for one exception) was found with paper chromatography than with paper electrophoresis.

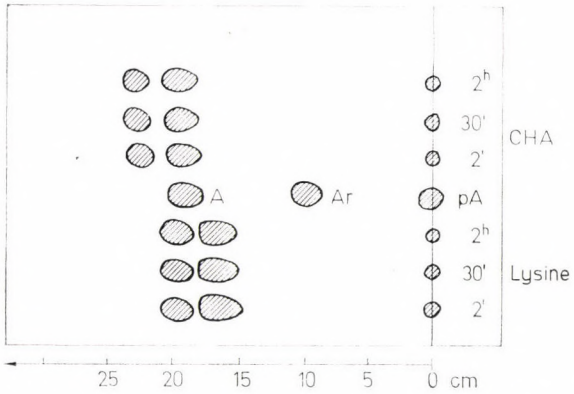

Fig. 2. Paper chromatographic separation of the products obtained by reacting periodate-oxidized AMP with an amine (for details see Fig. 1). A reaction mixture containing 0.18 μmole of AMP was applied to the paper. The run was performed in the solvent system n-butanol-water (86 : 14, by vol.) by the ascending technique

Table 2

Quantitative distribution of products separated by paper chromatography after a limited reaction of periodate-oxidized AMP with an amine

The experiment was performed with 5 μmoles of AMP in the presence of a 10-fold excess of periodate and a 20-fold excess of amine. Aliquots taken out after 2, 30 and 120 minutes, respectively, were reduced with ethyleneglycol and samples containing 0.18 μmole of AMP were applied to the paper. UV absorption of eluates obtained with 0.02 N HCl was measured at 260 mμ. The data are expressed as μmole and percentage quantities relating to a total of 1.5 μmoles. In the calculations the molar extinction coefficient of adenine + fragment was assumed to be equal to that of adenine ($\varepsilon_{260} = 13\,000\ M^{-1}\,cm^{-1}$)

	Elimination	Adenine		Adenine + fragment	
		μmole	% of total	μmole	% of total
CHA	2 minutes	0.30	20	0.65	38
	30 minutes	0.52	34	0.62	41
	120 minutes	0.71	47	0.54	36
Lysine	2 minutes	0.34	22	0.31	21
	30 minutes	0.45	30	0.65	43
	120 minutes	0.69	46	0.56	37

In the following experiments the intermediate still containing the fragment was separated from the cyclohexylamine reaction mixture by chromatography on DEAE-cellulose. Fig. 3/a shows that in the presence of large excess of CHA a sharp peak was obtained when the reaction mixture that had been incubated at 45°C was immediately applied to the column after a short (2 minutes) period of elimination and reduction and the column washed with distilled water. When elution was continued with a 0.01 M salt solution some more material was recov-

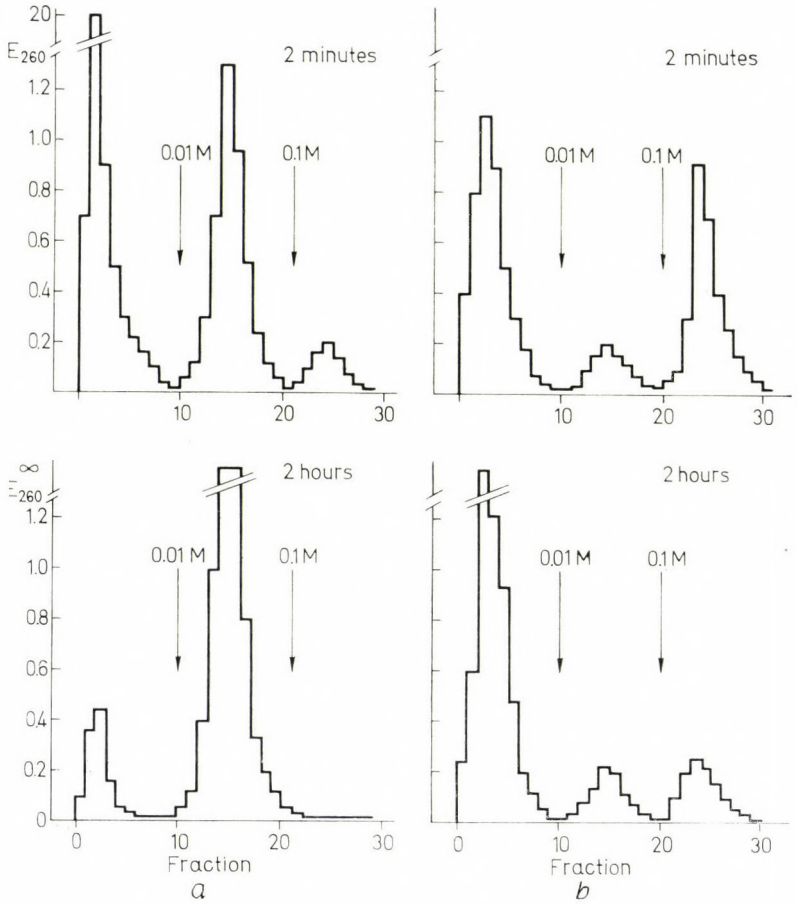

Fig. 3. DEAE-cellulose chromatographic separation of the products obtained by reacting periodate-oxidized AMP with an amine. A reaction mixture containing 2 μmoles of AMP was applied to a DEAE-cellulose column (0.8×15 cm). Elution was performed with water followed by 0.01 and 0.1 M NH_4HCO_3 solutions (pH 8.6). (The change of eluent is marked with an arrow). Three ml fractions were collected at a flow rate of 3 ml per 5–10 minutes. AMP was oxidized with a 10-fold excess of periodate. For the elimination
a) a 100-fold excess of CHA at 45°C or
b) a 20-fold excess of CHA at 30°C
was used

ered from the column as a distinct peak. Oxidized AMP was eluted with a 0.1 M salt solution. A comparison of the elution diagrams obtained with samples which had been subjected to elimination for 2 minutes and 2 hours, respectively, clearly shows that the amount of material eluted with water considerably decreased during the 2 hours period of elimination, while that of the substance eluted by the 0.01 M salt solution increased. The eluted substances were concentrated by vacuum evaporation and their UV spectra taken in acid and alkali. The substance in peak No. 2

was found to be adenine, while that in the first peak differed only slightly from it, with an absorption maximum between that of adenine and adenosine. In paper electrophoresis the substance of peak 1 migrated behind adenine and that of peak 2 together with adenine. Hence the substance eluted with water from the DEAE-cellulose column behaved like the component running behind adenine in paper electrophoretograms of the reaction mixtures when spotted directly. In the case of a slow reaction (Fig. 3/b) the amount of the substance eluted with water was the highest even after a reaction period of 2 hours. Therefore the substance eluted with water most probably is the intermediate.

Discussion

As described in our earlier communication (Tankó et al., 1967) when the dinucleoside monophosphate GpC is oxidized with periodate then subjected to elimination for a short period and the resulting products are chromatographed on DEAE-cellulose, a smaller peak appears before the expected Gp following the elution of cytosine. This observation led us to assume that, after the formation of a Schiff base with the amine, the initial step of the elimination reaction is the cleavage of the bond adjacent to the oxygen atom of oxidized ribose and that a fragment of the oxidized ribose remains on both the base and the phosphate. For this reason the phosphate will have one charge less than after complete elimination. Because of the resulting weaker binding force this intermediate will elute from the column earlier than Gp. Since the amount of cytosine eluted with 0.01 M salt is less than that of free Gp, it is possible that cytosine is present together with an intermediate containing the fragment. The model experiments carried out with AMP to separate this latter component confirmed the above assumption. By a better separation (paper electrophoresis, paper chromatography and DEAE-cellulose chromatography) of the products obtained by short and slow elimination it was possible to detect a second component in addition to adenine. This second component might be the base still containing the ribose fragment. Khym and Cohn (1961) have pointed out that the dialdehyde obtained from ribose forms with amine an intermediate of the Schiff base type whose stability depends on the pH and which decomposes below pH 7.0. We do not know whether or not our separated intermediate is in fact in the form of a Schiff base.

As to the different behaviour of the intermediate during paper chromatography, depending on whether the cyclic CHA or the open-chain diamino acid, lysine had been used in the elimination reaction, it should be kept in mind that, as pointed out by Hunt (1965) and Steinschneider and Fraenkel-Conrat (1966), the nature, steric structure and stability of the addition product are certainly influenced by the nature and structure of the applied amine. Such an effect was actually observed when lysine was used below pH 6.0 in the elimination reaction. With this compound, in contrast to CHA, it was impossible to detect the intermediate despite the limited reaction.

A comparison of the data in Table 1 and Table 2 reveals that a higher amount of free base was recovered from paper chromatograms than from paper electrophoretograms whereas the total amount of the base plus intermediate — with the exception of the 2-minute-samples — was approximately the same. This may be explained by the fact that while the paper electrophoretic run is completed 5 hours after the application of the substance, in the case of paper chromatography the amine is concentrated during its application to the paper and the drying process and all this, together with the long run, may cause a decomposition of the intermediate. This explanation is supported by the findings obtained with DEAE-cellulose chromatography. Here the reaction mixture was applied to the column immediately after dilution and elution started at once. In this way the intermediate was detectable even when elimination had been carried out at 45°C in the presence of a large excess of CHA. When a lower amine excess was used at 30°C the intermediate still occurred in large amounts after an incubation period of 2 hours. This suggests that the fragment-containing adenine eluted in the first peak is present as a Schiff base. Owing to its steric structure it may not bind on the column and can therefore be eluted with water.

A comparison of the speed of liberation of inorganic phosphate with that of the free base (Tables 1 and 2) indicates that for a single exception less base was liberated than inorganic phosphate in our earlier experiments (Tankó et al., 1967). Under optimum conditions the base was liberated more rapidly. Both results support the conclusion that the cleavage of the bond adjacent to the oxygen atom of oxidized ribose is the initial step in elimination, but indicate at the same time that it will depend on the experimental conditions which will be the faster process: the liberation of the base or the setting free of the new terminal phosphate.

We have found no reference in the literature on the mechanism of the cleavage of the Schiff-base type complex as suggested by us. Khym and Cohn (1961) observed only the nucleoside-like behaviour of the oxidized product, while recently Dugaiczyk and Eiler (1969) reported the formation of 5-formyl-uracil and 5-carboxy-uracil during the periodate oxidation and CHA elimination of pseudouridine-5'-phosphate. This last finding supports our own observation and assumption concerning the mechanism of elimination. As shown in the present paper prior to the liberation of the base, as an initial step to elimination the bond adjacent to the oxygen atom of oxidized ribose is cleaved in such a way as to leave temporarily a fragment of the oxidized ribose both on the phosphate and on the base.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (3), pp. 295—297 (1970)

Determination of the $^{85}Sr^{++}/Ca^{++}$ Ratio in Bone

(Short Communication)

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(Received November 10, 1969)

A method has been developed for the determination of the quantitative ratio between the amount of $^{85}Sr^{++}$ incorporated into the bone and that of other elements of the mineral phase of bone. The method is based on X-ray fluorescence spectrum analysis and on the measurement of radioactivity. The use of this procedure makes it possible to study not only the mineral metabolism of bone but also certain problems of its pathology.

Various methods have been used hitherto for the determination of the quantitative ratio between the amount of $^{85}Sr^{++}$ incorporated into the bone and that of other bone constituents (Charkes et al., 1968).

These methods, however, failed to reveal the quantitative ratio between the amount of incorporated $^{85}Sr^{++}$ and that of other elements constituting the mineral substance of the bone. Clarification of this problem would contribute to a better understanding of physiological mineral metabolism as well as of some pathological phenomena. In the case of certain bone lesions, demineralization as seen in X-ray pictures, is accompanied by either enhanced, or reduced radioactivity after the incorporation of $^{85}Sr^{++}$. An accurate determination of the $^{85}Sr^{++}/Ca^{++}$ ratio may provide an answer to the question whether it is the mineral substance or an inflammatory hyperaemia which is responsible for a higher radioactivity (Glauber et al., 1967).

The following method was worked out for the determination of the $^{85}Sr^{++}/Ca^{++}$ ratio in bone. The bones containing $^{85}Sr^{++}$ were extracted with a 27 % solution of ethylenediamine tetraacetate (EDTA) for 14 days. EDTA forms complexes with the earth metals in bone. As confirmed by X-ray diffraction tests after extraction no crystalline residue of the mineral substance of bone was present in detectable quantities. The extract was analyzed by two methods:

1) X-ray fluorescence spectrum analysis was performed to determine the quantity of Ca^{++} in the solution. In this test a manually operated universal Philips X-ray fluorescence vacuum spectrograph was used. The excitation radiation source was an X-ray valve with chromium anode operated at a potential of 30 kV

and a current of 30 mA. Pentaerythritol served as an analyzer crystal. The beam of rays was set parallel by means of the original light element collimator of the manufacturing firm. A gas-washed proportional counter valve with a side window made of 6 μm thick Mylar foil was the radiation detector. The voltage applied on the proportional counter was 1900 V. To improve the signal to noise ratio an amplitude discriminator with 4×21 V energy threshold and 20 V energy port was used. The measurements were carried out under a semi-vacuum system,

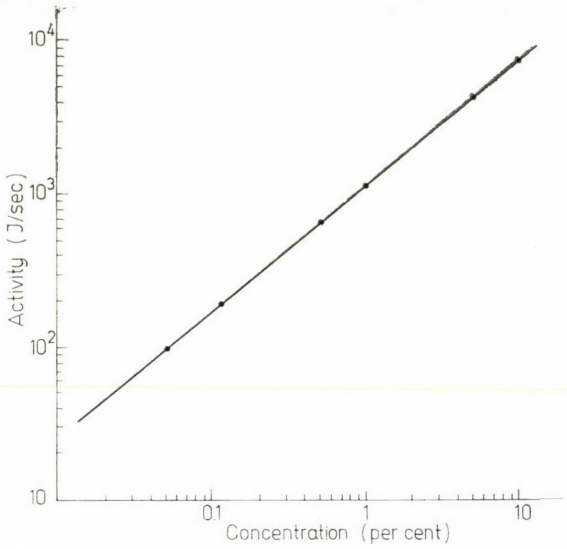

Fig. 1. Calibration curve to the determination of the Ca content of bone extracts. Ordinate pulse/sec; abscissa: the concentration in per cent values

i.e. the sample space was under normal atmospheric pressure. Pulse number was measured by recording with a Gamma type 5 decade line printing counter. The intensity of both the background and the Ca^{++} line was taken as the mean value of the pulse numbers measured automatically 500 times in succession. A calibration series of solutions was prepared from EDTA of the same concentration as used for extraction (Fig. 1). In determinations of Ca^{++} concentration the accuracy of the method was higher than ± 1 % standard deviation.

2) The quantity of $^{85}Sr^{++}$ in the solution was determined by radioactivity measuring. Two millilitres of the sample were transferred into a test tube and the radioactivity of the solution measured by means of the Frieseke-Hoepfner type FH 49 scaler and a hollow crystal of the type FH 421A. The radioactivity of the $^{85}Sr^{++}$ extracted from the bone was obtained by substracting the background value from the measured cpm value and by multiplying the difference by the dilution factor. Since the specific activity of $^{85}Sr^{++}$ was known the quantity of $^{85}Sr^{++}$ incorporated into the bone could be calculated. From the results obtained in this manner the weight ratio of $^{85}Sr^{++}$ to Ca^{++} in the sample was calculated.

This method was applied to 4-month-old Amsterdam "R" rats of 150 g body weight. The animals were killed 10 days after the administration of 20 μCi of $^{85}Sr^{++}$ and the femoral diaphysis investigated after the removal of the soft parts. In the sample the ratio of $^{85}Sr^{++}$ to Ca^{++} was found to be 1 : 280 000 (scattering of the results: 1.12). This test was intended to serve as an example. In other individuals, after the administration of different amounts of $^{85}Sr^{++}$, other ratios had been obtained. These results are quite different from those obtained from the determination by conventional procedures of the ratio of non-radioactive Sr^{++} and Ca^{++} introduced into the bone (Natelson, Shaid, 1961).

Our method has the following advantages:

1) It may be applied under appropriate conditions to any of the elements occurring in the bone (e.g. to the determination of the $^{85}Sr^{++}/P$ ratio).

2) After standardization it may be used with very small samples of bone removed by biopsy.

3) It facilitates the study of mineral metabolism.

4) It can be performed within a short period of time.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (3), pp. 299—303 (1970)

An Evidence for Deamidation of Prolactin Monomer

(Preliminary Communication)

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(Received April 27, 1970)

The nature of the heterogeneity of prolactin has been investigated by a number of workers (Squire et al., 1963; Sluyser, Li, 1964; Reisfeld et al., 1964; Lewis, Cheever, 1965; Cheever, Lewis, 1969). From indirect studies Cheever and Lewis (1969) have recently arrived to the conclusion that the gel electrophoretic components with the same molecular weight but different electrophoretic mobilities, of purified prolactin and growth hormone preparations from five species were deamidated forms of prolactins and growth hormones, respectively.

In the present paper direct evidence will be provided to show, that the main bovine prolactin components with identical molecular weights differ from one another only in the number of amide groups.

Prolactin was isolated from bovine pituitary glands by a modified version of the procedure of Sluyser and Li (1964). Fig. 1 illustrates the gel electrophoretic pattern (a) of the bovine prolactin preparation. A fraction of higher molecular weight was removed from the above preparation by gel chromatography on Sephadex G-75. The three main electrophoretic components were eluted as a single peak from Sephadex G-75 and were subsequently separated by chromatography on DEAE-cellulose (Fig. 1 b, c, d). (Details of the isolation of the bovine prolactin components have been described in another communication; Gráf et al., 1970.)

The crop sac stimulating activity of Components I, II and III was 57.6 IU, 63.6 IU and 55.3 IU, respectively (for test method see Grosvenor et al., 1958; Kurcz, 1966). Their weight average molecular weights were 24 000—26 000, according to ultracentrifugation experiments. On the basis of these data the components may be considered as monomer forms of prolactin. Their N-terminal amino acid residues proved to be identical (Thr). Amino acid analysis did not reveal any significant difference in the amino acid composition of the three peptides.

In order to compare the primary structures of these components, Components I and II were reduced by dithiothreitol and alkylated with ^{14}C bromo-acetic acid in 8 M urea. The N-terminal sequences of the reduced alkylated (RA) bovine prolactin components were analyzed by the Edman-dansyl procedure (Gray,

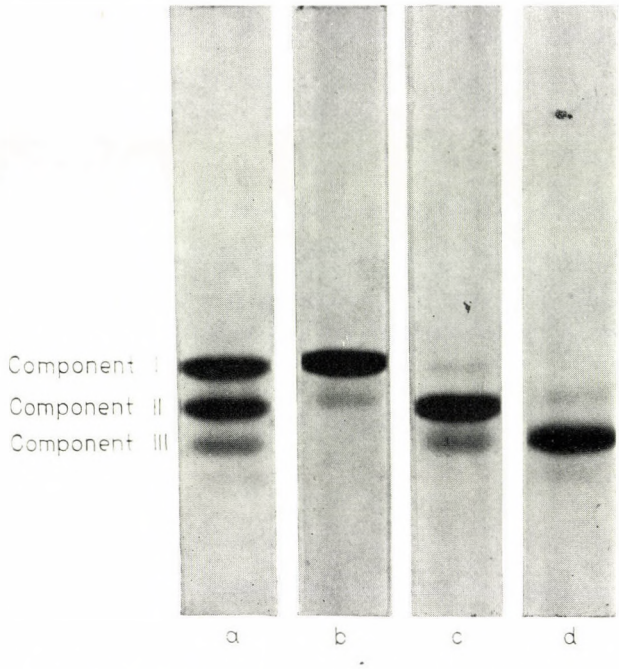

Fig. 1. Disc electrophoresis of bovine prolactin (a) and of Components I—III (b—d) separated by DEAE-cellulose column chromatography. Electrophoresis was performed at pH 9.0 in 7.5% polyacrylamide gel. Protein content of the samples applied was 100 μg (a) and 50 μg (b—d), respectively

1967) and the results showed the identity of the N-terminal sequences:

NH_2.Thr.Pro.Val.CysCM.Pro ...

The RA-Components I and II were hydrolyzed by trypsin in 0.05 M NH_4HCO_3 at 37°C with an enzyme to peptide ratio of 1 : 50 (w/w). The digested peptide mixtures were separated by two dimensional paper electrophoresis. Between the "fingerprints" of RA-Components I and II there were two significant differences: In the tryptic digest of RA-Component II the amount of a peptide (A in Fig. 2) was decreased and a new peptide fragment (B in Fig. 2) appeared as compared to that of RA-Component I. Fig. 3 shows radioautographs taken from the marked area of peptide patterns corresponding to Components I and II.

From the tryptic digest of RA-Component I we isolated by electrophoresis fragment A, while from that of RA-Component II fragments A and B. The amino acid composition of the three peptide fragments proved to be identical.

The complete sequence of fragment A was determined by the Edman-dansyl procedure. The positions of the amide groups were established by the comparative electrophoretic method described by Offord (1966) and Gray (1967).

The fragment has the following sequence:

NH_2.Thr.Pro.Val.CysCM.Pro.Asn.Gly.Pro.Gly.
.Asn.CysCM.Gln.Val.Ser.Leu.Arg.COOH

In spite of the identical amino acid composition of peptides A and B, peptide B showed a two times higher electrophoretic mobility than peptide A both at pH 6.5 and pH 5.0. This fact obviously means that the net charge of peptide B is — 2 instead of — 1, thus peptide B has one additional free carboxyl group

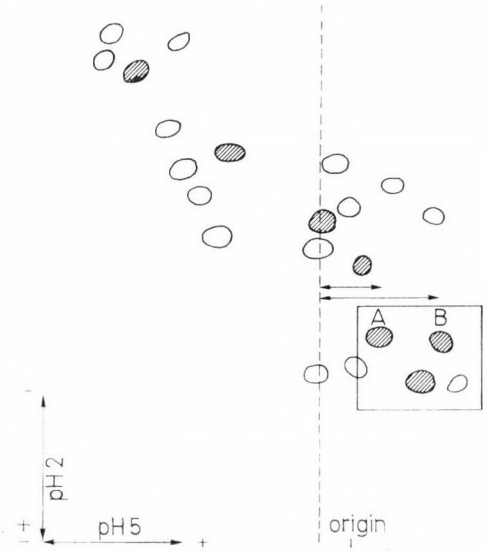

Fig. 2. Tracing of a two dimensional paper electrophoretogram of the tryptic digest of RA-Component II obtained after electrophoresis on Whatman 3 MM paper first at pH 5.0 (at 40 V/cm for 2.5 hr) and subsequently at pH 2.0 (at 60 V/cm for 2 hr). Radioactive spots are shaded. The dashed line, used as a reference in determining the mobilities of peptides A and B, shows the position of the neutral peptides

The results of sequence analysis indicate that peptide B has the same sequence as peptide A, but it contains aspartic acid in position six instead of asparagine, thus peptide B may be considered as the deamidated form of peptide A.

This finding, if related to the entire prolactin monomer, suggests that the formation of Component II is due to the deamidation of the sixth asparagine of Component I. Since the tryptic digest of homogeneous RA-Component II contains only 70 % peptide B (based on the total amount of peptides A and B) according to radioactivity measurements, only 70 % of Component II can result from the deamidation of the sixth asparagine. 30 % of Component II contains asparagine in position six. The excess negative charge in this 30 % fraction relative to Component I can derive from the deamidation of other residue(s), the position of which has not yet been established in the sequence of prolactin. Thus Component II (and probably Component III, too) is an isomer mixture of peptide molecules

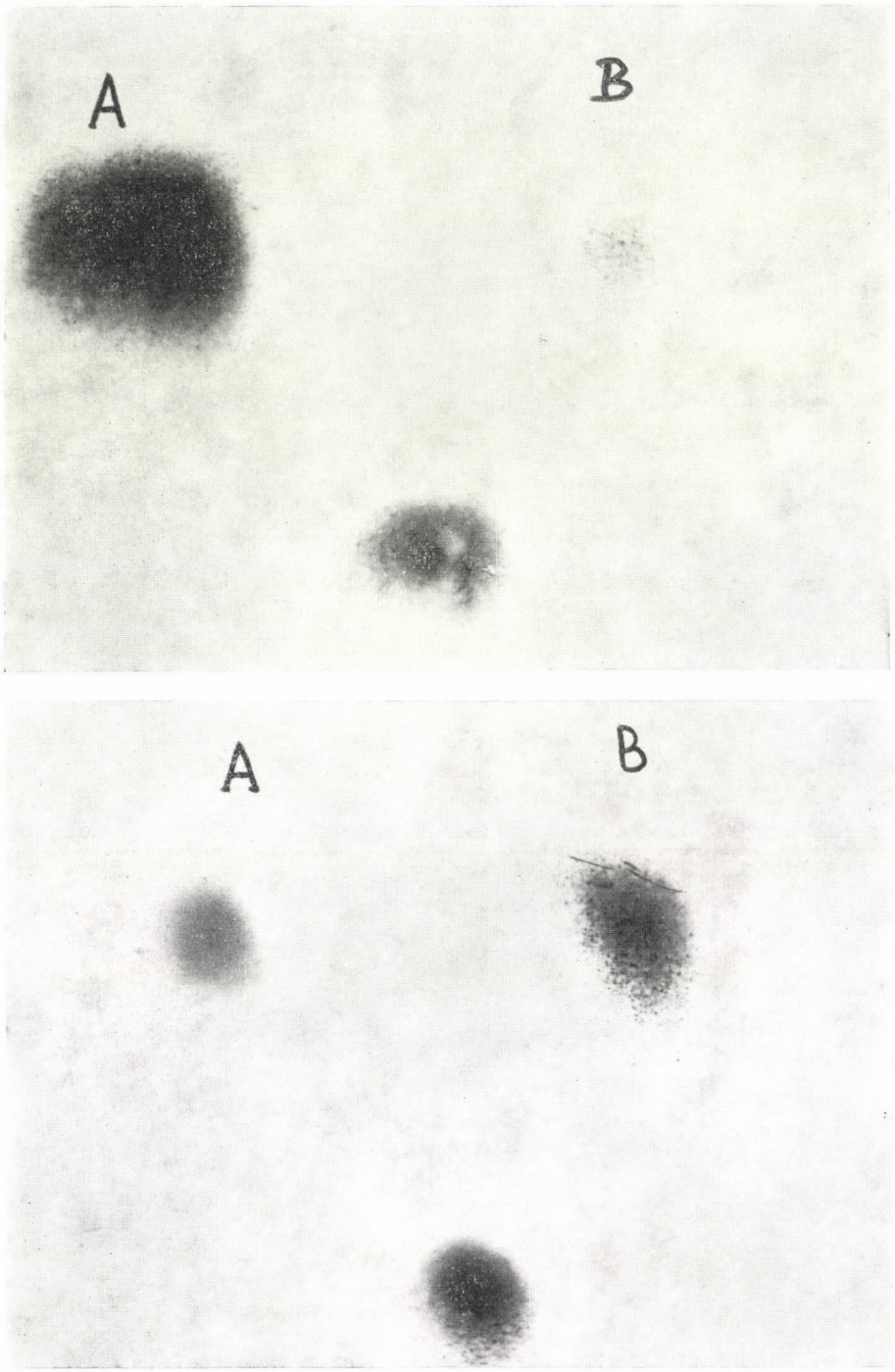

Fig. 3. Radioautographs of the "critical" area marked in Fig. 2. of the peptide patterns corresponding to RA-Component I (top), and RA-Component II (bottom), respectively

as far as the position of the free side chain carboxyls is concerned. A similar phenomenon has been observed also in the case of the deamidation of insulin (Sundby, 1962) and cytochrome C (Flatmark, 1966).

While our studies were in progress Li et al. (1969) have published the complete sequence of ovine prolactin. The position assigned by these authors to amides in the 1—16 N-terminal part of prolactin differs from that suggested by us. The reason of this discrepancy may be due to a species difference.

The authors wish to express their thanks to Dr T. Dévényi for the amino acid analyses, and to Dr P. Závodszky for the ultracentrifugal molecular weight determinations.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (3), pp. 305–307 (1970)

Amino Acid Sequence of Porcine γ-Lipotropic Hormone

(Preliminary Communication)

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(Received July 20, 1970)

γ-LPH* was prepared from porcine adenohypophysis as previously described (Gráf et al., 1969). It is a peptide hormone with lipolytic activity, consisting of 58 amino acid residues. Its analogy to the ovine γ-LPH (Chretien, Li, 1967) has become evident in the course of its sequence analysis (Gráf et al., 1970).

The N-terminal sequence of the porcine γ-LPH was analyzed by the Edman-dansyl procedure (Gray, 1967) and this gave the following results: Glu.Leu.Ala. Gly.Ala.Pro.Pro.Glu.Pro.Ala.Arg. The dansyl amino acid residues were identified by the method of Sajgó (1970). To determine the C-terminal residue, the hormone was hydrolyzed by carboxypeptidase A at pH 7.5 with an enzyme to peptide ratio of 1 : 20 (w/w) at 37°C for3 hours; the single amino acid residue liberated was identified in its dansyl form as Asp.

The porcine γ-LPH was digested with trypsin in 0.05M NH_4HCO_3 at 37°C for 3 hours with an enzyme to peptide ratio of 1 : 50 (w/w). Peptide fragments from the digest were purified by high voltage paper electrophoresis at pH 5.0 and pH 2.0, respectively. Usually one basic, two neutral and two acidic fragments were isolated. The homogeneity of the fragments was checked by end-group analysis. The sum of amino acids of these fragments accounted for that of undigested γ-LPH. Quantitative amino acid analyses were performed by the single column method of Dévényi (1968) in a Beckman–Unichrom analyzer.

The fragments were sequenced by the Edman-dansyl procedure. To estimate their amide contents electrophoretic mobility measurements were performed as proposed by Offord (1966). Only one of them was shown to contain an amide group which was located by the comparative electrophoretic method of Gray (1967).

The sequence of three out of the five main tryptic fragments proved to be identical with those of the corresponding segments of the ovine γ-LPH. One of the tryptic fragments differed only in one amino acid from the analogous fragment of ovine γ-LPH. Further species-differences were found to occur in an acidic

* LPH, lipotropic hormone.

tryptic fragment consisting of 23 amino acid residues. The sequence of 13 amino acids at the N-terminal portion of this acidic tryptic fragment revealed that it was the N-terminal tryptic fragment of porcine γ-LPH. This fragment was further digested with trypsin and papain. The trypsin resistant Arg.Asp bond in position 11.12 could be hydrolyzed only under special conditions (Cseh, Gráf, 1970). This Arg.Asp bond was also split by papain in 0.2 M pyridine-acetate, pH 5.5, containing 0.01 M cysteine and 0.005 M ethylene-diamine-tetraacetate with an enzyme to peptide ratio of 1 : 50 at 37°C for 2 hours. From the digests fragments with overlapping sequences could be isolated.

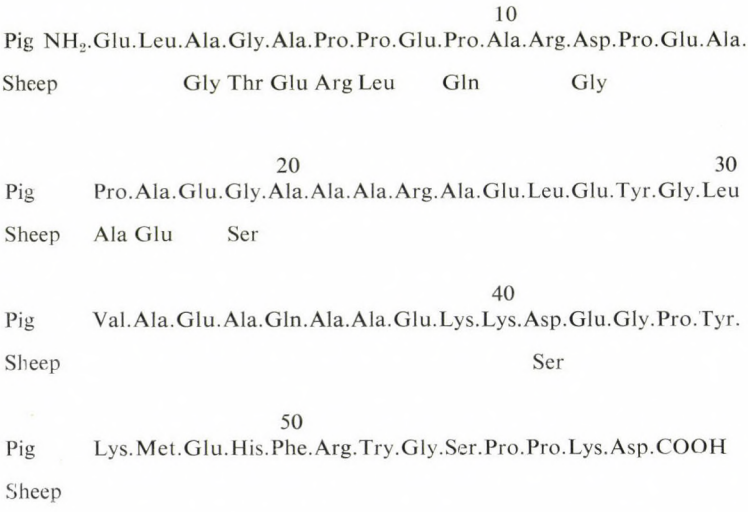

Fig. 1. Amino acid sequence of porcine γ-LPH. The differences from the sequence of ovine γ-LPH are indicated

From the results the complete amino acid sequence of porcine γ-LPH shown in Fig. 1 is proposed. Fitting of enzymatic fragments, i.e. the determination of their alignment within the procine γ-LPH molecule was partly accomplished by a comparison with the structure of ovine γ-LPH.

The porcine γ-LPH comprises the complete structure of porcine β-melanophore stimulating hormone at its C-terminal end, like the ovine analogues. Since all of the tryptic fragments of γ-LPH could be isolated from the tryptic digest of porcine β-LPH (Gráf et al., 1970) we assume that the sequence of porcine γ-LPH is identical with the $1-58$ sequence of porcine β-LPH as it has been shown previously for ovine LPHs (Chretien, Li, 1967).

Thanks are due to Dr Dévényi for the amino acid analyses.

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Note added in proof. The proposed sequence was recently confirmed by studying the structure of some fragments obtained by cyanogen bromide and chymotryptic cleavage. These results gave an additional evidence of the above alignment of tryptic peptides.

Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (3), pp. 309—311 (1970)

The Effect of Urea and Related Compounds on the Morphology and Energy Metabolism of Erythrocytes

(Preliminary Communication)

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Ponder (1954) has established that urea induces the fragmentation and spherocytic shape transformation of erythrocytes. It is known as well that the biconcave shape of erythrocytes is profoundly influenced by the energy metabolism of the cell (Nakao et al., 1961, Gárdos et al., 1966, Weed, 1967). We intended to study whether the morphological effects of urea and related compounds (thiourea, guanidine) were based on direct effects, or developed indirectly through the altered energy metabolism.

We have found that urea in relatively small concentrations (0.4—0.8 M) induced significant changes in the energy metabolism of erythrocytes. It brought about a prompt and significant fall in the ATP level without simultaneous inorganic phosphate release, and without a significant change in the $P_7 - P_0$ fraction (Table 1). We obtained similar results when thiourea was used. Guanidine, however, proved to be impermeable and did not influence the energy metabolism of the cell. The changes in the phosphate compounds elicited by urea and thiourea were followed. ATP level was found to be regenerated on the account of 2,3-DPG ($P_t - P_{180}$ fraction) when urea was used. In the presence of thiourea, however,

Table 1

Prompt effect of 0.6 M urea on the phosphate compounds of human erythrocytes (n = 4)

Phosphate compound (μmole/ml RBC)	P_0	$P_7 - P_0$	$P_t - P_{180}$	ATP
Control	0.54 (s = ±0.16)	3.80 (s = ±0.90)	6.95 (s = ±0.75)	1.08 (s = ±0.07)
0.6 M urea	0.49 (s = ±0.17)	3.88 (s = ±0.91)	7.38 (s = ±1.02)	0.72 (s = ±0.05)
p	<0.70	<0.90	<0.40	<0.01

in spite of 2,3-DPG breakdown, no ATP regeneration, but ATP breakdown occurred, with the simultaneous appearance of inorganic phosphate (Fig. 1). The examined permeable non-electrolytes reduced glucose consumption significantly on a 5 % level of probability. In some blood samples glucose consumption perfectly ceased to exist. There were characteristic changes in the morphology: the contour of the biconcave disk became crenated, the crenations grew, their stalk became thinner and thinner. Finally fragmentation occurred and the cell

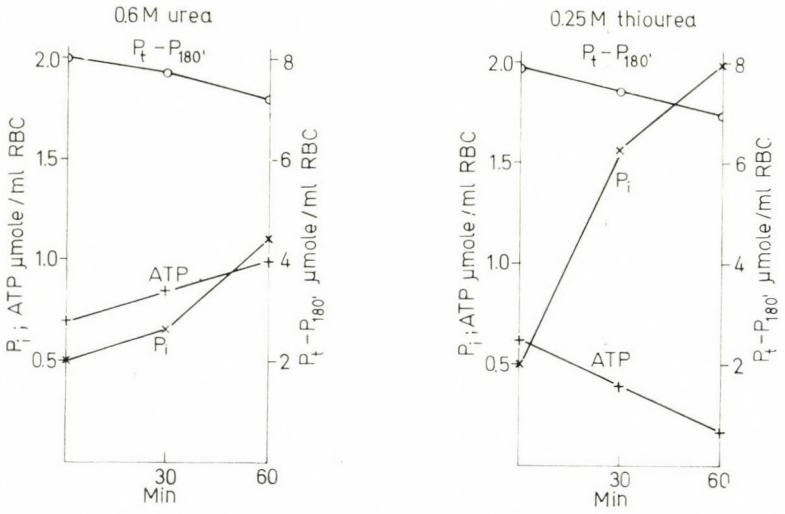

Fig. 1. The effect of 0.6 M urea and 0.25 M thiourea on the phosphate compounds of fresh human erythrocytes at 37°C. The solvent of the non-electrolytes was 0.16 M NaCl

turned into a microsphaerocyte. The regeneration of ATP in the presence of urea made it possible to study whether the changes in morphology and the alterations in glucose consumption catalyzed by the ATP-requiring hexokinase reaction were the consequences of the lowered ATP level. Fig. 2 clearly shows that these deviations developed directly, they were not improved by the normalization of the ATP level. The fact that the impermeable guanidine inducing no metabolic effects provoked fragmentation and shape changes as well, also refers to a direct morphological effect.

It is known that phosphate bonds of the guanidine type represent a high energy level and thus their formation occurs on the account of high energy phosphate compounds. The question may arise whether the prompt ATP decrease was not connected with the transport of the rapidly permeating non-electrolytes, whether these compounds were not phosphorylated on the account of ATP when they permeated the cell. Our results on erythrocytes preserved in ACD and rejuvenated by 1 mM adenine + 10 mM inosine before the investigation are inconsistent with this assumption. Under these conditions the prompt ATP decrease

upon the addition of urea diminished and finally disappeared during storage, while the compound permeated with an unaltered high speed. The investigations of Szelényi and Hollán (1968) lead to the same conclusion: thiourea permeates with an unchanged speed into erythrocytes depleted from ATP by iodoacetate.

The results show that though the investigated permeable non-electrolytes profoundly influence the energy metabolism of the cell, the basis of the morphological changes is to be sought rather in the disturbed non-covalent interactions

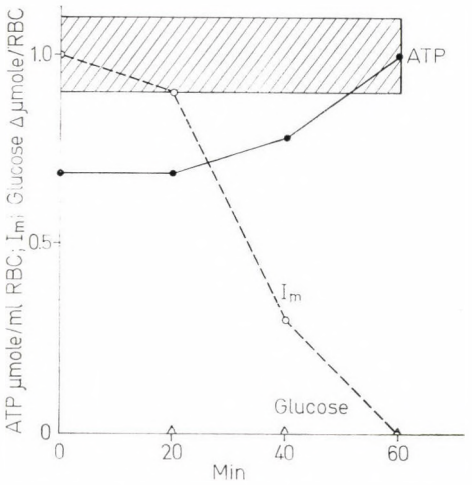

Fig. 2. The effect of 0.6 M urea on the ATP content, glucose consumption and morphological index (I_m) of fresh human erythrocytes at 37°C. Methods were used as described earlier (Gárdos et al., 1966)

within the membrane. If Sirs' (1969) conception is true, namely that the bonds between the membrane units are stronger on the outer surface than on the inner surface in the dimple region, then the effect of the so called H-bond reagents may be explained by their effect of weakening these external bonds.

For the skilful technical assistance thanks are due to Mrs Eva Irmai and Mrs Agnes Stokum.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (3), pp. 313–320 (1970)

A Stochastic Model for Stimulus-Irritation Processes

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(Received March 6, 1970)

The first part of this communication summarizes the consequences of the stochastic characteristics of the stimulus-irritation processes in the following two theses:

I. During a series of irritations the stimulus threshold is a probability-variable, the probability-distribution of which is determined by the changes in the stimulus threshold during a single irritation, and by the frequency of irritation.

II. The frequency of a series of irritations due to a series of stimuli is determined by the frequency of the stimuli, the strength of the stimuli and the probability distribution of the stimulus threshold.

The second part of this paper interpretes characteristic features of frequency transmission in terms of the above theses, and deals with them in a quantitative way.

1. Introduction

The operating mechanism of irritable systems is constituted by physico-chemical processes. But some of the characteristics of the behaviour of these systems can be interpreted without considering this, for they may be regarded as consequences of the stochastic nature of the processes.

Considering the strength of stimuli and the frequency of their arrival the following types of stimulation parameters can be distinguished:

a) A remarkable part of the stimuli is smaller than the resting value of the stimulus threshold; further on, the frequency of irritation is small when compared with the maximum irritation frequency determined by the duration of the absolute refracter state. In such cases the stochasticity takes its origin first of all from the accidental nature of the strength of stimuli. The behaviour of irritable systems under such conditions (e.g. the summation in time of stimuli of subthreshold strength) is interpreted adequately by the model which gives the conditions of stimulation with the probability distribution of the stimuli by their strength (Széphalmi, 1969).

b) The size of stimuli is near the resting stimulus threshold, or it is greater than that and, further on, the frequency of irritation is near the maximum frequency of irritation. At this stage not all of the stimuli even above the resting stimulus

threshold induces irritation, i.e. the carrier of the stochastic nature is the temporal distribution of stimulus arrival. The present communication deals with this case.

c) The strength of stimuli is comparatively high and the frequency of irritation is comparatively low. Under such conditions the stochastic nature of the stimulation process is overshadowed[1], the events of irritation become deterministic: every stimulus induces an irritation.

d) In a general case both the strength and the time of arrival of the stimuli must be treated as a probability variable. The model reflecting this case must contain also preceding special cases as limiting cases, in the same way as also model a) and model b) contain case c).

It should be mentioned that, even though the model to be treated here draws its conclusions from the condition of the functional unit of irritable systems adapted in successive points of time, by applying the ergodic hypothesis (see e.g. Peterson, 1961), the interpretation can be extended to the behaviour at a certain point of time of an irritable system consisting of more functional units.

2. The structure of the model

The model to be treated is a stochastic model of the stimulus-irritation processes in such a case when the stimuli are not lower than the stimulus threshold belonging to the subnormal state of irritation[2] and when the frequency of irritation is near the maximum frequency of irritation.

It is the aim of the model to determine such a relationship in which the frequency of irritation is obtained as a function of the frequency of stimuli and the strength of stimuli.

We remark that the expression "stimulus" is used in a general sense, meaning e.g. the influence of the cell passing on the irritation during the transmission of irritation; furthermore, when using the word "frequency", we do not suppose a regular periodicity either.

The parameter characterizing the momentary state of the functional unit of irritable systems is the momentary value of the stimulus threshold (under this we mean the minimum strength of stimulus which could just induce an irritation at the given moment). So the time-course of the irritation can be well represented by the change in time of the stimulus threshold. The basis for the description of the changes in stimulus-threshold during the frequent irritation is given by the changes of stimulus threshold due to one single irritation and by those following this, respectively. This change — by which we can unambiguously characterize an irritable object from the point of view of our investigations — is fixed by a

[1] Not considering the natural "widening" of the resting stimulus threshold which can be interpreted as the central limit-distribution-theses of the probability theory and which follows the normal probability distribution.

[2] This limiting factor is needed because the present model does not contain the time summation of subthreshold stimuli.

time-function. We denote this by $f(t)$, and, at present the only reservation is that a positive function of one value should belong to $t > 0$ (the moment of the beginning of irritation is $t = 0$).

Let the examined irritable object be reached by a stimulus series of n frequency and of s strength; the response to these stimuli is a series of irritations of N frequency. Under such conditions the irritations follow each other with an average difference in time of $t_N = 1/N$. The changes in time of the stimulus threshold during the series of stimuli can be described as follows: the parts of the $f(t)$ function beginning at $t = 0$ and ending, on an average, at $t = t_N$ follow each other. So the momentary value of the stimulus threshold is a probability variable, the value of which at a certain given point of time depends on the time of the last irritation preceding the given moment. Its probability distribution — which, if the frequency of irritation is N, is denoted by $P_N(x)$ and interpreted in the usual way (e.g. Rényi, 1962) — is given by the following Equation:

$$P_N(x) = \frac{(\Delta t)_N}{t_N}, \tag{1}$$

where t_N is the average time interval between the successive irritations, and $(\Delta t)_N$ is that part of this interval for which the condition $f(t) \leqq x$ is valid.

Equation (1) indicates that the probability distribution of the stimulus-threshold during a series of stimuli is determined by the changes of the stimulus-threshold during a single irritation and the following ones, respectively, and by the frequency of irritation together. In the case of every concrete $f(t)$ and N the $P_N(x)$ can be determined also numerically with the aid of (1).

According to the interpretation of the probability distribution the value of $P_N(x)$ at an arbitrary point x means a probability for the momentary value of the stimulus-threshold to be smaller than x at a certain point of time. From this follows that this probability depends also on the frequency of irritation; this is indicated by the index N.

A stimulus of s strength, arriving at an arbitrary point of time, induces an irritation if the actual stimulus threshold at the moment of its arrival is lower than s. The value of the stimulus threshold is a probability variable with $P_N(x)$ distribution, and so also the induction of irritation is a random event which can be characterized by a probability value. For the sake of simplicity it is supposed here that the moment of arrival of a given stimulus is independent of the time of the last preceding irritation. This supposition is especially justified when the stimuli come from different sources; but besides, not every stimulus induces an irritation, and so this approximation does not cause a greater inaccuracy even if the stimuli follow each other at regular intervals of time.

Let $p(s, N)$ denote the probability of the fact that a stimulus of s strength stimulates a system the irritation-frequency of which is N. As it follows from the above-mentioned considerations:

$$p(s, N) = P_N(x)|_{x=s}. \tag{2}$$

On the other hand, according to the classic interpretation of the probability:

$$p(s, N) = \frac{N}{n} . \tag{3}$$

From the comparison of (2) and (3) it follows that:

$$N = n \cdot P_N(s), \tag{4}$$

where $P_N(s)$ is the value of the probability distribution of the stimulus-threshold belonging to N irritation frequency at the point s. Equation (4) is a relation of general validity between the strength of the stimulus, the frequency of the stimulus and the frequency of irritation; the specialities treated in detail in the Introduction prevail only in the $P_N(s)$ definition: in the case of this model this means the application of Equation (1).

The model treated here uses only the $f(t)$ function for the deduction of Equation (4), which can be interpreted as follows: the changes of the stimulus threshold during and after the irritation determine unambiguously the frequency of the series of irritation induced by the stimulus series (given by the frequency and by the strength of the stimulus).

3. Conclusions that can be drawn from the model

The model built up in paragraph 2, is concretized in two steps. The first thing to do when applying the model is to give the character of the function $f(t)$ characteristic of the irritable cell to be examined, e.g. as follows: when an irritation appears, the system gets into an absolute refracter stage; the duration of this is denoted by t_0. This stage is represented by a stimulus threshold value of $k = \infty$, corresponding to the fact that the stimuli effecting the system in this state are ineffective, whatever their size is. During the following relative refracter state (the duration of which is denoted by t_1) the stimulus threshold decreases from the initial k_1 value to k_0. The refracter state is followed by a supernormal stage of irritation of t_2 duration, and by a subnormal stage of irritation of t_3 duration, respectively. During these two periods of time the stimulus threshold is lower and higher, respectively, than the k_0 value; with a minimum value of k_2 and a maximum value of k_3, respectively. After the subnormal state of irritation the state of the system can be considered a resting one with a k_0 threshold value, unchanging in time, which is the resting stimulus threshold of the system[3].

On the basis of paragraph 2 we can state that such a concretization of the $f(t)$ function already determines the bases of the main features of the behaviour

[3] The condition of the 1st footnote is valid also here.

of the system. For a numerical analysis we must give the numerical values of $k_0, \ldots, k_3, t_0, \ldots, t_3$ parameters. Further on, the resting value of the stimulus threshold (k_0) and the duration of the absolute refracter state (t_0) figure as units of the strength of stimulus and of the time, respectively; the other parameters should be considered in comparison to these. As the duration of the absolute refracter state is considered as the unit of time, the following values of frequency are compared with the maximum frequency of irritation of the system.

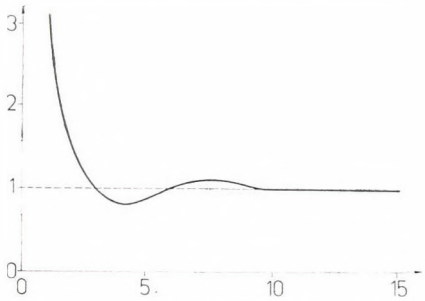

Fig. 1. The schematic pattern of the changes of the stimulus threshold during and afte a single irritation. Abscissa—time, ordinate—stimulus threshold

Fig. 1 is the hypothetic concretization of the $f(t)$ function, with the following parameters: $k_1 = 3.0$; $k_2 = 0.8$; $k_3 = 1.1$; $t_1 = 2$; $t_2 = 3$; $t_3 = 4$; and with $k_0 = 1$; $t_0 = 1$ as units of measurement.

It is generally clumsy to give the $f(t)$ function analytically. The graphical way of presentation applied here needs only elementary algebraic operations in the following calculations; therefore, they are not treated here in detail.

Fig. 2 shows the density function of probability of the stimulus threshold in the case of different frequencies of irritation chosen arbitrarily. The functions have been determined by using Equation (1), with a histogram method.

It can be seen in Fig. 2 that the probability distributions of the stimulus threshold belonging to different frequencies of irritation usually show an essential difference both from each other and the normal distribution. With the decrease of the frequency of irritation the probability distribution of the stimulus threshold — according to the present model — approximates to the δ-function of Dirac appearing with the resting value of the stimulus threshold. If we omit the neglection referred to in the 1st footnote, a normal distribution appears instead of the δ-function. Thus, the approximation of the probability distribution of the stimulus threshold with a normal (or logarithmically normal) distribution can be applied only in limited cases, i.e. with low frequencies of irritation. With the present parameters the one-twentieth part of the maximum frequency of irritation can be considered to be a low frequency of irritation.

The relation between stimulus parameters and frequency of irritation is characterized by Fig. 3 calculated on the basis of Equation (4). This figure shows

the quotient of the frequency of irritation and the frequency of stimuli (further on we call it the factor of frequency-transmission) at a quantitative level, in the case of different parameters of stimulation.

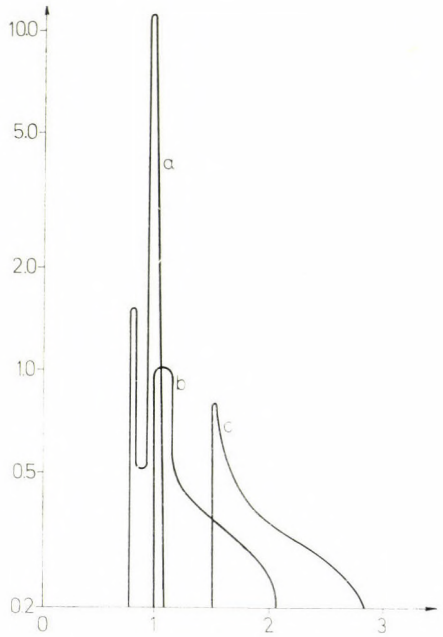

Fig. 2. The probability density function of the stimulus threshold during an iterative periodic irritation process, in the case of different frequencies of irritation. The value of the frequency of irritation: *a*: 0.05; *b*: 0.33; *c*: 0.50
Abscissa – stimulus threshold, ordinate – probability density value on logarithmic scale

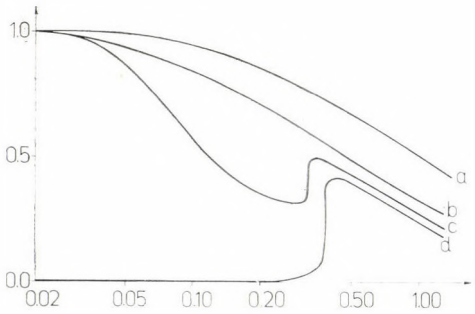

Fig. 3. The dependence of the frequency-transmission factor on the stimulation parameters. The values of stimulus strengths: *a*: 3.0; *b*: 1.5; *c*: 1.0; *d*: 0.9. Abscissa – the frequency of stimulus on logarithmic scale; ordinate – the quotient of the frequency of irritation and frequency of stimulus

Discussion

On the basis of Fig. 3 we enumerate some otherwise generally known phenomena of frequency transmission arising from the model. They are given by the model without the consideration of any biological hypothesis behind those known up to now; this can be interpreted in such a way that these phenomena are not active (or, to put it in another way, are not specific) biological phenomena, but the mechanical consequences of the stochastic nature of the phenomenon treated here. Examining the question from the other side, the model naturally cannot contain the features of the stimulus — irritation processes outside this sphere, e.g. the phenomenon of a series of irritations appearing under the effect of one single stimulus (Ernst, 1968). Such a limitation of the range of reference of the model is in accordance with the fact that the values of the frequency-transmission factor calculated from the model are never greater than 1.

The frequency-transmission phenomena arising from the model are as follows:

1. In the case of any stimulus fixed frequency the frequency-transmission factor increases with increasing strength of stimuli (a — d curves).

2. If the strength of the stimulus is equal to, or greater than, the resting stimulus threshold, the frequency-transmission factor approximates to 1 when the frequency of stimulation decreases (a — c curves); see c) paragraph of the Introduction.

3. If the strength of the stimulus is under the resting stimulus threshold, the frequency-transmission factor approximates to 0 with the decrease of the frequency of stimulation (d curve). This can be considered as an explanation for the noise-straining ability of the irritable systems, arising from the model.

4. In the case of any fixed stimulus strength the frequency-transmission factor approximates with the increase of the frequency of irritation to 0 (a — d curves). This remark follows naturally from the fact that the frequency of irritation is a limited one.

5. If the strength of the stimulus is greater than the maximum stimulus threshold at subnormal state of irritation, the frequency-transmission factor decreases monotonously with the increase of stimulus frequency (a, b curves). If the strength of the stimulus falls between the maximum stimulus threshold at subnormal state of irritation and the minimum stimulus threshold belonging to the supernormal stage of irritation, the frequency-transmission factor shows a maximum at a certain frequency of stimulation (c, d curves). The (local) maximum of the frequency-transmission factor occurs at a stimulation frequency of $n = 0.33$. This extreme value appears also in the case when not the frequency-transmission factor but the frequency of irritation is examined as a function of the frequency of stimulation. With the present parameters the duration of the refracter state is 3 units; so the local maximum of the irritation frequency appears in the case

of a stimulation frequency calculated as a reciprocal value of the refracter state. This is the stochastic interpretation of the Vedenski inhibition as concluded from the model.

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The Dielectric Characteristics of Bound Water

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(Received February 28, 1970)

In order to investigate the dielectric characteristics of the bound water the dielectric constants of dried gelatine powder and gelatine solutions were measured at 2.6 GHz frequency in a temperature range from −7 to 20°C. The decrease of the dielectric constant due to cooling is proportional to the quantity of the frozen water. The dielectric constant of the non-frozen water — in contrast to the value of about 3—4, expectable on the basis of the "ice-berg" hypothesis — is higher than 14. The high dielectric constant of the hydrated gelatine is an indirect proof of the charge-movement on the surface of proteins.

Introduction

According to earlier measurements concerning high-frequency and microwave conductivity the ionic conductivity in protein solutions is decreased, partly because of the ion-binding of proteins, and partly because of the interaction of protein and water (Ernst, 1970; Masszi, Örkényi, 1967, 1967a; Masszi, 1969). King and Medley (1949) suppose that the water-binding of polyelectrolytes decreases the dielectric constant of water, which leads to a decrease of the dissociation of electrolytes. According to the "ice-berg" hypothesis a low value can be expected for the dielectric constant of bound water, but the investigations performed in our institute contradict to the "ice-berg" hypothesis. Pócsik (1967, 1969) has pointed out that the density of bound water is higher than that of free water. In our present experiments the microwave dielectric constant of gelatine solutions at room temperature and that of frozen ones were measured in order to examine the dielectric characteristics of bound water.

Methods

The measurements were made at a frequency of 2.6 GHz ($\lambda = 11.52$ cm) with two different methods:

a) With materials of low conductivity – such as dried gelatine powder, gelatine-toluene mixture, ice – the dielectric constant was determined on the basis of measuring the position of the standing wave minimum. Fig. 1 shows the principle of this measurement. The material to be measured was placed in a specially

prepared coaxial measuring cell (Masszi, Örkényi, 1967a), and the distance between the entrance plane of the cell and the first minimum position was determined. On the basis of the feedline theory, in the case of a cell which is open circuited at the end, with materials of neglectably low conductivity the following equation can be written:

$$\frac{Z_{on}}{Z_o} \operatorname{ctg} \frac{2\pi \cdot l \cdot n}{\lambda} = \operatorname{tg} \frac{2\pi x_n}{\lambda} \tag{1}$$

where Z_{on} is characteristic impedance of the cell containing the material of n refraction-index, Z_o is that of the standing wave meter, λ is the free space wave

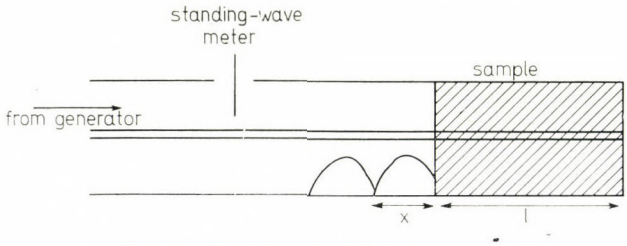

Fig. 1. The scheme of the measurement

length, l is the length of the cell, and x_n is the distance between the entrance plane of the cell and the first minimum position in the case of a cell filled with a material of n refraction-index.

First, the minimum positions were determined with an empty cell and then, after filling the cell with the material to be examined, the shift of the minimum position, Δx. In equation (1), in the case of an empty cell, $n = 1$, and so x_1 can be calculated; further on:

$$x_n = x_1 + \Delta x, \tag{2}$$

from which n was calculated on the basis of equation (1) and, from this the dielectric constant was determined on the basis of the $\varepsilon = n^2$ relation. In consequence of the negligably low conductivity high standing wave ratios occurred, which rendered it possible to determine the minimum positions with an accuracy of 0.1 mm. As equation (1) refers to an ideal case, — for detailed deduction see the Appendix — the accuracy of the approximation was checked by measuring materials of known dielectric constants. According to these measurements the error is 10 per cent at the most.

b) With materials of medium conductivity — such as are 0—50 per cent gelatine solutions at 20°C, or 20—50 per cent frozen ones, the dielectric constant was determined from the measurement of the standing wave-ratio according to Lertes et al. (1966). The principal connection corresponds to that of the preceding

method (Fig. 1). The measuring cell applied here was as long, as to make the wave refracted from the end of the cell to be attenuated completely, and it did not influence the value of the standing-wave-ratio. In this case, with a coaxial waveguide:

$$n = a\left[\frac{r^2 + l}{2r} + \sqrt{\left(\frac{r^2 + l}{2r}\right)^2 - \left(1 + \frac{\kappa^2}{a^2}\right)}\right] \tag{3}$$

where $a = \frac{Z_{o1}}{Z_o}$, r is the standing wave-ratio, κ is the microwave absorption coefficient of the investigated material. With the investigated materials

$$\frac{\kappa^2}{a^2} < 1, \tag{4}$$

and so equation (3) can be simplified to the

$$n = ar \tag{5}$$

form.

The value of a was calculated from the geometrical data of the waveguide, and it was determined also experimentally by measuring a material of known dielectric constant (distilled water). The approximation applied in equation (5) was controlled experimentally, by measuring the attenuation (Masszi, Örkényi, 1967a).

An $l = 2$ cm long cell was applied with measurements of a type, and an $l = 8$ cm long one with measurements of b type. The temperature was stabilized with a liquid-bath, with an accuracy of 0.1 °C. With freezing experiments the sample was kept at a low temperature for 18 hours before the experiments.

Results

For determining single components of the gelatine-water-ice system the following values of dielectric constants were measured.

Water (20 °C)	78
Ice	2.9
55 per cent gelatine powder + 45 per cent air	2.4
55 per cent gelatine powder + 45 per cent toluene	3.0
Toluene	2.5

In order to determine the own dielectric constant of gelatine the approximating formula

$$\varepsilon = p\varepsilon_1 + (1 - p)\cdot\varepsilon_2 \tag{6}$$

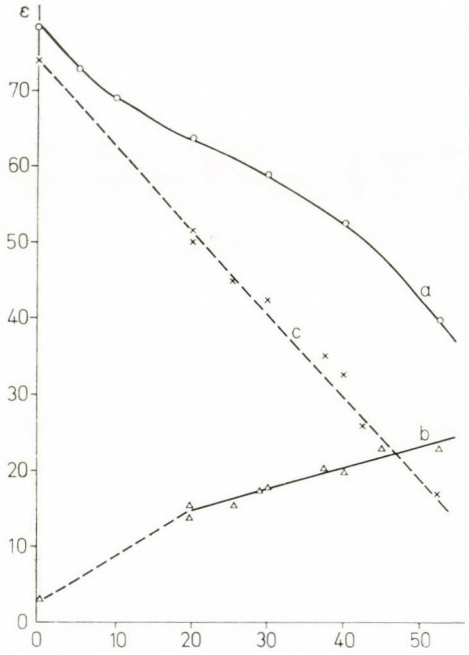

Fig. 2. The dependence of the dielectric constant on the concentration of gelatine (*a*) at 20°C (*b*) at −6°C, (*c*) the difference of the dielectric constants measured at 20°C and −6°C

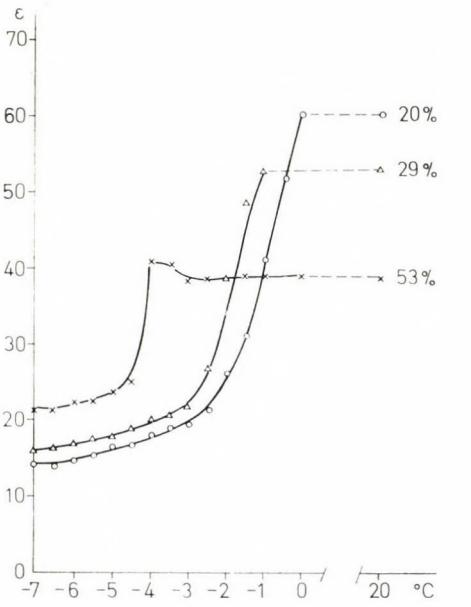

Fig. 3. The temperature-dependence of the dielectric constant. With gelatine solution of o−o 20 per cent, △−△ 29 per cent, ×−× 53 per cent

was applied (Szkanavi, 1953), where ε means the dielectric constant of the mixture, ε_1 and ε_2 mean that of its components, while p and $(1-p)$ are the rate of the volume of 1. and 2. components, respectively. On the basis of the gelatine-air mixture the dielectric constant of dried gelatine is 3.6, on the basis of the gelatine-toluene mixture it is 3.4.

The curve *a* in Fig. 2 shows the concentration dependence of the dielectric constant of gelatine solutions of 20 °C and curve *b* that of gelatine solutions frozen at —6 °C. The difference of the dielectric constants measured at 20 °C and —6 °C, i.e. the decrease of dielectric constant due to the freezing out of water, shows a diminution approximately proportional to the concentration, as shown by the curve *c* of Fig. 2.

When the samples are slowly warmed from —7 °C, with a speed of about 1 °C/hour, the dielectric constant will reach the value corresponding to room temperature by an initially slow and then abrupt rise (Fig. 3).

Discussion

Fig. 3 refers to the fact that at low temperature the water-binding going together with swelling hinders the freezing out of water to an increasing extent. On the basis of the data of Tanner (1966) gelatine solutions at —6 °C become concentrated up to a water content of 0.5 g water/g gelatine, and with further cooling this quantity of water changes only to a slight degree. Similar result has been obtained also by the calorimetric investigations of Mrevlisvili and Privalov (1967). According to the density measurements of Pócsik (1969) 0.9 g water/g dry material does not freeze out from the muscle at —7 °C. On the basis of the above data the nonfrozen water can be considered as bound water, for which, on the basis of the "ice-berg" hypothesis the following approximation may be valid:

$$\varepsilon_{\text{bound water}} \approx \varepsilon_{\text{ice}} \approx 3$$

According to our measurement:

$$\varepsilon_{\text{gelatine}} < 4$$

so, after freezing,

$$\varepsilon_{\text{solution}} < 4$$

can be expected for the dielectric constant of the solution; in contrast to this the dielectric constant of the frozen samples is greater than 14 and it increases with increasing concentration. So the measured data contradict the "ice-berg" hypothesis.

According to the electron microscopic investigations of Luyet and Rapatz (1958) well-definable intermolecular ice-crystalls come about under the effect

of freezing in the gelatine solutions on the basis of which, at first approximation, the frozen samples can be considered as a mixture of ice and gelatine solution. On the basis of equation (6), the dielectric constant at —6 °C (ε_{-6}) is:

$$\varepsilon_{-6} = p_w \cdot \varepsilon_{\text{ice}} + (1 - p_w) \cdot \varepsilon_{\text{solution}} \tag{7}$$

and, after warming, at 20 °C (ε_{20}):

$$\varepsilon_{20} = p_w \cdot \varepsilon_{\text{water}} + (1 - p_w) \cdot \varepsilon_{\text{solution}} \tag{8}$$

where p_w is the proportion of the volume of the frozen water to the whole volume and $\varepsilon_{\text{solution}}$ is the dielectric constant of the concentrated solution.

From equations (7) and (8):

$$p_w = \frac{\Delta\varepsilon}{\Delta\varepsilon_w} \tag{9}$$

where $\Delta\varepsilon = \varepsilon_{20} - \varepsilon_{-6}$, and $\Delta\varepsilon_w = \varepsilon_{\text{water}} - \varepsilon_{\text{ice}} = 75$. Table 1 shows that the quantity of the frozen water decreases with increasing concentration and that the

Table 1

The decrease of the dielectric constant under the influence of freezing ($\Delta\varepsilon$); the proportion of the volume of the frozen water to the whole volume (p_w); the concentration of the non-frozen solution (c per cent); the dielectric constant of the non-frozen solution ($\varepsilon_{\text{solution}}$)

	Gelatine concentration in per cent			
	20	30	40	50
$\Delta\varepsilon$	52	42	33	18
p_w	0.70	0.57	0.45	0.24
c per cent	68	70	72	70
$\varepsilon_{\text{solution}}$	37	35	34	28

solutions concentrate to a concentration of approximately 70 per cent. This value agrees well with the data of Tanner (1966). The dielectric constant of the residual, non-frozen gelatine solution ($\varepsilon_{\text{solution}}$) has a value of about 35. Starting with the fact that this value is given by the dielectric constant of bound water and of gelatine in an additive way, the dielectric constant of bound water is 120 to 130. From this high microwave dielectric constant value, — if we do not suppose the bound water to have characteristics similar to ferroelectric — we can conclude that gelatine cannot be decomposed to the sum of two materials of different dielectric constant; and the protein-water interaction and the charge movement along the protein play an important part in the determination of the value of the dielectric constant.

Appendix

With the deduction of equation (1), we started with the transformation equation, according to which the value of impedance at distance x from the termination is, with a waveguide terminated with a Z_t impedance:

$$Z = Z_o \frac{Z_t + jZ_o \operatorname{tg} \frac{2\pi n}{\lambda} x}{Z_o + jZ_t \operatorname{tg} \frac{2\pi n}{\lambda} x} \tag{10}$$

where Z_o is the wave resistance of the waveguide, $j = \sqrt{-1}$. With a cell of l length, open circuited, $Z_t = \infty$ and so at the entrance of the cell the impedance — according to (10) — is:

$$Z_c = \left(-j \operatorname{ctg} \frac{2\pi}{\lambda} nl\right) Z_{on} \tag{11}$$

where Z_{on} is the wave-resistance of the cell filled with a material of n refraction index;

$$Z_{on} = \frac{Z_{ol}}{n} \tag{12}$$

(Almássy, 1967), where Z_{ol} is the wave-resistance of the empty cell, which can be calculated on the basis of the geometrical data. Its value is: 94 ohm (Masszi, Örkényi, 1967a).

In the standing wave-meter connected to the cell, the impedance is $Z = 0$ at the minimum position and so, — from equation (10) — at the x_n first minimum position:

$$0 = -jZ_{on} \operatorname{ctg} \frac{2\pi}{\lambda} nl + jZ_o \operatorname{tg} \frac{2\pi}{\lambda} x_n, \tag{13}$$

where $Z_o = 50$ ohm is the wave-resistance of the standing wave-meter. From equation (13), we can conclude to relation (1), and solving it for x_n we obtain:

$$x_n = \frac{\lambda}{2\pi} \operatorname{arc\,tg} \left(\frac{Z_{ol}}{nZ_o} \operatorname{ctg} \frac{2\pi}{\lambda} nl\right) \tag{14}$$

On the basis of equation (14), taking $\varepsilon = n^2$ into consideration, the $-\Delta x = f(\varepsilon)$ calibration curve was adapted, from which the ε, corresponding to the measured Δx value, was determined (Fig. 4).

At the measurements the following sources of error can occur:

1. With the open circuited waveguide, radiation and stray capacity can occur. A $\frac{\lambda}{\sqrt{2.1}}$ long coaxial leading section filled with teflone, open at the end, having an inner diameter of 3 mm and an outer one of 7 mm, was connected to the end of the cell. In this case no radiation occurs at the end of the waveguide, and the stray capacity increases the electric length of the waveguide sector with

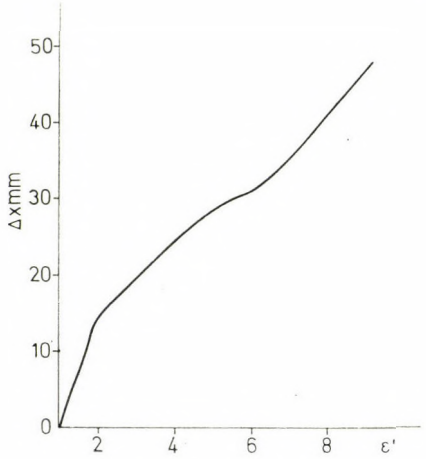

Fig. 4. The $-\Delta x = f(\varepsilon)$ function

1 mm (Marcuvitz, 1948). The waveguide section was shortened by 1 mm, and so, according to equation (10), at the end of the cell $Z_t = \infty$.

2. On each side of the cell, at the cell-waveguide transition, a leap-capacity occurs which can cause a shift of the minimum positions. On the basis of Whynnery's and Jamieson's (1944) calculations the value of leap-capacity can be esteemed to be 0.05 *pF*, which corresponds to a real susceptance of 8 mohm^{-1} cm^{-1}. By the repeated applications of equation (10) the minimum positions, calculated by considering the leap-capacities, were determined. These appeared as 1—2 mm smaller than the values calculated on the basis of equation (14). As it was the difference between the minimum positions that was determined during the measurements, the error is smaller than 2 mm.

3. The loss, occurring in the material to be measured, neglected at the deduction of equation (14), can influence the minimum position. With the investigated materials the standing-wave-ratio is the same as that measured with an empty cell which means that the dielectric loss is negligable in comparison to the loss caused by the wall-currents.

At the deduction of equation (3), we started with the assumption that at a cell length with which the wave reflected from the end of the cell would com-

pletely damped:

$$Z_t = \frac{Z_{ol}}{n^*} \tag{15}$$

(Istvánffy, 1957), where

$$n^x = n - j\kappa \tag{16}$$

is the complex refraction index. On the basis of equation (15) the reflection factor:

$$\Gamma = \frac{a - n^x}{a + n^x}, \tag{17}$$

where $a = \frac{Z_{ol}}{Z_o}$, and the absolute value of the reflection factor:

$$\Gamma_0 = \sqrt{\frac{(a-n)^2 + \kappa^2}{(a+n)^2 + \kappa^2}} \tag{18}$$

Equation (18) when raised up to the second power becomes:

$$\Gamma_0^2 = \frac{a^2 - 2an + n^2 + \kappa^2}{a^2 + 2an + n^2 + \kappa^2} \tag{19}$$

which is a quadratic equation for n, the solution of which is

$$n_{12} = a\left[\frac{1+\Gamma_0^2}{1-\Gamma_0^2} \pm \sqrt{\frac{1+\Gamma_0^2}{1-\Gamma_0^2} - \left(1 + \frac{\kappa^2}{a^2}\right)}\right] \tag{20}$$

By replacing equation

$$\Gamma_0 = \frac{r-1}{r+1}, \tag{21}$$

$$n_{12} = a\left[\frac{r^2+1}{2r} \pm \sqrt{\frac{r^2+1}{2r} - \left(1 + \frac{\kappa^2}{a^2}\right)}\right] \tag{22}$$

In equation (22), if the discriminant is positive, $n > a$, if it is negative, $n < a$. As $a = 1.9$, with our measurements it is enough to take only the positive sign into consideration.

The relative error made with the application of equation (5):

$$\frac{n - ar}{ar} = -\frac{1}{2}\left(1 - \frac{1}{r^2}\right)\left[1 - \sqrt{1 - \frac{4\kappa^2}{\left(r - \frac{1}{r}\right)^2 a^2}}\right] \tag{23}$$

Considering the fact that if x is positive

$$+\sqrt{1-x} > 1-x, \tag{24}$$

$$\left|\frac{n-ar}{ar}\right| < \left(1-\frac{1}{r^2}\right)\frac{2\kappa^2}{\left(r-\frac{1}{r}\right)^2 a^2} \tag{25}$$

Equation (25) indicates that n can be determined independently of κ with a standing wave-ratio measurement. E.g.: in the case of a gelatine solution of 50 per cent $\kappa \approx 0.8$ and $r^2 = 12$, the mistake made with the application of equation (5) is smaller than 3 per cent. With distilled water this value decreases to 0.4 per cent.

The following sources of error can occur with the measurement:

1. The wave reflected from the end of the cell is not damped completely. For the field-strength amplitude reflected from the end of the cell we can write:

$$E \leq W_0 e^{-\frac{2\pi}{\lambda} l\kappa} \tag{26}$$

where the equation is valid only with fitted cells. With distilled water $\kappa \approx 0.6$ and, on the basis of (26), $\frac{E}{E_0} \leq 0.07$. If the condition concerning the cell-length is fulfilled, the standing wave-ratio and the minimum position is independent of the fact whether the cell is short-circuited or open-circuited. This offers a simple possibility of checking, which was used with every measurement.

2. The susceptance of 0.8 $\text{mohm}^{-1}\,\text{cm}^{-1}$ occurring in the front of the cell can influence the value of reflection coefficient. Taking this susceptance into consideration equation (18) can be written as

$$\Gamma_0 = \sqrt{\frac{(a-n)^2 + (\kappa - 0.8\,10^{-3} Z_{ol})^2}{(a-n)^2 + (\kappa - 0.8\,10^{-3} Z_{ol})^2}}$$

and, according to this, the effect of leap-capacity appears as if the value of κ decreased with ~ 0.08. According to equation (25) the change of κ to this extent does not influence the value of r.

3. The condition for determining the standing wave-ratio is the knowledge of the characteristic of the rectifier crystal. A previously calibrated IHS-IN 23 crystal diode of quadratic characteristic was used with the measurement.

The author is indebted to Dr. I. Torma for his valuable remarks concerning the mathematical treatment of the experimental results.

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The Influence of $\bar{\beta}$-Radiation of Tritium on the Excitation and Ion Content of the Striated Muscle

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(Received May 8, 1970)

The effect of β-radiation of tritium (maximum energy 18 KeV) on the excitation of the striated muscle was investigated. Excised sartorius muscles of the frog *Rana esculenta* were kept in Ringer solution containing tritium with a specific activity of 10 mCi/ml at a temperature of 2°C. The stimulus threshold, the amplitude of action potential, K^+- and Na^+-content and the resting electric resistance of the muscles were measured at fixed intervals.

The stimulus threshold of muscles absorbed a dose of a few thousand rads was found to diminish significantly when compared to that of the control muscles. The amplitude of action potential changed in an opposite way. The muscles treated with tritium had not any considerable K^+-loss and Na^+-gain while their resting resistance was always higher than that of the controls kept in normal Ringer solution under similar conditions.

The effects enumerated above can be interpreted on the basis of Ernst's semiconductor hypothesis (cf. Ernst, 1963).

Introduction

The considerable radiation resistance of muscle is accentuated by papers of radiation biology dealing with muscle (Hollaender, 1950; Schreiber, 1957). The idea about the radiation resistance of muscle is based, theoretically, on the fact that muscle tissue does not consist of mitotic cells; on the other hand, many experimental data prove that the muscle is a machine almost indestructible as far as its mechanical function is considered.

The opinion about similar features of nerve tissue has recently changed considerably (Lebedinsky et al., 1958); for recent years studies proved that excitation processes in nerves are very sensitive to radiation and, what is more, there are some results which may be considered as positive radiation effects (Bachofer, Gantereaux, 1960). This respect — i.e. that a positive radiation effect had been found — was taken into account also in interpreting our own results. Many investigations followed the above mentioned ones, and they drew attention to new aspects of radiation effects on muscle and nerve tissue (Hugh, 1958; Ernst et al., 1959; Bergeder, Hockwin, 1960; Bergström et al., 1962; Tigyi, 1964).

On the other hand, on the basis of Ernst's semiconductor hypothesis, one could expect ionizing radiation to have a strong influence on the excitation processes of the muscle, for the properties of semiconductors are sensitive to radiation and muscles are assumed to have semiconductor characteristics.

Methods

Excised sartorius muscle was used as experimental object. With regard to the low energy of the tritium it was applied in the form of tritiated water as a component of Ringer solution, serving, thereby as an internal, incorporated radiation source. The muscles to be irradiated were kept in closed glass dishes containing tritiated normal Ringer solution at a temperature of 2°C, while the control ones were kept in an inactive normal Ringer solution with other conditions left unaltered. The specific activity of the tritiated solution was 10 mCi/ml; thus, the radiation dose (E) calculated (Whitehouse, Putman, 1953) as the product of a constant ($k = 2.22$), of specific activity, of average radiation energy, of irradiation time and of muscle weight was:

$$E = 2.22 \cdot 0.01 \text{ Ci/ml} \cdot 6000 \text{ eV} \cdot 24 \text{ hours} \cdot 0.2 \text{ g} \sim 700 \text{ rad/day}.$$

The absorbed dose was supposed to be homogeneously distributed inside the muscle. Control measurements were performed at intervals of 24 hours: the muscles were taken out of the tritiated solution, warmed up to 20°C, and then put (at room temperature) onto platinum electrodes with a diameter of 0.5 mm mounted in a plexiglass holder. The stimulator was an electronic square wave generator constructed in this institute.

The details of the experimental procedure are summarized as follows:

1. The stimulus threshold was measured by recording the appearance of the action potential on the screen of a cathode-ray oscilloscope; the duration of electric stimulus was 0.1 ms.

2. The amplitude of action potential was determined by photos taken of action potentials elicited by stimuli of 2 V and of 0.1 ms.

3. In order to analyse the ion content, the muscles were dried until constant weight and, after weighing, we incinerated it in an electric furnace at 400°C. The obtained ash was dissolved and its inorganic ion content was measured with the method of flame photometry.

4. In order to measure the longitudinal and transversal resistance a special vessel was made to which platinum plate electrodes with a width of 3 mm were attached. The stability of the contacts was guaranteed by a covering insulating plate burdened by a weight of 20 grams. The length and width of muscles were measured in order to check there was no shrinkage or swelling. The transversal and longitudinal resistance of muscles could be measured by this method with

an accuracy of 1 per cent. The measuring was performed with a Wheatstone-bridge which was balanced by a parallel circuit of a resistor–condenser system, the supply source was an a.c. generator of 10^4 Hz (Fig. 1).

5. The changes of water content of muscles were checked by measuring their weight during the time of experiment.

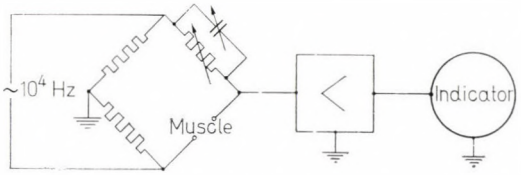

Fig. 1. The scheme of the resistance-measuring apparatus. Frequency: 10^4 Hz

Results

Excitability: It was surprising that the stimulus threshold of muscles irradiated for about a fortnight remained lower than that of the non-irradiated controls. If muscles absorbed a dose of a few thousand rads, the increase of excitability was significant at a 5 per cent level in Student's "*t*" test. The increase of excitability went on proportionally to the absorbed dose and, under the effect of a dose of 5—6000 rads equivalent to an 8-day dose, a very high degree was reached. This trend is shown in Fig. 2, which gives the average data on 20 muscles in per-

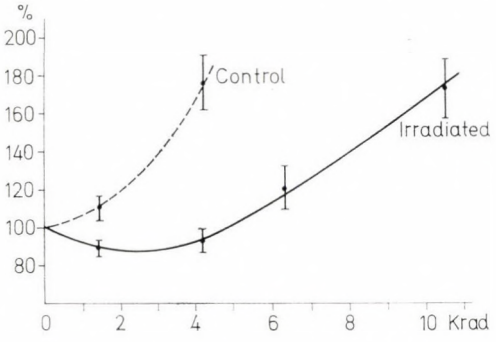

Fig. 2. The stimulus threshold (given as the percentage of the initial values) plotted against the absorbed dose. Dose rate: 700 rads/day

centage of the initial values. (An overwhelming part of the control muscles was no longer excitable than for a week; thus, their statistical evaluation would be unreal.)

Moreover, a considerable percentage of the treated muscles after irradiation with 2000 rads displayed a transient decrease of the stimulus threshold. This averaged to 10 per cent and was significant at a probability level of $P = 0.05$,

considering in particular, that no corrections were made for taking into account the individually differing lifetime of muscles.

The amplitudes of action potentials of treated muscles, elicited by stimuli of 2 V and of 0.1 ms, were generally larger than those of the control muscles. The amplitude of action potential augmented during the first period of irradiation, and later diminished. For the standard deviation was rather large, this change

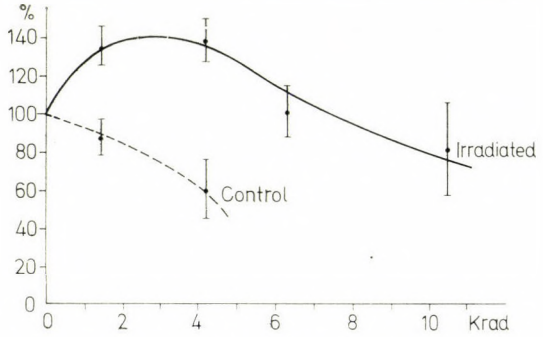

Fig. 3. Amplitudes of action potentials plotted against the absorbed dose. Amplitudes are given as the percentage of the initial values. Dose rate: 700 rads/day

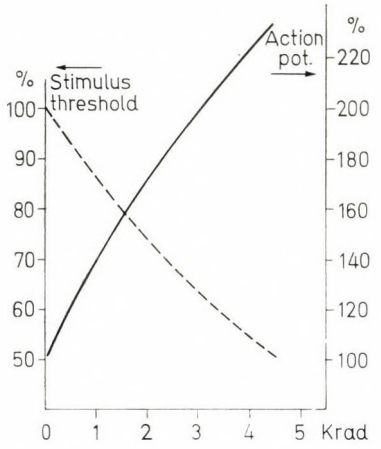

Fig. 4. Action potential amplitude (full line) and stimulus threshold (dashed line) plotted against the absorbed dose. The curves represent the rate of the data of treated muscles and of the control ones; both of them are given as the percentage of the initial values

became significant only when the absorbed dose exceeded 4000 rads ($P = 0.05$); even if its relative values were considered (Fig. 3).

Comparison of the effects, both rather considerable when compared with the control pairs, reveals a close negative correlation between stimulus threshold and action potential also during the first period of irradiation (i.e. during the first week). The correlation coefficient was $r = -0.85 \pm 0.08$ at a dose of 4000 rads.

Since only a small part of control muscles was excitable during the second period, no relative values were computed over irradiations of 5000 rads (Fig. 4).

Ion content: Since the change of the ion content is generally known as one of primary reactions to irradiation, the K^+- and Na^+-ion content of the muscles were determined. Unexpectedly, the changes in K^+- and Na^+-ion content and in the ratio of K^+/Na^+ were significantly lower in the case of treated muscles

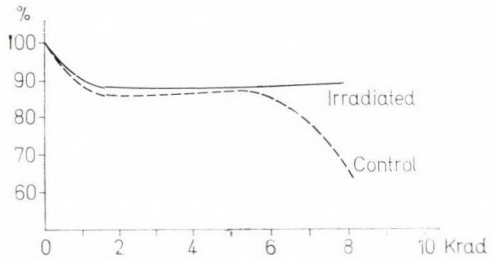

Fig. 5. K-content plotted against the absorbed dose. Average values of 30 muscles expressed in percentage of the initial values

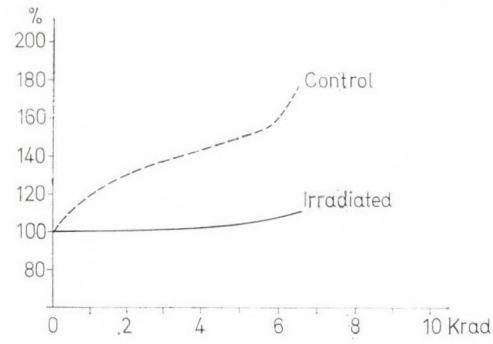

Fig. 6. Na^+-content plotted against the absorbed dose; average value of 30 muscles in percentage of the initial values

than in the control ones. The potassium content of irradiated muscles (in terms of mg K per g of dry muscle) was approximatively constant after the first 48 hours, while that of the control muscles distinctly decreased after a week (Fig. 5).

Similarly, the Na^+-ion content of treated muscles remained practically unaffected, whereas the Na^+-uptake of the untreated controls increased soon and considerably (Fig. 6).

The absorbed radiation dose seemed to prevent the change of the ion content and in such a way by keeping the K^+/Na^+ proportion at a normal level, it may enable the treated muscles to survive for a time twice as long as unirradiated ones.

Electric resistance: The longitudinal component of the resting impedance of a muscle could be interpreted as an indicator of structural changes of muscle

tissue. Therefore it seems important that it was over the control throughout the experiments. The difference was significant at a level of $P = 0.05$, as proved by a two-sample t-test (Fig. 7 and Table 1).

No considerable change of transversal resistance was observed. There was no need for making any correction for changes of length and width, since the water content of muscles, as checked by measuring their weight changed to any greater extent but at the end of experiments.

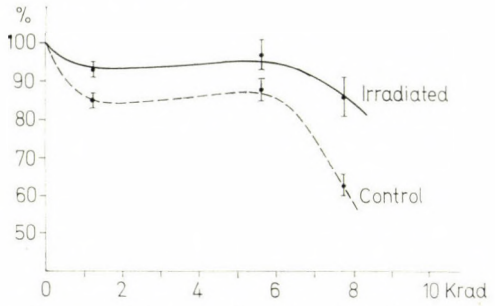

Fig. 7. Longitudinal resistance of the muscles plotted against the absorbed dose. Average value of 30 muscles in percentage of the initial values

Table 1

Longitudinal electric resistance of irradiated and control muscles at three characteristic phases of a fortnight-long incubation (average of 30 experiments)

Irradiated	Control
~1500 rad: 2370 ± 50 Ω	2120 ± 50 Ω
~6000 rad: 2490 ± 100 Ω	2180 ± 60 Ω
~7000 rad: 2190 ± 100 Ω	1580 ± 50 Ω

Discussion

If we synthetize the data of the change of ion content and the active and passive electrical changes, a simple hypothesis could be suggested at first: the membrane permeability of the muscle diminishes, i.e. the packing of the membrane increases. At first sight this conclusion appears to be backed by the inhibition of the shift of K^+- and Na^+-ion content and of the electric resistance. However, a deeper analysis does not confirm this hypothesis:

a) the transversal electric resistance does not differ from that of the controls

b) this hypothesis can not account for the increase of excitability.

The fact, that the irradiation does not influence the transversal resistance, but it does the longitudinal one, supports the opinion, that the effect is due to the

change of the longitudinal (fibrillar) structure — at least in part — and that the fibre membrane remains unaffected. This idea is in accordance with the opinion of our institute on the bound K^+-content of muscle and the semiconductor hypothesis of excitability (Ernst, 1963).

There are some other data according to which the isotope-mass effect of tritium may play a part in producing the above-presented effects, therefore new experiments to be performed with deuterium are now in progress in our laboratory.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (3), pp. 341—348 (1970)

Semiconductor Property of Frog Sartorius Muscle

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(Received May 8, 1970)

The temperature dependence of membrane resistance and that of injury potential of frog sartorius muscle fibres were investigated in the temperature range of 16°C—43°C. The membrane resistance decreased exponentially when the muscle was warmed up from 16°C to 24°C. The computed activation energy was found to be greater by one order than what could be due to an ionic conduction. The resistance did not depend on temperature over 24°C.

The injury potential increased up to 31°C ($Q_{10} = 6$ mV), it was constant between 31°C and 37°C and decreased abruptly ($Q_{10} = 45$ mV) over this temperature.

Introduction

Excitable tissues have to be closely associated with semiconductor properties as it was first suggested by Ernst (1955, 1956). Thermoelectric and photoelectric effects were found in nerve and muscle, and they were interpreted in terms of the semiconductor character of these tissues (Lakatos, 1969a, 1969b). Wei (1969) supposed quite hypothetically on the basis of a few experimental results (Segal, 1968; Cohen, Keynes, 1968; Fraser, Frey 1968) that the membrane of a giant nerve axon could operate like a *pnp* junction transistor. Also our working hypothesis has been based on an analogy. When the current across a nerve membrane is plotted against the potential difference (Cole, Curtis, 1941), there is a remarkable similarity between this curve and that of a semiconductor p — n junction as far as the shape of the curve and the order of parameters are concerned. It may be supposed that this similarity is due to a common physical basis; in other words, the cell membrane has semiconductor characteristics. It was the aim of our experiments to check this assumption. A very typical feature of semiconductors is their resistance decrease with increasing temperature; this was shown on dried frog muscle (Lakatos, 1962).

The experiments to be presented here were carried out on excised frog muscles the membrane resistance of which was measured as the function of temperature, while other conditions were kept constant.

Methods

The experiments were performed with sartorius muscle fibres of the frog *Rana esculenta*. The excision of the sartorius muscle was carried out under dissecting microscope in order to avoid any injury of the surface fibres. The preparation was laid on a thin glass slide with its visceral side upwards and attached to the slide in a slightly stretched state by its tendons with plastic clamps. The muscle prepared in this way was put into a Petri dish containing 100 ml of Ringer solution and mounted in the apparatus shown in Fig. 1.

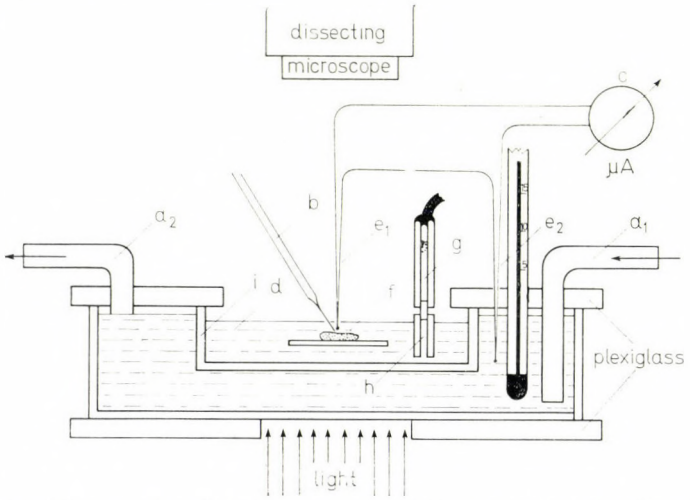

Fig. 1. Scheme of the experimental arrangement. a_1: the influx and a_2: the efflux of the water controlling the temperature; *b*: microelectrode; *c*: galvanometer; *d*: Ringer solution; e_1 and e_2: junctions of the thermocouple; *f*: the muscle (cross-sectional view); *g*: indifferent calomel electrode; *h*: agar-agar bridge; *i*: Petri-dish. For further explanation see text

Glass microelectrodes having resistances of 5 to 15 Mohm were used to perform the measurements; their tip diameter was less than 1 micron. Saturated normal calomel electrodes were used as unpolarizable ones, their total resistance not exceeding 6 Kohm.

The temperature of the preparation was controlled by an ultrathermostat (Type U 10 MEDINGEN). The surface temperature of the muscle was measured with a thermocouple. The measuring was started after stabilisation of the temperature as indicated by the thermoelement. The absolute value of the temperature of the muscle was obtained as the resultant of the temperature taken by the mercury thermometer and the temperature difference measured by the thermoelement; the accuracy of the measurement was $\pm 0.5°C$. The thermal steady state took 20 min to set in.

The membrane resistance was measured by the so-called "one microelectrode method" (Schanne et al., 1966) which is similar to that used for settling the inner

resistance of a battery. Corresponding to this the electromotive force of the "battery", i.e. the injury potential (E_m), and the voltage drop (U_{k1}) on a standard resistance (R_k) of 20 Mohms were measured. The resistance could be calculated from the above mentioned data on the basis of Ohm's law, as follows:

$$R_b = \left(\frac{E_m}{U_{k1}} - 1 \right) R_k, \tag{1}$$

when the 10^{12} ohm input impedance of the cathode-follower is considered as infinitely great in comparison with the other resistances in question. The resistance R_b represents the sum of the membrane resistance (R_m) and of the resistance of the microelectrode (R_e). R_e was therefore determined in every measurement by connecting it with an outer voltage E_t nearly equal to the injury potential (Fig. 2) while the microelectrode was not in contact with the muscle but with the Ringer solution. In this way the error caused by the non-linearity of the current-voltage characteristics of a microelectrod (Krischer, 1968) was avoided. If a voltage drop in the resistance R_k is U_{k2} then:

$$R_e = \left(\frac{E_t}{U_{k2}} - 1 \right) R_k \tag{2}$$

and the membrane resistance:

$$R_m = R_b - R_e \tag{3}$$

Substitution of (1) and (2) into (3) results in:

$$R_m = \left(\frac{E_m}{U_{k1}} - \frac{E_t}{U_{k2}} \right) R_k \tag{4}$$

The injury potential and the voltage in R_k were recorded through a high stability DC cathode-follower by a Kipp and Zonen micrograph (Type BD 1) (Fig. 2).

The accuracy of measurement was determined by substituting for R_b a 3 Mohm resistor. Its resistance could be measured in a reproducible way; the standard deviation was ± 0.05 Mohm. This value was not influenced by using as R_k a resistor of less than 20 Mohms. That means that the relative accuracy of the measurement was ± 2 per cent.

The measurements covered the temperature range from 16°C to 43°C. The procedure was begun at a temperature below 20°C. After the membrane resistance had been measured 5 times at the least, the temperature was increased in 5°C to 10°C steps up to a temperature of 43°C, at which the last measurements were performed. When a new temperature was set in, the Ringer solution was refreshed. According to this method each muscle was investigated at 4 or 5 temperature levels; the average duration of the whole procedure was 4 hours.

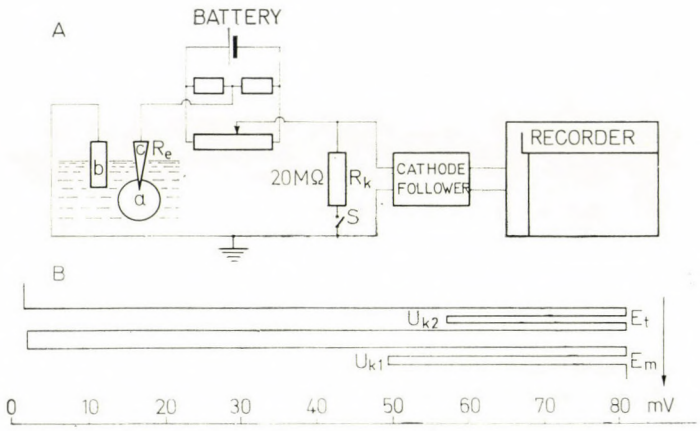

Fig. 2 A. Electronic scheme of the measurement. *a*: muscle fiber (cross-sectional view); *b*: indifferent electrode; *c*: microelectrode. When the switch *S* is on, the voltage drop in standard resistance R_k is recorded; B. Plot recorded by the micrograph, according to which $R_m = 4.9$ Mohm. The record chart moved in the direction shown by the arrow at a speed of 720 mm/h

Results

14 muscles were investigated, all at 26 different temperatures within the temperature range mentioned above. On the average the membrane resistance of 30 fibres was determined at each temperature level.

Control experiments based on two considerations were carried out. On the one hand it seemed necessary to settle whether the alterations in membrane resistance were due to the progress of time or not; on the other hand, the reversibility of these changes was checked. In order to investigate the first question the muscles were kept at 20°C for 10 hours; on the average, their membrane resistance increased by 0.3 Mohm per hour. (The measurements were not corrected by this value.) The reversibility was checked, as follows: after the muscles had been investigated at gradually increasing temperatures and their membrane resistance measured between 30°C and 40°C they were cooled relatively quickly to the initial temperature (about 18°C) and their membrane resistance was measured again. The data obtained are summarized in Table 1. As it can be seen, though the resistances generally increased upon the effect of the cooling, they remained 2 or 3 Mohms lower than their average initial values.

If the muscles were investigated under the condition of increasing temperature the average membrane resistance abruptly decreased from 10.3 Mohm to 1.5 Mohm in the range 16°C—24°C. Above 24°C there was no significant change in the membrane resistance, but when the temperature exceeded 42°C this relatively constant range was followed by an increase up to 3.5 Mohm (Fig. 3).

Table 1

Data obtained in control experiments concerning reversibility
Each number represents the average of 5–10 experimental values

No. of the muscles	Before heating		At heating		Cooled again	
1	16°C	10.7 MΩ	30°C	2 5 MΩ	16°C	6.0 MΩ
2	17°C	8.4 MΩ	38°C	1.8 MΩ	16°C	7.5 MΩ
3	16°C	5.4 MΩ	37°C	2 2 MΩ	16°C	3.3 MΩ
4	17°C	9.0 MΩ	21°C	3.0 MΩ	17°C	4.6 MΩ

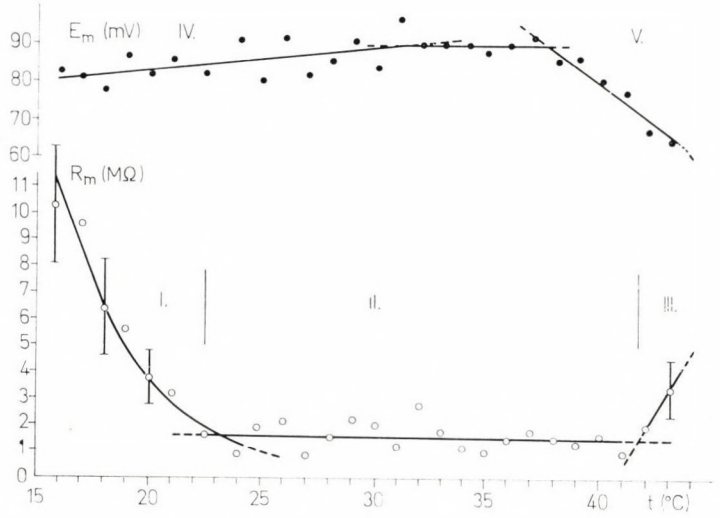

Fig. 3. Dependence of the membrane resistance (○) and of the injury potential (●) on the temperature. The full lines are the regression lines fitted to the sample points over the different temperature ranges. The regression coefficients (slopes) are $\frac{2 \text{ ev}}{k\,^{\circ}\text{C}}$, $0.017 \frac{\text{Mohm}}{^{\circ}\text{C}}$, $0.6 \frac{\text{mV}}{^{\circ}\text{C}}$ and $-4.5 \frac{\text{mV}}{^{\circ}\text{C}}$, for the sectors I., II., IV. and V., resp.

The decrease of the membrane resistance had an exponential character in the range I. Since d.c. was used in the measurements the imaginary components of the membrane resistance could be left out of consideration. Therefore, the alterations of resistance were mainly due to the changes of the amount of carriers. Their dependence on the temperature in semiconductors is described by the relationship

$$R = R_0 e^{E/kT} \tag{5}$$

where E is the activation energy of charge carriers, k is the Boltzmann's constant and T is the absolute temperature. The logarithm of equation (5) is:

$$\log R = \log R_0 + \frac{E}{kT} \tag{6}$$

If $\log R$ is plotted against $\frac{1}{T}$, the relationship is represented by a straight line, the slope of which is $\frac{E}{k} = B$. The results were treated in this way, and regression line was fitted to the sample points, the slope was the regression coefficient (B). The

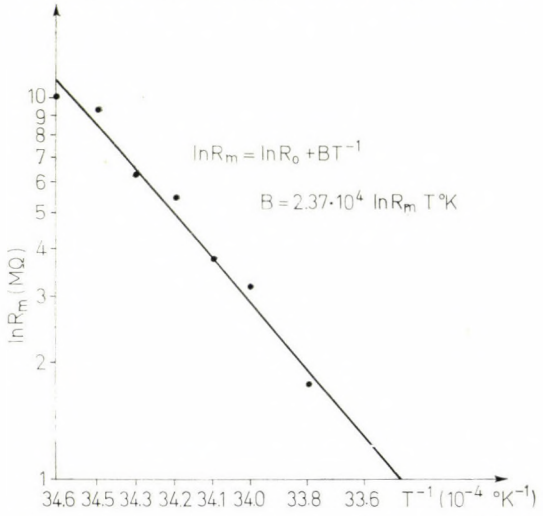

Fig. 4. Logarithmus of the membrane resistance over the range 16°C—24°C plotted against $\frac{1}{T°K}$. The full line is the regression line, B: the slope (see text)

activation energy is given by the product kB; and $E = 2$ ev activation energy could be computed from our experimental result (Fig. 4). The standard deviation of this activation energy was ± 0.1 ev — reckoned from that of the regression coefficient.

Discussion

This activation energy of 2 ev is by nearly an order greater than that needed by the ions of Na^+ and K^+ for permeating the membrane of squid giant axon (0.42 ev and 0.07 ev, resp. Hodgkin, Keynes, 1955) and of human red cell (0.87 ev and 0.65 ev resp. Glynn, 1956; Solomon, 1952). The voltage source of the measurements was the injury potential itself which would be a concentration potential

of K^+ according to the ion-theory of bioelectricity, it means that the carriers penetrating the membrane ought to be K^+ and/or Cl^- ions. The result that the activation energy is 2 ev contradicts this, but is in accordance with the experimental results of Eley and Spivey (1960); they measured the d.c. conductivity of proteins, which was found to be 2—3 ev. The details of their investigation made it clear that the conduction of proteins had a pure electronic character (at least when they are thoroughly dried); protons or other ions did not take part in the conduction process. The activation energy of d.c. conductivity of dried muscle with less than 2 per cent water content is 3.2 ev, and 2 ev if the muscle has a little bit higher water content (but not more than 5 per cent). Potassium ion requires an activation energy of 0.65 ev for permeating synthetic lipid membrane (Bangham et al., 1965). The low frequency conductivity of KCl was measured in gelatine solution of 8.5 per cent and an activation energy of about 0.2 ev was derived (Masszi, Örkényi, 1967). Considering all the above mentioned data the activation energy of 2 ev computed from the reported measurements does not fit in with the hypothesis according to which potassium, chloride or other ions would be the only charge carriers. Though Wei's hypothesis (1969) according to which the membrane behaves like a transistor, is not verified by the present experiments, these afford a chance to assume that the charge carriers penetrating the membrane may be electrons or holes.

For the membrane resistance practically does not depend on the temperature in the range of 24°C—41°C, it may be supposed, that as a consequence of the afore-said hypothesis, the impurities causing conductivity are saturated above 24°C. However, this assumption requires further investigations (particularly considering the time-dependence of membrane resistance).

The increase of resistance at 42°C or 43°C could not be attributed to the increase of membrane resistance, because general coagulation of muscle proteins could be observed at this temperature.

It is, however, worthy of attention that no correlation exists between the change of injury potential and that of the membrane resistance over the investigated temperature range, apart from temperatures above 40°C where the resistance increases and the injury potential decreases abruptly. This problem requires further experimental investigations.

The author is indebted to Professor J. Tigyi for raising the problem and for his encouragement during the work.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (3), pp. 349–363 (1970)

On the Application of the Phenomenological Treatment of Irreversible Thermodynamics to Contracting Muscle[1)]

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(Received April 15, 1970)

Current phenomenological theories of muscle contraction have been critically evaluated. In view of the existing discrepancies both with experiment and the theory of irreversible thermodynamics an attempt has been made to derive a new phenomenological formulation of the energetics of the input-output relation of contracting muscle. Extending Caplan's (1966) theory the present concept allows one of the phenomenological coefficients to vary both with the load and affinity. The rates of heat production and mechanical work for frog sartorius muscle at 0°C can be quantitatively predicted, provided a rate of energy storage is assumed which is a constant fraction of the chemical energy rate over the whole range of loads.

Introduction

As early as 1931 Onsager presented a concept according to which all flows and forces in a system are linearly dependent upon each other. The resulting *phenomenological equations* can be written as follows

$$J_1 = L_{11} X_1 + L_{12} X_2 + \ldots + L_{1n} X_n,$$

$$J_2 = L_{21} X_1 + L_{22} X_2 + \ldots + L_{2n} X_n,$$

$$\vdots$$

$$J_n = L_{n1} X_1 + L_{n2} X_2 + \ldots + L_{nn} X_n.$$

To each simultaneous flow or process $J_1, J_2 \ldots J_n$ in a system one can assign a conjugate force $X_1, X_2 \ldots X_n$; the sum of all products of $J_i X_i$ should always have the dimensions of entropy production or decrease in free energy with time. For a given flow J_1 to be driven by the forces $X_2 \ldots X_n$, the "coupling" or "cross" coefficients $L_{12} \ldots L_{1n}$ have to differ from zero. Onsager (1931) has shown that at least for processes close to equilibrium the matrix of the coupling coefficients is symmetric, or that

$$L_{ik} = L_{ki} \quad (i \neq k).$$

In contrast to other chemical reactions which show a nonlinear relationship between reaction velocity and affinity (Prigogine, 1961) the chemical reactions

[1] From a lecture given on April 15th, 1970 at the University of Pécs.

involved in muscular contraction have been described by linear phenomenological equations (Caplan, 1966, 1968; Wilkie, Woledge, 1967; Bornhorst, Minardi, 1969). Muscle has been considered as a linear energy converter, which couples a spontaneous process serving as energy source to a non-spontaneous process which represents the mechanical plus thermal energy output. The total rate of energy output of contracting muscle is given by

$$E = fv + \alpha v + Q_m. \tag{1}$$

The muscle shortens with a characteristic velocity v at any particular load f, the product of both parameters fv is the mechanical power output. The rate of the thermal output during active contraction has been subdivided arbitrarily into two fractions (Hill, 1964a, 1964b); according to Hill's definition *shortening heat* (αv) is the *extra* heat liberated during actual shortening over and above the socalled *maintenance heat* (Q_m), the latter heat fraction is still produced when an active muscle is only allowed to develop tension.

Current phenomenological theories of muscular contraction

In 1965 Kedem and Caplan (Kedem, Caplan, 1965) considered a system in which two irreversible flows were coupled. For energy conversion to take place Kedem and Caplan assumed that process 1 was driven by the spontaneous coupled process 2. Each of the flows J_1 and J_2 is assumed a linear function of the conjugate forces

$$\begin{aligned} J_1 &= L_{11}X_1 + L_{12}X_2 \leqq 0 \\ J_2 &= L_{21}X_1 + L_{22}X_2 \geqq 0 \end{aligned} \tag{2}$$

With respect to muscular contraction process 1 has been considered to be the shortening against an external load, which is driven by the coupled chemical reaction (process 2). Thus

$$\begin{aligned} J_1 &= -v, \\ X_1 &= f, \\ J_2 &= w, \\ X_2 &= A, \end{aligned}$$

where v is the shortening velocity >0 ($J_1 < 0$), f is the mechanical force, w is the reaction velocity and A is the chemical affinity.

Kedem and Caplan introduce a degree of coupling q for the energy conversion

$$q_{12} = \frac{-L_{12}}{\sqrt{L_{11}L_{22}}}, \qquad q_{21} = \frac{-L_{21}}{\sqrt{L_{11}L_{22}}} \tag{3}$$

where $0 \leq q_{12}q_{21} \leq 1$.

For $q = 0$ the two processes are independent of each other. The closer the value of q approaches unity the tighter is the coupling between the two processes, at $q_{12}q_{21} = 1$ the system is completely coupled. To convey information about the system and its mode of operation Caplan (1966, Fig. 2) displays the phenomenological equations in form of an input-output diagram. The output or $f - v$ diagram can be incorporated into the input or $A - w$ diagram, whereby the value of q^2 has a geometrical significance as any line drawn in the diagram is divided in the ratio $q^2/(1 - q^2)$ by the axes (see Fig. 1). For $L_{12} \neq L_{21}$ q^2 would be replaced by $q_{12}q_{21}$. As coupling tends to zero, the output space shrinks towards a single line. Geometrically $A_0 = w_m = 1$ (see Fig. 1)[2]. According to Caplan (1966)

$$f_0 v_m = A_m w_0 q^2/(1 - q^2) \tag{4}$$

As becomes obvious from Fig. 1, *vectorial* multiplication is required for f_0 and v_m, a fact to which we will return later.

In the same publication Caplan (1966) postulates the existence of an active regulator, which controls the input in response to the load (Fig. 2). The quantities A and w are varied with the load (or force) and shortening velocity in such a way that the relation between f and v is a rectangular hyperbola. While the operation mode of the energy converter is described by equation (4), the regulator function is

$$f/f_0\, h + vh/v_m = 1 - fv/A_m w_0 \tag{5}$$

where h represents an arbitrary programming function of the regulator. If h is a constant, it must be unity; it can never become zero as this would require zero output at a finite load.

Defining the following quantities

$$\xi = f/f_0$$

$$\gamma = v/v_m$$

$$\varepsilon = fv/A_m w_0$$

$$\kappa = q/\sqrt{1 - q^2} \tag{6}$$

the converter function (eq. 4) becomes

$$\varepsilon = k^2 \xi\gamma \tag{7}$$

[2] Throughout the text the subscript 0 refers to the socalled isometric case, the subscript m refers to the condition where the muscle shortens under zero load with maximal shortening velocity.

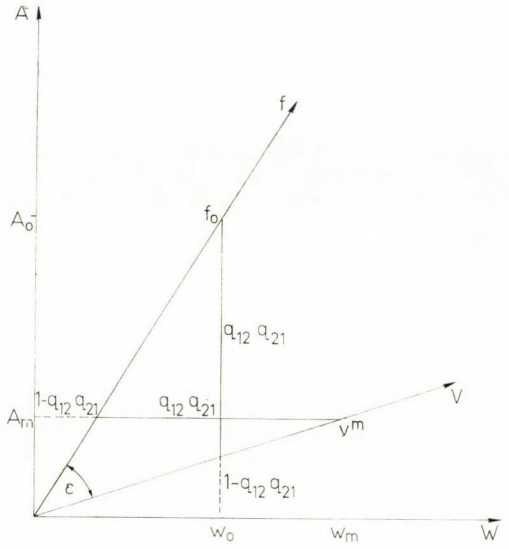

Fig. 1. Geometrical input output diagram

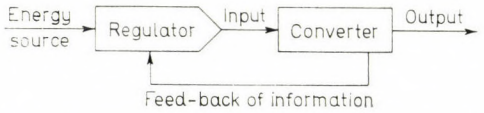

Fig. 2. Bloc diagram of the self-regulated energy converter

and the regulator function (eq. 5) assumes the form

$$\xi/h + \gamma h + \varepsilon = 1 \tag{8}$$

Equations (7) and (8) can be represented in form of the three-dimensional output or $\varepsilon - \xi - \gamma$ space, either as hyperbolic-parabolic area or as a plane for the case that h is unity (Fig. 3).

The intersection of the two surfaces in the $\xi - \gamma$ plane for the case that h equals unity leads to the force-velocity relation derived empirically by Hill (1938):

$$(f + a)(v + b) = (f_0 + a)b = (v_m + b)a.$$

Following the definition of k it can be shown from equation (7) that the converter surface becomes steeper with higher degrees of coupling. The curvature of the force-velocity-hyperbola is determined by the degree of coupling: the greater the degree of coupling the greater the curvature. The two muscle-specific constants

a and b of Hill's force-velocity relation are redefined in terms of equation (4).

$$a = A_m w_0 / v_m = f_0 (1 - q^2) / q^2$$
$$b = A_m w_0 / f_0 = v_m (1 - q^2) / q^2 \tag{9}$$

Thus

$$q^2 = \frac{f_0}{f_0 + a} = \frac{v_m}{v_m + b} \tag{10}$$

In 1967 Wilkie and Woledge made an attempt to decide whether muscles are energy converters of the type described by Caplan's theory. For this purpose

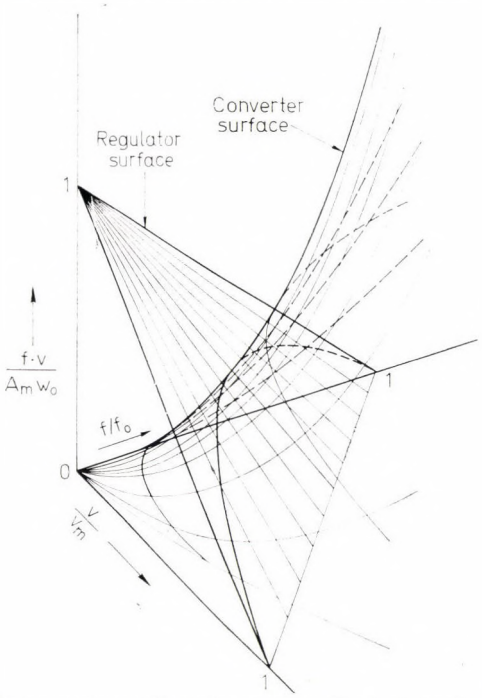

Fig. 3. Regulator and converter surfaces in a normalized three-dimensional output space. The regulator surface is drawn for the case $h = 1$ (after Caplan, 1966, Fig. 7)

these authors derive the appropriate equations for the changes of A and w (Wilkie, Woledge, 1967, equs. 18—21).

Wilkie and Woledge (1967) suggest that w can be directly compared with the observed total energy output (heat + work), as the in vivo enthalpy $\bar{\Delta} H$ of the chemical reaction which supplies the energy for muscular contraction is + 11.0 kcal/mole. If the free energy change per mole, $\bar{\Delta} F$, is set equal to the

affinity A, the variations in A required by Caplan's (1966) theory can be estimated from the ratio $Y = \overline{\Delta} F/\overline{\Delta} H = A/\overline{\Delta} H$.

For frog sartorius at 0°C $a/f_0 = b/v_m = 0.25$ (Hill, 1938), hence the scalar product $f_0 v_m = 16\ ab$. As the rate of maintenance heat at the point of peak isometric tension development is ab (Hill, 1964b), the chemical reaction at this point $w_0 A_0$ is then $1/16 f_0 w_m$.

As for contracting muscle (Ernst, 1963; Baskin, Paolini, 1967) volume and pressure changes will be negligible ($\simeq 10^{-5}$), the difference between internal energy and enthalpy, or between Gibb's and Helmholtz' free energy, can be forgotten. For the molar enthalpy change Wilkie and Woledge write

$$H w_0 = \frac{1}{16} f_0 v_m.$$

By substituting this expression in equation (11)

$$\frac{w}{w_0} = \frac{f}{f_0} + \frac{1}{q^2} \frac{f_0 v_m}{A_0 w_0} \cdot \frac{v}{v_m} \tag{11}$$

which is their eq. (18) and defining $A_0 = Y \overline{\Delta} H$, these authors derive that

$$\frac{w}{w_0} = \frac{f}{f_0} + \frac{1}{q^2} \cdot \frac{16}{Y} \cdot \frac{v}{v_m} \tag{12}$$

However, Wilkie and Woledge (1967, Figs 2, 3) predict variations of chemical flux or free energy changes as functions of muscular force which are considerable at variance both with Caplan's (1966) theory and experimental results.

To derive a quantitative expression for the thermal output of contracting muscle Caplan (1968) considers the whole muscle as a closed system and writes the first law of thermodynamics as follows

$$\dot{H} = \dot{Q} + fv \tag{13}$$

In expression (13) $\dot{H}$ is the rate of decrease of enthalpy and $\dot{Q}$ the observed rate of heat production of muscle. However, there are considerable dangers involved if the first law is uncritically applied to muscle as this would ultimately imply that muscle should do work upon heating it. Caplan's (1968) phenomenological description of the total energy output of contracting muscle, based on the assumption of constant affinity, requires that the sum of the heat liberated per unit shortening and of the load $(\alpha + f)$ should remain constant. This is incompatible with experimental observations as Hill (1964a) has shown that the shortening heat constant α for frog sartorius muscle depends on the load to the effect that $\alpha = 0.16 f_0 + 0.18 f$.

A further attempt to compare Caplan's phenomenological theory with experimentally observed chemical data has been made by Bornhorst and Minardi (1969). As it is impossible to measure the rate of the chemical reaction during steady states of shortening because of technical difficulties, Bornhorst and Minardi integrate the theoretical rate equations with time to obtain relations among actual quantities instead of rates. However, following Bornhorst and Minardi's predictions the product of affinity and chemical rate Aw would have a minimum at medium loads. As according to Caplan Aw the rate of the chemical input should be identical with the rate of the energy output, the theoretical predictions of their Figs 4 and 5 disagree with experimental results obtained on muscle, which show a maximum at medium loads.

In view of the existing discrepancy between the heat data and all previous phenomenological theories Bornhorst and Minardi (1970) formulate a new irreversible thermodynamic theory. This theory differs in that the chemical affinity is constant and the coupling coefficients characteristic of the whole muscle are now variable.

In contrast to earlier authors, Bornhorst and Minardi (1970) apply the thermodynamic theory to detailed changes at the ultrastructural level. The following postulates are made: a) the myosin cross-bridges (Huxley, 1969) are linear energy converters, b) the number of operating (or active) cross-bridges m decreases towards higher shortening velocities and c) the coupling coefficients are a function of the number of operating cross-bridges.

Bornhorst and Minardi obtain the variation of the coupling coefficients of the whole muscle in terms of the number of operating cross-bridges and the coupling coefficients of the individual cross-bridge

$$L_{11} = L^i_{11}/m, \quad L_{12} = L^i_{12}, \quad L_{21} = L^i_{21}, \quad L_{22} = L^i_{22}\, m \tag{14}$$

Bornhorst and Minardi (1970) write the first law for muscle as

$$w\bar{\Delta}H = \dot{Q} + fv \quad \text{for } f > 0 \quad v > 0$$

where $\dot{Q}$ is the rate of heat production.

For the heat rate under isometric conditions $\dot{Q}_0$ it follows $w_0, = \dot{Q}_0/\bar{\Delta}\, H$. Using the definition of Wilkie (1960), where Y is defined as $A/\bar{\Delta}\, H$[3], Bornhorst and Minardi obtain the expression

$$\frac{\dot{Q}}{f_0\, v_m} = \frac{a/f_0}{1 + a/f_0}\left[\frac{\dot{Q}_0}{f_0\, v_m}\left(1 + \frac{f_0}{a}\frac{f}{f_0}\right) + \frac{1}{Y}\left(1 - \frac{f}{f_0}\right)\right] - \frac{fv}{f_0\, v_m} \tag{15}$$

The total energy rate $(\dot{Q})f_0 v_m + fv(f_0 v_m)$ would then be a linear function of the load.

A comparison of their theoretical predictions with the total energy rate observed experimentally for frog sartorius at 0°C is shown in Fig. 4. It is obvious

[3] This is justified as in view of the studies of Ernst and his collaborators (1963) volume changes during contraction are as little as $1 - 3 \times 10^{-5}$ in frog muscle.

that the experimental results deviate markedly from the theoretical curve at all loads smaller than $0.5\,f_0$. However, to get even this limited agreement the authors have to assume that Y has a value as low as 0.651. In contrast values of 0.9—1.3 have been estimated by Wilkie and Woledge (1967) for the driving chemical reaction in muscle.

Values for the ratio of free energy to enthalpy ($\Delta F/\Delta H$) less than 1.0 can never be true for an over-all coupled reaction in a thermodynamically closed system or for a driving chemical reaction in which entropy is actually produced.

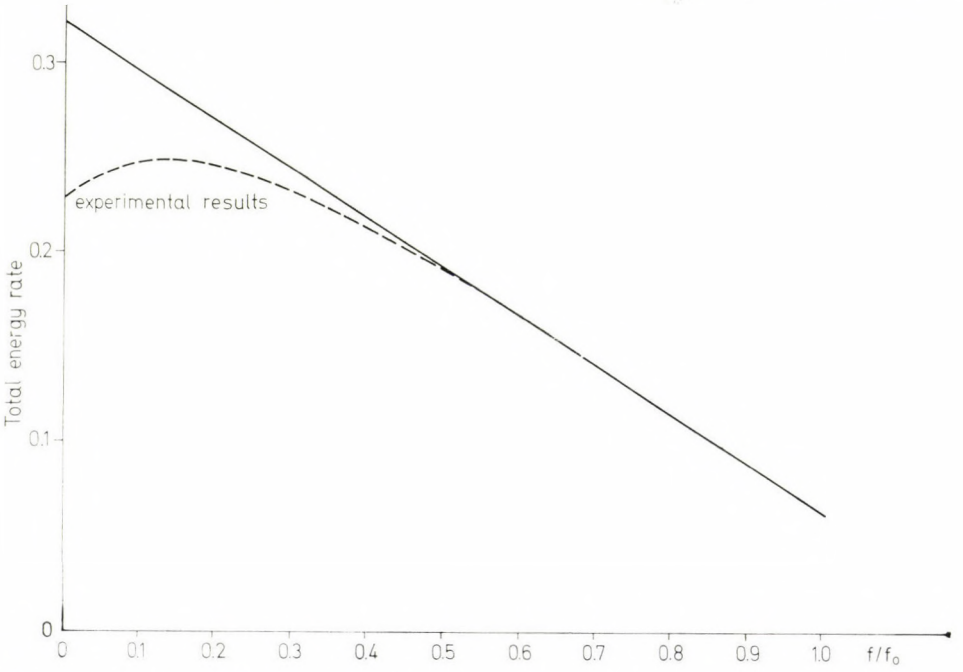

Fig. 4. Comparison of theoretical and experimental normalized energy rates for frog sartorius muscle. The continuous curve has been calculated by Bornhorst and Minardi (1970) using equation (15)

As for any driving chemical reaction such as in muscular contraction the second law of thermodynamics requires that the entropy term has to be $\Delta S \geqq 0$, it follows that $Y = A/\Delta H \geqq 1$. Alternatively, an entropy flow into the surroundings, which represents a reversible heat production, cannot be true as muscle would then have to do mechanical work merely upon heating the system.

A general criticism applicable to all previous models is the assumption that Onsager symmetry is valid in the case of contracting muscle. However, the Onsager relation does not hold for muscle as follows from a numerical estimate of the coupling coefficients, using the equations $L_{12} = -v_m/A_m$ and $L_{21} = -w_m A_0/f_0 A_m + w_0/f_0$. For frog sartorius at 0 °C characteristic values are $f_0 = 50$ g, $v_m =$

$= 6$ cm/sec, and an affinity A_0 of 11.0 kcal/mole has been estimated for isometrically contracting muscle (Wilkie, Woledge, 1967). As $A_m = 0.844\ A_0$ (see below) and making the assumption that the chemical input of the energy converter Aw is equal to the total energy output $(fv + \alpha v + Q_m)$, the values for $L_{12} = -15.1$ nmole/g-sec and $L_{21} = -3.1$ nmole/g-sec can be calculated. If the affinities A_m and A_0 were the same, the difference in the numerical values of L_{12} and L_{21} would even be greater. Only in the most unlikely case that A_m is as low as 2.5 kcal/mole are the two coupling coefficients identical. The difference still persists if an energy storage rate is included in the total energy output.

An irreversible thermodynamic concept of muscle with variable coupling coefficients and variable affinity

In view of the existing discrepancies between the various phenomenological theories and the experimental data an attempt is made to approximate the energetic data of frog muscle at 0 °C by a new model.

From the data given in Fig. 4 (and for $Y_m \simeq Y_0 \simeq 1$) it follows that

$$\frac{A_m w_m}{A_0 w_0} \cong 3.56 \tag{16}$$

Let us first consider the theoretical–geometrical case $A_0 = w_m = 1$ and hence with equation (4) $A_m w_0 = 1 - q_{12} q_{21}$, for $f_0 \times v_m = q_{12}\ q_{21}$. From equation (10) for $a/f_0 = 0.25$ the value of $q_{12} q_{21}$ is 0.8, thus $w_0 = 0.2/A_m$.

Equation (16) becomes then for $A_0 = 1$

$$A_m = 0.844 \tag{17}$$

According to Caplan's theory $A_0 \times w_m$ is unity. The product $f_0 w_m = 16\ ab$ is obtained through scalar multiplication and is hence independent of q. The scalar quantities for frog sartorius are $f_0 = 4\ a$ and $v_m = 4\ b$. As indicated in Fig. 1 these scalars are longer than A_0 and w_m, respectively. In fact using equ. (17) the scalar multiplication of the input parameters gives a value for $A_0 w_m = 11.9\ ab$, which is less than $16\ ab$. This result together with eq. (17) leads to

$$A_0^* w_0^* = 2.82\ ab \tag{18}$$

$$A_m^* w_m^* = 10.04\ ab \tag{19}$$

If one compares expressions (18) and (19) with the total energy rates under isometric conditions and for zero load shortening, it becomes obvious that the rate of energy input Aw is 2.82 times greater than the output rate.

In experiments in which the thermoelastic changes of active and passive rigor muscle have been investigated, it has been demonstrated that muscle can store energy, which is used to support mechanical work in the late phase of the muscle twitch (Chaplain, 1970). The postulate is then that this energy reservoir is filled with a certain rate, which for $f = 0$ should be 10.04 ab — 3.56 ab = = 6.48 ab, i.e. 0.645 $A_m w_m$. For $f = f_0$ the energy storage rate is 2.82 ab — ab = = 1.82 ab, i.e. 0.645 $A_0 w_0$.

The question which needs to be answered is, whether Aw as predicted by Caplan's theory always leads to 0.645 Aw as energy storage rate.

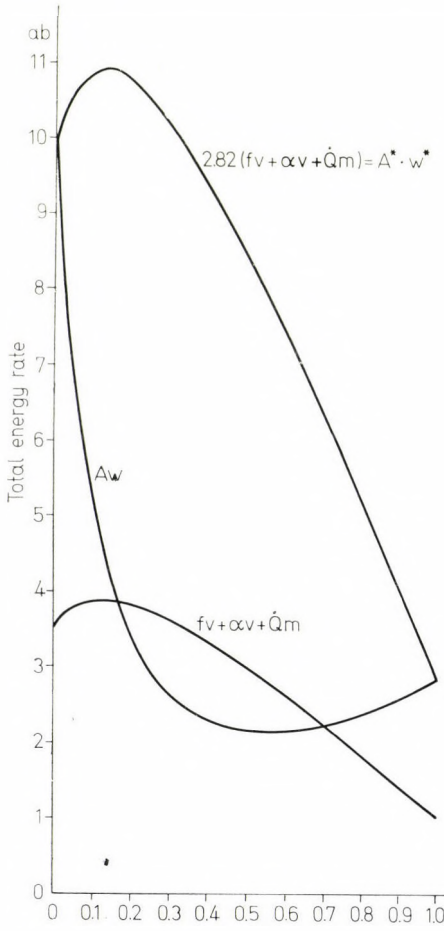

Fig. 5. Comparison of theoretical and experimental energy rates. The quantity $fv + \alpha v + \dot{Q}_m$ is the total rate of energy production observed experimentally for frog sartorius muscle at 0°C (Hill, 1964a, b); Aw is the rate of the chemical energy input calculated by Caplan, and A^*w^* is the rate of chemical energy predicted for the case that the coefficient L_{22} varies with the load and affinity

Plotting w/w_m and A/A_0 as a function of f/f_0, gives rise to curves, which are similar in shape to those published by Caplan (1968; Fig. 4) and by Bornhorst and Minardi (1969; Figs 4, 5). However, if $(fv + \alpha v + \dot{Q}m)$, $2.82\,(fv + \alpha v + \dot{Q}m)$ and Aw are plotted as functions of f/f_0, Fig. 5 reveals a discrepancy between the curves of Aw and $(fv + \alpha v + \dot{Q}m)$. Even the assumption of an energy reservoir is not particularly helpful in this respect as there is little reason to believe that the system depletes a pre-existing energy reservoir at medium loads, while at lower and higher loads the reservoir is actually filled.

Therefore an attempt will be made to extend Caplan's theory by introducing variable phenomenological coefficients, an approach previously suggested by Wilkie and Woledge (1967). Reasonably only one coupling coefficient L_{22} is varied. A nonlinear relation exists, as the coefficient L_{22} is assumed to vary both with the affinity A and the external load f/f_0.

The task set is to obtain an adequate description of the function $2.82\,(fv + \alpha v + \dot{Q}m)$, denoted subsequently as A^*w^*.

The ratios of $A^*w^*/A_0^*w_m^*$ agree at $f = 0$ and $f = f_0$ with Aw/A_0w_m in Caplan's (1966) theory. At medium loads (or velocities) the values of $A^*w^*/A_0^*w_m^*$ are *higher* than the corresponding values of Aw/A_0w_m given in Table 1.

Table 1

f/f_0	v/v_m	w/w_m	A/A_0	Aw/Aw_m	Aw	$\frac{A^*w^*}{A_0^*w_m^*}$
0	1.000	1.000	0.844	0.844	10.04 *ab*	0.844
0.10	0.643	0.666	0.642	0.428	5.10 *ab*	0.914
0.20	0.445	0.492	0.576	0.283	3.36 *ab*	0.905
0.30	0.318	0.389	0.568	0.221	2.63 *ab*	0.856
0.40	0.231	0.326	0.595	0.194	2.30 *ab*	0.791
0.50	0.167	0.285	0.641	0.183	2.17 *ab*	0.711
0.60	0.118	0.260	0.699	0.182	2.16 *ab*	0.624
0.70	0.079	0.245	0.767	0.188	2.24 *ab*	0.532
0.80	0.048	0.237	0.840	0.199	2.37 *ab*	0.436
0.90	0.022	0.235	0.918	0.216	2.56 *ab*	0.338
1.00	0	0.237	1.000	0.237	2.82 *ab*	0.237

The phenomenological equations and their expressions for the isometric and unloaded isotonic case have been rewritten as

$$-v = L_{11}f + L_{12}A^* \tag{20}$$

$$w^* = L_{21}f + L_{22}(A^*, f/f_0)A^* \tag{21}$$

$$v = 0 = L_{11}f_0 + L_{12}A_0^* \tag{20a}$$

$$w_0^* = L_{21}f_0 + L_{22}(A_0^*, 1)A_0^* \tag{21a}$$

$$-v_m = L_{12}A_m^* \tag{20b}$$

$$w_m^* = L_{22}(A_m^*, 0)A_m^* \tag{21b}$$

Naturally, $q_{12}q_{21}$ will change with $L_{22}(A^*, f/f_0)$. With Caplan the coupling is always the same at all points on the force-velocity hyperbola. Deviating from Caplan's postulate, it has been tested whether a better agreement with experimental observations is possible if one were to assume that the degree of coupling changes along the force-velocity hyperbola. To arrive at A^*/A_0^* and w^*/w_m^* equations (20, a, b) can be used

$$\frac{A^*}{A_0^*} = \frac{f}{f_0} + 0.844\,\frac{v}{v_m} \tag{22}$$

Using equations (17), (20a), (21), (21b), (22) and the expression $q_{12}\,q_{21} = 0.8$ we can write

$$\frac{w^*}{w_m^*} = \frac{L_{22}\,(A^*, f/f_0)}{L_{22}\,(A_m^*, 0)} \cdot \frac{v}{v_m} + \frac{1}{0.844}\,\frac{f}{f_0}\left[\frac{L_{22}\,(A^*, f/f_0)}{L_{22}\,(A_m^*, 0)} - 0.8\right] \tag{23}$$

The variation in $A^*w^*/A_0^*w_m^*$ with f/f_0 follows from $Aw = 2.82\,(fv + \alpha\,v + \dot{Q}m)$ and $A_0^*w_m^* = 11.9\,ab$. The functional dependence of A^*/A_0^* on f/f_0 is given by equation (22) and the force-velocity relation. Thus w^*/w_m^* and the ratio of $L_{22}\,(A^*, f/f_0)/L_{22}\,(A_m^*, 0)$ are exactly predetermined.

Let us split $L_{22}(A^*, f/f_0)$ into two additive terms, one term $L_{22}^{\wedge}$ dependent on the affinity A^* and one $L_{22}^{\sim}$ dependent on f/f_0.

As the linear relation between flow and affinity is strictly valid only when $A/RT \ll 1$ (Prigogine et al., 1948) and as $A^*/RT \cong 18$ for the present system is in fact much greater than unity, an inverse proportionality between $L_{22}^{\wedge}$ and A^* is assumed

$$L_{22}^{\wedge}\,(A^*) = \frac{K}{A^*} \tag{24}$$

This implies that the reaction velocity no longer increases with increasing affinity, if the chemical reaction takes place without any mechanochemical coupling (see Fig. 6).

$L_{22}^{\sim}$ has a maximum at about $0.3\,f/f_0$ which parallels the maximum of work output (Hill, 1964b). As $\tilde{w}^* = L_{22}^{\sim}\,.\,A^*$ a high value of $L_{22}^{\sim}$ would imply a high rate of the chemical reaction. In fact the data summarized in Table 1 indicate that for $f/f_0 = 0.3$ the affinity has its minimum. However, the product of A^*/A_0^* and $L_{22}^{\sim}(f/f_0)$ also has its maximum at $0.3\,f/f_0$ as illustrated in Fig. 7. The concave curve in Fig. 8 between $f/f_0 = 0$ and $f/f_0 = 1$ is the force-velocity hyperbola of Caplan's output diagram. The same curve reflects in terms of Caplan's input space ($A^*/A_0^* - w^*/w_m^*$—diagram) the relationship between affinity and reaction velocity. If L_{22} is made to vary, the relation between both parameters is no longer determined by the force-velocity curve. Only as far as the dependency of A^*/A_0^* upon f/f_0 is concerned Caplan's original hyperbola is still obtained. The relation

between w^*/w_m^* and f/f_0 (and hence w^*/w_m^* and A^*/A_0^*) no longer follows Caplan's predictions as in indicated in Fig. 8 by the upper curve drawn from $f/f_0 = 0$ and $f/f_0 = 1$. The fact that two curves are running from $f/f_0 = 0$ to $f/f_0 = 1$ cannot be reconciled with Caplan's theory.

Further, Caplan assumes that the Hill hyperbola describes the behaviour of an autonomic linear energy converter with constant degree of coupling. If L_{22}

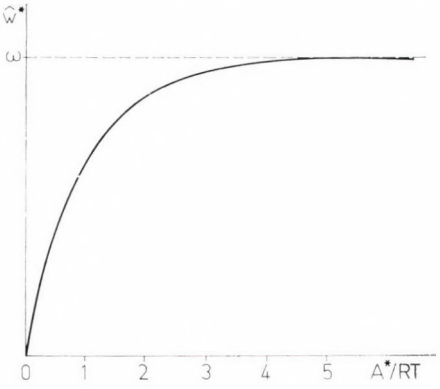

Fig. 6. Relationship between the affinity-dependent term $\hat{w}^*$ and the ratio of A^*/RT. The reaction velocity approaches ω, the extreme value of the reaction velocity for the condition most distant from equilibrium (see Haase, 1963 p. 125). The appropriate equation is $w^* = \omega(1 - e^{-A^*/RT})$

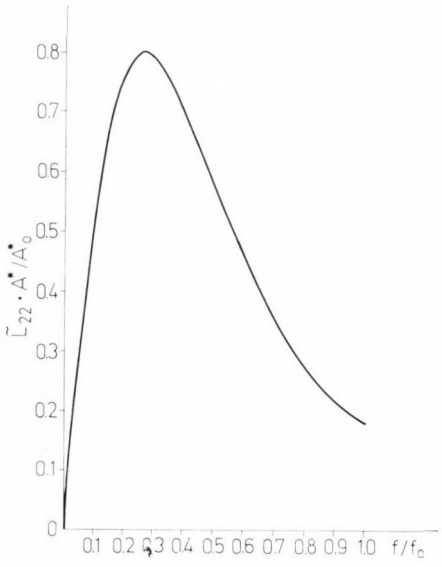

Fig. 7. Relationship between the normalized load-dependent term of the reaction velocity $\tilde{w}^*$ and the fractional load f/f_0

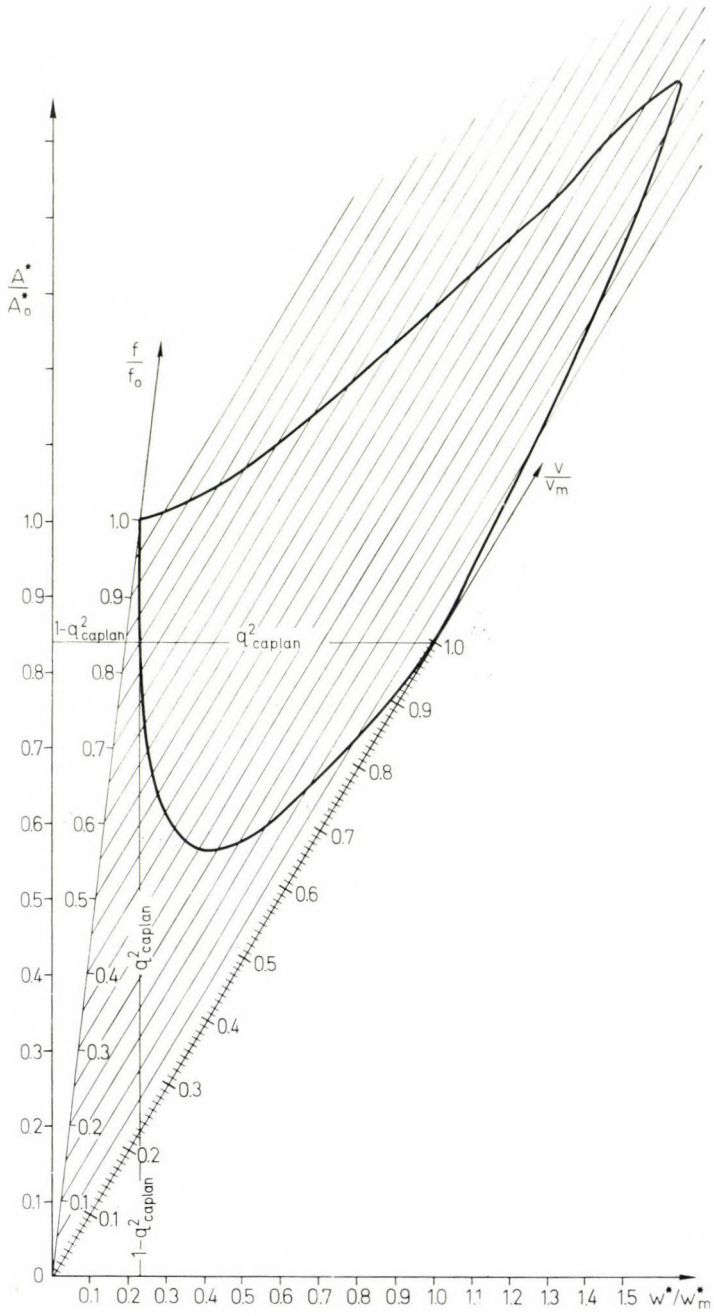

Fig. 8. Input-output diagram. The hyperbola between $f/f_0 = 1$ and $v/v_m = 1$ corresponds to A^*/A_0^* (see equ. 22) and remains the same as that predicted by Caplan's theory. The upper curve between $f/f_0 = 1$ and $v/v_m = 1$ corresponds to w^*/w_m^* (see equ. 23)

is no longer constant this means that $q_{12}q_{21}$ will change as a function of f/f_0. At any rate the present treatment which assumes one of the phenomenological coefficients to be variable together with variable affinity, is consistent with the theory of irreversible thermodynamics as $L_{11}L_{22} \geqq L_{12}L_{21}$ or in other terms $L_{12}L_{21}/L_{11} \leq L_{22}$.

In summary, if the cross coefficient L_{22} is considered as a variable it is possible to derive the function 2.82 ($fv + \alpha v + \dot{Q}_m$) shown in Fig. 5. The resulting curve exceeds the total energy output of frog sartorius muscle by a factor of 2.82. It is not possible with the geometrical form of the phenomenologi al theory to make A^*w^* and $fv + \alpha v + \dot{Q}_m$ congruent, as the scalar product of f_0v_m is 16 *ab* with $q = \frac{1}{\sqrt{1 + a/f_0}}$ and the vectorial product of $\vec{f_0} \times \vec{v_m}$ is 9.52 *ab* while $A_0w_m = A_0^*w_m^* = 11.9$ *ab*. If one feels that the calculated value of A^*w^* is in fact a realistic choice one needs to postulate the existence of an energy reservoir, with a constant fraction of energy being stored at a rate equal to 0.645 A^*w^*. Other possibilities such as the variation of L_{21} or even of two coefficients such as L_{21} depending on f/f_0 and L_{22} upon A^* have been investigated. However, it became apparent that no useful meaning can be attached to these results.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (3), pp. 365—371 (1970)

Water Circulation Caused by Hindered Diffusion and Unequal Distribution of Solutes

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(Received April 16, 1970)

The experimental system consists of three chambers. The chambers are separated from each other by two pieces of parchment of the same area. The parchments are covered with perforated plexi plates on their outer side, one on a big, the other on a small area. There is a watery solution of colloid and anorganic substance in the space among the two membranes. Outside the membranes there is water on both sides. A water flow comes about in the system towards the membrane covered on a big area. This flow is due to the large-surface covering plate because this hinders the diffusion of the solute perfused through the membrane on a larger area than does the small-surface covering plate. Through the system the water is able to flow from the side chamber of higher concentration to the one of low concentration. In the side chamber placed in the direction of water-stream a higher hydrostatical pressure can be measured than that on the other side.

At every perforation of the plexi covering plates a water circulation comes about through the parchment between the middle chamber and the side chambers. The circulating water carries the solutes with itself depending on the permeability; therefore, there comes about a selective, unequal distribution of solutes between the different spaces. In the side chamber at the membrane covered on a large area the inorganic material becomes relatively opulent, as compared to the other side chamber. The explanation of the mechanism of active ion transport seems to be possible.

Introduction

Metabolism is a basic feature of the living material. Through this the living cells maintain a combination and concentration different from their surroundings through energy investment. E.g. the K^+ concentration of the cell is higher, its Na^+ concentration is lower than that of its surroundings. Certain kinds of cells produce solutions different from their own inner concentration through energy investment. The mechanism of the formation of the difference from the environment is being examined also nowadays. It seems to be necessary to find a simple mechanism through which the whole sphere of problems can be solved.

A literary data of fundamental importance is Ernst's (1929) recognition about the connection of energetics, structure and function. Koefoed-Johnsen and Ussing (1953) demonstrated with living membranes the fact that the degree of streaming is a function of membrane structure. Further on it is known that ions pass along separate channels through the membranes of erythrocytes. In the opin-

ion of Pappenheimer (1953) the hydrodynamical streaming and the diffusion happen through the capillary walls along channels containing water. According to Bálint's (1965) text-book, the moisture produced by living cells contains solutes besides the water.

The aim of the model experiment to be described here is to prove that in an appropriate structure it is possible to utilize the osmotic working ability of an appropriate metabolic product to a degree which seems to be suitable for ensuring the streams of solutions in biological objects and for keeping on the above mentioned differences in concentration, respectively. The three-chamber system already known from the literature is most suitable for this purpose because of its simplicity. After Pfeffer's theoretical work Lepeschkin (1906, 1908, 1908a, 1909), and then Curran and McIntosh (1962), and later Ogilvie et al. (1963) and the author (Homola, 1962) also worked with such a system. In these experiments the chambers were separated from each other by two membranes of different permeability. Differently from the above mentioned experiments the author (Homola, 1966) built two equal parchment pieces into the three-chamber system, and covered the two parchment pieces on both sides with perforated impermeable plates on areas of different size. This experimental apparatus was somewhat complicated because of covering the membranes on both sides. In the experiments to be described here the membranes are covered only on one side. So the system became more suitable for demonstrating a simple but important fundamental biological process.

Methods

The sketch of the experimental arrangement can be seen in Fig. 1. The three chambers of the apparatus were separated from each other by two parchment-membranes — m_1 and m_2 — of equal area (225 cm^2 each). The membranes were covered with plexi plates perforated with holes of a diameter of 3.2 mm along the side-chambers.[1] The plexi plate covering membrane m_1 had ten times more

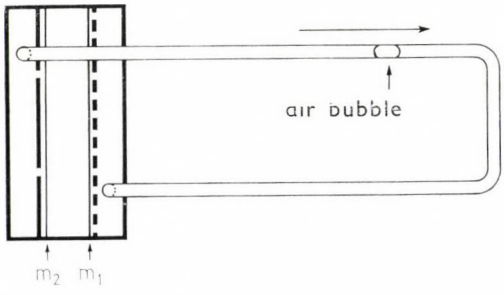

Fig. 1

[1] During the experiment the parchment membranes rested on the plexi plates. (On the basis of a measurement to be described later we could conclude to the presence of narrow gaps between the parchment and the plexi plate.)

perforations than the one covering membrane m_2. So membrane m_2 was covered by the plexi plate on an area of 215 cm^2, while membrane m_1 was covered only on an area of 125 cm^2. Also the other parts of the apparatus were made of plexi glass. The volume of the middle chamber was 290 ml, while the side chamber beside m_1 was of a volume of 128 ml, and the one beside m_2 was of 103 ml. The change of the volume of the chambers during the experiment was hindered by stiffeners and supporters.

The middle chamber of the apparatus was entirely closed during the experiments after filling it up and after making it airless. In the series of experiment the middle chamber was filled up with uncleaned gum-arabic solution of 20 per cent. (The freezing-point decrease of this solution was $\Delta = 0.21°C$; the ash-content of 1.00 gram of the dry gum-arabic powder used for making the solution was 0.033 g.) The two side chambers were filled with distilled water.

In one of the experimental series the volume and speed of the water-stream coming about through the system were measured by the author. Fig. 1 shows the apparatus viewed from above. The side-chambers were connected to one another by a U-shaped calibrated glass tube filled with water, containing a bubble of air. The volume and speed could be determined from the wandering of the bubble. During the experiment all chambers were closed outward.

In an other series of experiment the author measured the difference in pressure between the side-chambers with the aid of a mercury difference manometer. The atmospheric value of the pressure in the middle chamber was measured with another apparatus. This latter measurement was always done at the end of the experiment, and so it did not disturb the main experiment.

The measurements were done at room temperature. After each experiment the apparatus was emptied and rinsed several times. A new experiment was done only after checking the intactness of the membranes, or, — if necessary — after building in new membranes.

In a further series of measurement all three chambers were filled with water. Water was pumped continuously into the middle chamber at a pressure of 650 torr. While doing this, the side-chambers were open above. Through the membrane covered on a small area, there streamed a quantity of water 1.9 times larger than hrough the membrane covered on a large area, during an equal period of time.

Results

The volume and speed of the water streamed through the apparatus were measured in 15 experiments. In every experiment the water was transported from the membrane covered on a small area to the membrane covered on a large area in the membrane system. The average of the volume of perfusion for one experiment was 2.76 ml (1.50 to 3.48 ml) during the first day. The speed of perfusion was 0.11 ml/hour. It was the first day when the speed of perfusion was the largest.

From this time on it decreased, and then — changing with each experiment — the direction of the water transport stopped in 1 or 2 weeks and then it turned back. After the side-chambers were rinsed and filled up with distilled water the water transport went on again in the original direction with the speed experienced at the beginning of the experiment.

The determination of the concentration of the solute diffused into the side-chambers was done in 7 experiments. In three of them the side-chambers were separated from each other by the mercury of the difference-manometer in order to measure the difference in pressure for 1 or 2 days before the determination of the concentration. In a further experiment the side chambers were closed for 2 days, in another for 19 days before measuring the concentration. In two experiments the two side-chambers were connected to each other through a measuring tube for 6 and 15 days, respectively, before measuring the concentration. It was found in each case that the dry material content was 2 to 11 times higher in the side chamber beside the less-covered membrane than in the other one.

After the experiments lasting 15 and 19 days, respectively, was determined not only the dry-material content but also the ash-content of the dry material. In the experiment lasting 15 days the water flow stopped on the 14th day, and then its direction turned. This was the time when the concentration was measured in the side-chambers and, according to this, the dry-material content was 2.8 times higher outside the less-covered membrane (5.4 per cent), than on the other side (1.9 per cent). But the ash-content of the dry-material was 1.3 times higher outside the membrane covered on the larger area (4.5 per cent) than on the other side (3.6 per cent). Similar result was obtained from the concentration measurements of the side-chambers in the 19 day's experiment. Here, the dry-material content was 2.3 times higher outside the membranes covered on a small area. The percentage of the ash content of the dry-material was 1.4 times higher outside the membrane covered on a large area.

Ten experiments served for measuring the difference in pressure between the side chambers. The movement of the mercury in the manometer in every experiment indicated the fact that the water streamed from the membrane covered on a small area to the membrane covered on a large area through the membrane system. The difference in pressure reached its maximum 1 day after beginning the experiment, and then it decreased. According to the average of the 10 experiments the maximum of the difference in pressure was 154 torr (122—200 torr).

The pressure in the middle chamber was measured in 7 cases at the end of the experiments. The mean value of the measurements was 653 torr (471—775 torr). (The atmospheric pressure of the side-chambers was not measured, only the differences in pressure.)

If 0.25 M solutions of $K_4Fe(CN)_6$, then of Na_2CO_3, and then of NaCl were put into the middle chamber, the change in the pressure difference between the side chambers was 100, 95 and 25 torr, respectively, during 1 hour. The time periods necessary for the experiments were in similar proportion to each other.

Discussion

It is known that biological membranes let through not only water but more or less also the solutes in it. The membrane generally decreases the free diffusion rate of the solutes. Pappenheimer (1953) wrote about "restricted diffusion" because of the diffusion-decreasing effect of the pores of the membrane. Kedem and Katchalsky (1958) introduced Staverman's σ reflection coefficient as a selectivity coefficient in the mathematical description of the streams penetrating the membrane.

In a previous paper the author (Homola, 1966) called attention to the role of hindered diffusion along the membrane. The following sequence of ideas throws a light on its essence:

Parchment lets through the inorganic solutes and, according to the measurements, it also lets through gum-arabic to some degree. If the solution were closed between two equal fixed parchment membranes, and the membranes were not covered by covering plates, and, further on, there were a lot of water on both sides, there would not come about a water stream through the system. The potential difference existing in the difference of concentration would equalize without work, the solute would diffuse away into its environment. But if the free diffusion of the solute is hindered by applying a perforated covering plate of larger area on the one membrane than on the other, a volume-work comes about in this structure. Notably, there is essentially a very narrow gap between the membrane and the covering plate that is in connection with the water outside the membrane. (This is referred to by the data of perfusion with pure water, when only 1.7 times less water passed through the parchment covered on the larger area instead of the expectable 10 times less water.) A part of the diffused solute remains in the gap along the membrane. The osmotic pressure difference is smaller on the two sides of the covered membrane parts than on the two sides of the uncovered membrane parts. Therefore water flows from the solution into the gap, and from there into the outer water-space.[2] *The solute hindered in its free diffusion will be diluted along the membrane, and this is why work can come about.* This work can be enormous. Its size depends on the degree of dilution. Not considering the friction-, etc. losses, if the solute would be diluted infinitesimally, the work would be numerically similar to the work produced by gas molecules of the same number as the diluted parts of solute if it enlarged isothermally from a volume corresponding to the concentration to an infinite volume. It is possible that in the living system this significant energy is supplied by the metabolism, by producing molecules appropriate for the structure as necessary. In the apparatus this energy brings about the stream of water from the side chamber of higher concentration to the side chamber of lower concentration and also the larger pressure measured in the side chamber along the parchment covered on a larger area.

[2] In the apparatus the whole area of the gaps is larger with the parchment covered on a larger area and, therefore, the stream of the water is dominating in this direction.

The above data show that, in an absolute value more organic and inorganic material flowed through the parchment covered on a small area than through the other one. The experience that the stream of water in the apparatus stopped after a week or two and then turned towards the membrane covered on a small area is in connection with this. The ash content of the dry material was relatively higher outside the membrane covered on a large area. The relative opulence of the inorganic material outside the membrane covered on a large area is the result

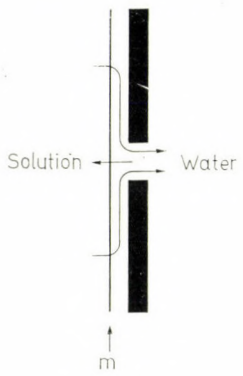

Fig. 2

of a transport process connected with a constant water circulation. Its mechanism is as follows:

The stream of water happens not only through the whole system from the membrane covered on a small area to the one covered on a large area, but there are also constant water circulations on the surface of both membranes around every perforation of the covering plates. At the perforations of the covering plate the solution is in touch with the water through the membrane, and here water flows into the solution because of osmosis (Fig. 2). On the area of the covered membrane the solute flows into the gap and, according to the described mechanism, water flows from the solution through the gap into the water space. In the gap the water streaming out carries with itself the smaller inorganic parts more easily than does the large colloids. This selection becomes more effective on the membrane covered on a larger area, because the whole surface of the gaps is larger here than on the other side. Because of the continuous water circulation through the membrane the water is continuously exchanged between the chambers. This is in accordance with several literary data, according to which the continuous exchange of water in living cells is quicker than that of any other material.

The inorganic part of the solute streamed into the side chambers was determined only in two experiments. Nevertheless the mechanism of water circulation offers a possibility to make the selective ion-accumulation in the cell more understandable, e.g. K^+-accumulation. Theoretically, the accumulation of certain ions can come about so that the water streaming into the cell carries the ion into it

but, at the place where the water flows out, the membrane has another kind of structure and, therefore, the water does not carry the ion out of the cell. The water and the ions for which this part of the membrane is permeable can get into the cell through the gaps in the protein. Both the smaller K^+ and the larger Na^+ can get into the cell here. The lipids of the cell membrane constitute a layer hindering the outward diffusion, along which osmotically effective metabolic product flows which brings about a water flow through the channels in the protein layer beside the lipids. According to the schematic conception these channels go completely through the protein layer. The water carries both the Na^+ and K^+ into these. The mouth of outflow of the channels lets the Na^+ ion through, though strongly restricts it (restricted diffusion). The Na^+ lingering in the channels hinders the passing through of K^+ (hindered diffusion). The accumulated K^+ diffuses back into the cellular moisture through smaller secondary channels in the appropriate phase of operation. After all the pumping out of Na^+ ion and the accumulation of K^+ ion happen here.

This roughly schematic conception does not make it necessary to suppose a "carrier material", and explains the more important physiological and pathological changes in concentration which come about in the case of ion lack.

The formation of water-circulation and unequal distribution of solutes need no system of two membranes. The two membranes-, three chambers-system made it possible to demonstrate the effect. However water circulation and the unequal distribution of solutes can come about also between two water spaces in the case of an appropriate permeable membrane, a structure hindering the diffusion and the continuous flow of an appropriate solute.

The actuality of this paper is proved by Csáky's (1969) communication about biological transport in epithelial cells, which was read by the author after writing the present article. The author agrees with the conclusion that the flow of water can bring about the accumulation of certain solutes in the water spaces inside the cell.

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Book Review

Two volumes on the optical rotatory properties of biological macromolecules:

M. W. Wolkenstein: Optische Eigenschaften und Struktur der Fermente (Optical Properties and Structure of Enzymes). W. Beier (editor), Fortschritte der experimentellen und theoretischen Biophysik, Vol. 10, VEB Georg Thieme, Leipzig. This book is the German translation of a part of the author's former book entitled The Physics of Enzymes which was published in Russian in 1967.

The author's earlier activities were concentrated on the theoretical and experimental investigation of synthetic polymers and this interest has recently turned to the biological macromolecules. His book consists of a discussion of selected topics concerning the relations between structural and functional properties of proteins rather than a systematic analysis of the subject given by the title of the book.

In the first three chapters the interpretation of the optical rotatory power and the application of optical rotatory dispersion measurements to the investigation of the conformation of proteins, particularly enzymes, are discussed. The following part is devoted to the different properties of enzymes, e.g. the polyelectrolyte nature of proteins and its influence on the polypeptide chain conformation. Data are also presented which were obtained in the author's laboratory on dehydrogenases. In addition, two other basic problems, the connection between the cooperative interactions within the protein molecule and the kinetics of enzymes, as well as the allosteric properties of some enzymes are analyzed.

The most original part of the volume deals with the magneto-optical properties (Faraday-effect) of hemoglobin. This phenomenon has been thoroughly studied in the author's laboratory in order to elucidate the background, both theoretical and experimental, of the effect. Interesting results are presented which were obtained by optical rotatory dispersion measurements in magnetic field on hemoglobin and myoglobin.

The two appendices are concerned with the theory of graphical treatment of enzyme kinetics and the anomalous dispersion of magneto-optical effect, respectively. The list of references contains more than 300 references. Interesting and original ideas are suggested in the discussion of the different topics mentioned above.

B. Jirgensons: Optical Rotatory Dispersion of Proteins and Other Macromolecules, Molecular Biology, Biochemistry and Biophysics Series, Vol. 5. Springer Verlag, Berlin—Heidelberg—New York, 1969.

The author and his associates have published numerous papers in the last decade on the optical rotatory dispersion of proteins and other macromolecules, as well as other books on the physical chemistry of macromolecules. The present volume is a survey book of practical problems and results of optical rotatory dispersion measurements.

The introduction shortly summarizes some general features of protein structure. The theoretical background and semi-empirical treatment of optical rotatory dispersion data are also discussed in this part.

Chapters IV to VII are devoted to the analysis of the periodic organization of polypeptide chains by investigating the optical activity of proteins and synthetic compounds (polyamino acids).

Although there are plenty of data in the literature which strongly suggest the existence of a definite correlation between structural pattern and rotatory properties of proteins, the interpretation of the experimental data is still not satisfactory. In some cases there is a fairly good agreement between data obtained by X-ray diffraction and optical rotatory dispersion, but in others the situation seems to be less clear-cut.

This conclusion is also supported by numerous data and figures relating to proteins both with ordered (α-helical) and unordered structure. On the other hand, the experimental evidence convincingly shows that the optical rotatory parameters are very sensitive indicators of different structural alterations of proteins.

The last two chapters summarize the data obtained with proteins which have been for some reason neglected by structural investigations until now, e.g. nucleoproteins, histones, glycoproteins and lipoproteins.

Dr. Jirgensons' book is a concise review of the topic and a useful introduction for the reader who is interested in the application of spectropolarimetry for the investigation of protein and polypeptide structure.

P. E.

Printed in Hungary

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

A kézirat nyomdába érkezett: 1970. VI. 17. — Terjedelem: 9,75 (A/5) iv, 50 ábra

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**Für Studierende der Medizin,
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Von **Eckhart Buddecke**
o. Prof. für Physiologische Chemie
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Groß-Oktav. Mit mehr als 400 Formeln, Tabellen und Diagrammen. XXXII, 499 Seiten. 1970. Plastik flexibel DM 27,50

Die progressive Zunahme des biochemischen Fachwissens erfordert eine überschaubare und zusammenfassende Darstellung der Biochemie als Hilfsmittel für den Unterricht. Der Grundriß der Biochemie von E. Buddecke gliedert den Wissensstoff in die Kapitel „Stoffe und Stoffwechsel", „Stoffwechselregulation" und „Funktionelle Biochemie der Organe und Gewebe" mit dem Ziel, durch knappe Darstellung gesicherter Fakten und gezielte Stoffauswahl dem Leser einerseits eine rasche Information zu bieten, andererseits jedoch auf die vielfältigen Beziehungen und Anwendungsmöglichkeiten der Biochemie zur klinischen Chemie und Molekularpathologie hinzuweisen, um damit dem Studierenden der Medizin und Zahnmedizin sowie der anderen Gebiete der Naturwissenschaften das Verständnis klinischen Fachwissens zu erleichtern.

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15. XII. *1970* Index: 26.030

VOLUME 5
NUMBER 4
1970

Acta biochimica et biophysica

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (4), pp. 375–384 (1970)

Effect of the Cereus Lytic Enzyme on the Chemical Bonds of the Bacterial Cell Wall

S. CSUZI

Institute of Medical Chemistry, Semmelweis University, Budapest

(Received April 7, 1970)

The lytic enzyme preparation obtained by us from B. cereus appears to be a muraminyl-alanine amidase. This is supported by the fact that glucoseaminopeptides, peptides and saccharides containing glucoseamine and muramic acid could be isolated from the cell wall of B. cereus digested with the enzyme. The same saccharides as well as glucoseaminopeptides were released from Micrococcus lysodeikticus cell wall by the same enzyme. It is known that in this cell wall muramic acid is attached to an ala.glu.(gly).lys peptide. These results are in good agreement with the muraminyl-alanine amidase activity supposed.

Teichoic acid free B. cereus cell wall could be degraded to the same product as the intact cell wall.

The insoluble cell wall seems to be the only substrate for the lytic enzyme, as efforts to prepare soluble substrates were unsuccessful.

Peptides isolated from B. cereus cell wall treated with the lytic enzyme consist of ala, glu, and DAP* amino acids with molar ratios of 3:2:2 and 2:2:2, respectively. The saccharides obtained are built up from equimolar quantities of muramic acid and glucoseamine. These are therefore the basic elements of B. cereus cell wall structure.

Introduction

According to Weidel's concept (Weidel, Pelzer, 1964) the cell wall is regarded as a bag-shaped macromolecule built up from glucoseaminopeptides. The enzymes that split peptides of this structure were classified by Strominger (Strominger, 1967) into two main groups, namely muraminyl- alanine amidases and peptidases. A member of the former group is the Streptomyces amidase purified and studied by Ghuysen (1960, 1961, 1962) in great detail. This enzyme splits only the soluble form of substances containing muraminyl-alanine bonds. Muraminyl-alanine amidase activity was also detected in the extract obtained from Flavobacterium (Kato, Strominger, 1968) and in the autolysin associated with the cell wall of B. subtilis (Young et al., 1964). The peptidases split the cell wall peptides at different positions, two such enzymes isolated from Streptomyces albus (Petit et al., 1966; Ghuysen et al., 1966) can release alanine N terminal from the cell wall peptides. As described previously (Csuzi, 1964, 1968) we have obtained from one of the B. cereus strains a lytic enzyme which dissolves the

* Abbreviation: DAP = diaminopimelic acid.

cell wall of certain B. cereus strains. An increase in the quantity of alanine N terminal groups was demonstrated (Csuzi, 1969) and it was supposed that the enzyme had amidase activity.

One possible way for the identification of bonds split by the purified lytic enzyme(s) is the isolation and analysis of the products obtained through the action of the enzyme on a glucoseaminopeptide polymer (cell wall) or on its oligomer.

Materials and Methods

Determination of the dry matter content of cell wall suspensions

One ml of cell wall suspension was diluted to 10 ml with distilled water and centrifuged. The pellet was resuspended in 1 ml of distilled water. 0.5 ml of this suspension was dried at 100 °C until a constant value was reached.

Determination of the dry matter content of solubilized cell walls

An aliquot of the solution obtained after enzymatic digestion of cell walls was acidified to pH 3.1 with acetic acid and centrifuged. The supernatant was lyophilized and from the weight of the residue the amount of salts contained in the reaction mixture was subtracted.

Chromatography of solubilized and acidified solutions of cell wall fragments

An aliquot of the solubilized cell wall constituents at pH 3.1, containing about 160–200 mg dry weight substance, was chromatographed on a column of Dowex 50×2, 1.8×150 cm, with a volatile pyridine acetic acid buffer. Elution was carried out by a linear gradient from 0.2 M at pH 3.1 to 2.0 M at pH 5.0. Ninhydrin and indole reactive groups were determined alternately (Moore, Stein, 1948; Dische, Borenfreund, 1950) in 1 ml aliquots of the fractions.

Extraction of teichoic acid from B. cereus cell walls

The extraction was achieved by Archibald's method (Archibald, Baddiley 1965) and checked by measuring the decrease of total phosphate and dry weight of the cell wall from 0.26 μM/ml to 0.02 μM/ml and from 2.0 mg/ml to 1.66 mg/ml, respectively. The remaining insoluble cell wall material was centrifuged and the pellet was resuspended and washed three times in distilled water. This material is referred to as teichoic acid-free cell wall.

Hydrolysis of B. cereus cell wall by HCl

Cell wall of bacteria was prepared as described previously (Csuzi, 1964) except that of Micrococcus lysodeikticus. In the latter case the proteins and peptides attached to the cell wall were digested with pepsin (0.5 mg/ml) at pH 3.0 instead of trypsin. The strains of B. cereus and the cultivation method were

described previously (Csuzi, 1968). Micrococcus lysodeikticus strain was kindly supplied by Dr Zabos.

Amino acids and amino sugars were determined by an automatic amino acid analyzer (Moore, Stein, 1948).

Experimental

The observation that the quantity of alanine N terminal group increases (Csuzi, 1969) in the cell wall treated with our lytic enzyme suggests that the glucoseaminopeptides are split into peptides and saccharides. The identification of these products is possible if the cell wall is not contaminated with exogeneous

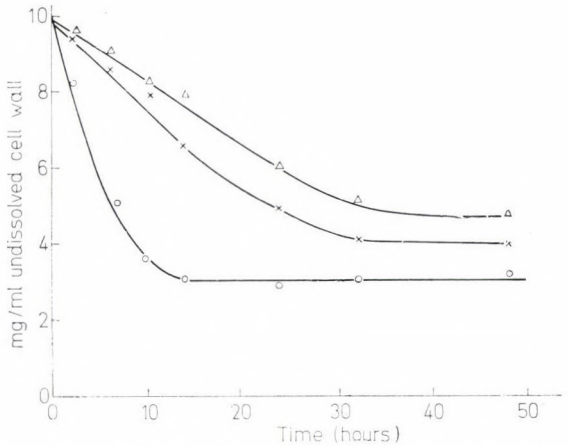

Fig. 1. Degradation of B. cereus 130, B. cereus 569 and Micrococcus lysodeikticus cell walls by the lytic enzyme. 10 mg/ml cell walls was incubated with 100 μg/ml lytic enzyme in 0.01 M phosphate buffer at pH 7.8, in the presence of a few drops of toluene. Samples (1 ml) were taken at intervals and the lytic enzyme inactivated in a boiling water bath for 5 minutes. The dry weight of the samples was determined (see Methods) and referred to as undissolved cell wall. Undissolved cell wall from: B. cereus 130 = ○−○−○; B. cereus 569 = x−x−x; Micrococcus lysodeikticus = △−△−△

peptides. The amino acid and glucoseamino sugar content of our cell wall preparation is shown in Table 1. Of the amino acids only ala, glu, and a typical cell wall amino acid, DAP were present in the hydrolysate. In addition, two amino sugars, glucoseamine and muramic acid were detected. The molar ratio of the amino acids ala, glu and DAP was found to be 3:2:2. These results show that the purity of the cell wall meets the requirement described above.

In order to isolate products obtained by the enzymatic hydrolysis of the cell wall, a suspension of 10 mg/ml cell wall was degraded by the cereus lytic enzyme. The time needed for degradation was determined on the basis of the decrease of the dry weight of undissolved cell wall in a long term incubation with the enzyme. As shown in Fig. 1 the degradation process of B. cereus 130

Table 1

Amino acids and amino sugars in the cell wall of B. cereus

	mmoles of amino acids and amino sugars per g cell wall
Glucoseamine	0.36
Muramic acid	0.38
Alanine	0.48
Glutamic acid	0.35
Diaminopimelic acid	0.36

cell wall came to an end after 8–10 hours, while that of B. cereus 569 and Micrococcus lysodeikticus only after 20–24 hours. The peptides and glucoseaminopeptides of the solubilized B. cereus cell wall were separated by chromatography on a Dowex 50×2 column and detected by the ninhydrin and indole reactions, respectively (Fig. 2 and Fig. 3). The substances represented by peaks A and B can be easily detected by indole and less effectively by ninhydrin but those represented by peaks E and F only by ninhydrin. The quantitative composition of the separated products is given in Table 2. In peaks A, B and C glucoseamine

Table 2

Amino acids and amino sugars in the separated fractions of B. cereus cell wall solubilized by the lytic enzyme

The fractions represented in Fig. 2 were lyophilized and analyzed for amino acids

	mmoles of amino acids and amino sugars per g substance of					
	peak A	peak B	peak C	peak D	peak E	peak F
Glucoseamine	2.16	1.86	0.87	0.91	0	0
Muramic acid	1.83	1.40	0.76	1.09	0	0
Alanine	0	0.62	2.0	0.49	2.25	1.50
Glutamic acid	0	0.49	1.44	1.36	1.52	1.58
Diaminopimelic acid	0	0.45	1.52	1.19	1.68	2.04

and muramic acid can be found in equimolar ratios. The peak A material consists of the two amino sugars only, whereas in peak B amino acids can also be detected. A molar ratio of 8:8:3:2:2 found for glucoseamine, muramic acid, ala, glu, and DAP, respectively, is compatible with the quantities of components given in Table 2. Peak C is similar to peak B in the ratio of its amino acids, i.e. the molar ratio is 3:2:2 for ala:glu:DAP. The material under peak D also contains equimolar quantities of muramic acid and glucoseamine, but the amino acids ala, glu, and DAP are present in an unusual 1:2:2 molar ratio. Peaks E and F contain material composed only of amino acids, their ala, glu and DAP molar ratios are 3:2:2

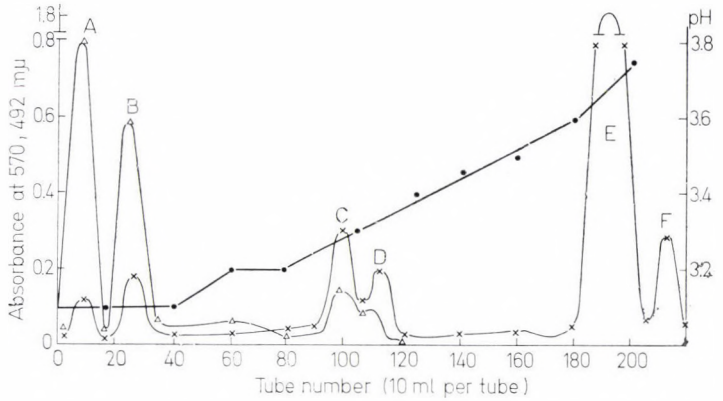

Fig. 2. Chromatography of products from B. cereus 130 cell wall solubilized by the lytic enzyme. Cell wall of B. cereus 130 was treated with the lytic enzyme for 48 hours (see Fig. 1), subsequently acidified to pH 3.1 and centrifuged. 180 mg of the soluble components of the cell wall (in 30 ml) were applied to the Dowex 50×2 column. pH = ●—●—●; A_{570} mμ of ninhydrin-reactive substances = x—x—x; A_{492} mμ of indole-reactive substances = △—△—△

and 2:2:2, respectively. The data of Fig. 2 and Table 2 represent the separation of cell wall fragments into peaks consisting of either only peptides (peaks E and F) or saccharides (peak A) suggesting that (1) the substrate is composed of glucose-aminopeptides and (2) its muraminyl.alanine bond was split by the lytic enzyme.

The peptides in the cell wall of Micrococcus lysodeikticus are known to be attached to the saccharide chain through a muraminyl.alanine bond, therefore the isolation of peptides and saccharides from this cell wall treated with our lytic enzyme, would mean the splitting of the muraminyl.alanine bond. As it

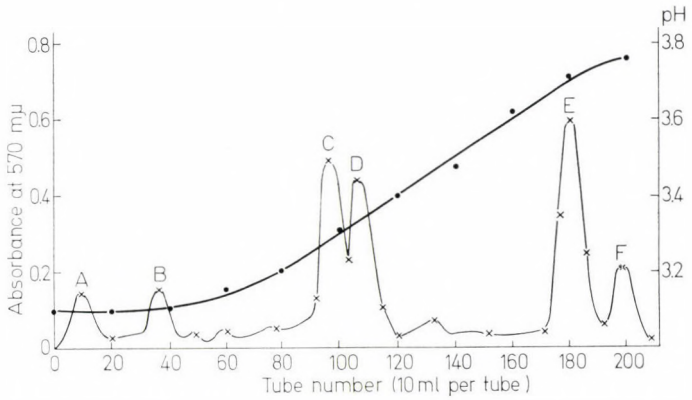

Fig. 3. Chromatography of B. cereus 569 cell wall solubilized by the lytic enzyme. The cell wall was incubated with the lytic enzyme as described in Fig. 1, subsequently acidified and centrifuged. 170 mg of soluble components were chromatographed. pH = ●—●—●; A_{570} mμ of ninhydrin-reactive substances = x—x—x

can be seen in Fig. 4 only three substances were found. The amino acids and amino sugars of the substances separated under peaks A, B and C are presented in Table 3. The substance of peak A consists of glucoseamine and muramic acid in an equimolar ratio. That of peaks B and C are glucoseamine, muramic acid, ala, glu, lys and gly in a molar ratio of 10:10:4:2:2:2 and 1:1:8:4:4:4, respectively. The fact that a pure saccharide (peak A) and a glucoseaminopeptide

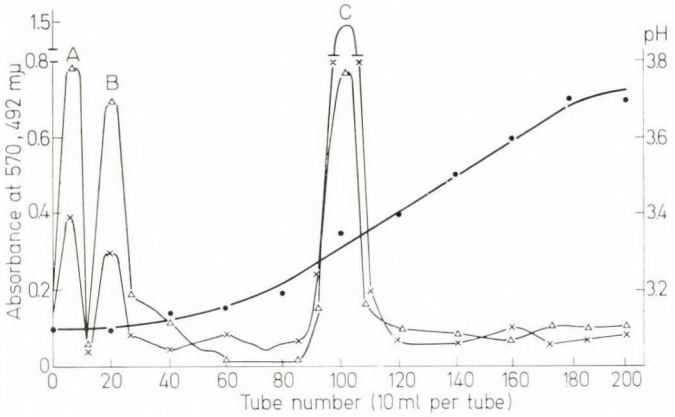

Fig. 4. Chromatography of Micrococcus lysodeikticus cell wall digested with the lytic enzyme. The cell wall was incubated with the lytic enzyme as described in Fig. 1, subsequently acidified to pH 3.1 and centrifuged. 170 mg (in 40 ml) of soluble components were applied to the Dowex 50×2 column. pH = ●—●—●; A_{570} mμ of ninhydrin-reactive substances = = x—x—x; A_{492} mμ of indole-reactive substances = △—△—△

(peak C) mainly composed of amino acids have been isolated supports the suggestion that muraminyl.alanine bonds were disrupted by our lytic enzyme.

The cell wall preparation examined in our experiments was composed of glucoseaminopeptide and teichoic acid. The data presented in Fig. 2 and Table 2 suggest only the degradation of glucoseaminopeptide, however the fragments isolated might be contaminated by teichoic acid. The cell wall preparation was freed from teichoic acid (cf. Methods) and then digested with the lytic enzyme. As it can be seen in Fig. 5 the chromatography led to a similar pattern as represented in Figs 2 and 3. The substance of peak E was analyzed and ala, glu and DAP could be detected in the same molar ratio as in the non-extracted cell wall (cf. Table 2). This experiment shows that no fragment formed from teichoic acid in experiments presented in Figs 2 and 3.

The next question is whether less polymerized forms of the glucoseaminopeptide obtained by the degradation of cell wall saccharide chains are a sensitive substrate for our enzyme. As shown in Fig. 1 the B. cereus 130 cell wall was degraded most rapidly. It is reasonable to suppose that this cell wall contains the largest quantity of sensitive bonds. The glycosidic bonds were hydrolyzed by HCl to produce soluble oligomers of glucoseaminopeptides. Pure glucoseaminopeptides were also isolated by chromatography and are represented in Figs

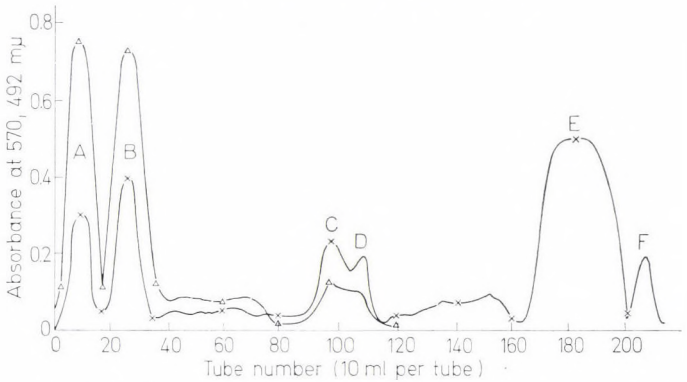

Fig. 5. Chromatography of products of teichoic acid-free cell wall of B. cereus 130 solubilized by the lytic enzyme. Cell wall free from teichoic acid (8 mg/ml) was incubated with 100 μg/ml lytic enzyme in 0.01 M phosphate buffer at room temperature for 48 hours in the presence of a few drops of toluene. Subsequently the solution was acidified to pH 3.1, centrifuged and 30 ml of the supernatant containing 165 mg acid soluble cell wall fragments were applied to the Dowex 50×2 column. A_{570} mμ of ninhydrin-reactive substances = x−x−x; A_{492} mμ of indole-reactive substances = △−△−△. The fractions under peak F were collected and their amino acids were determined. Ala, glu and DAP could be detected in quantities of 1.8, 1.0 and 1.1 m moles/g dry weight, respectively

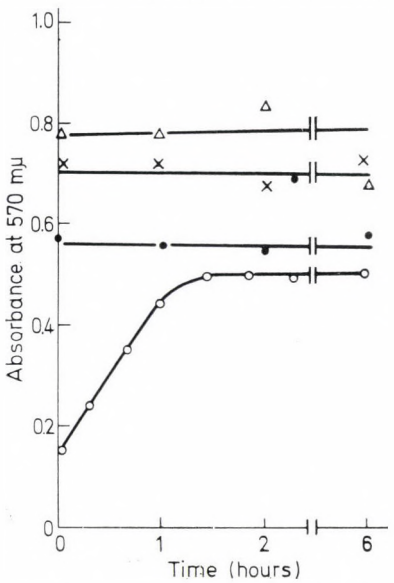

Fig. 6. Ninhydrin-reactive groups in substances of peaks B, C as well as in acid-hydrolyzed and nonhydrolyzed cell wall of B. cereus. Reaction mixtures a, b, c and d consisting of 8 mg/ml of substance of peak B, 2 mg/ml substance of peak C, 2 mg/ml substance of HCl-hydrolyzed cell wall and 2 mg/ml nonhydrolyzed cell wall, respectively, were incubated with 10 μg/ml lytic enzyme in 0.01 M phosphate buffer at pH 7.8. The substances of peaks B and C were obtained by lyophilization from fractions corresponding to peaks B and C in Fig. 2. Samples of 0.1 ml were taken for the detection of ninhydrin-reactive groups. After developing the colour the insoluble cell wall fragments were centrifuged and the absorbancy measured at 570 mμ. a = △−△−△; b = ●−●−●; c = x−x−x; d = ○−○−○

2, 3 and 4. Substances of peaks B and C (cf. Fig. 2 and Table 2) and that of HCl-hydrolyzed cell wall (cf. Methods) were treated by the lytic enzyme, but as it can be seen in Fig. 6 no further increase in ninhydrin reacting substances could be detected. At the same time equal concentrations of the B. cereus cell wall and the enzyme led to an increase in ninhydrin reacting substances.

As the cell wall of Micrococcus lysodeikticus is degraded by our lytic enzyme (cf. Fig. 4 and Table 3), another possibility to look for a soluble and less polymerized substrate would be to study the effect of the lytic enzyme on the lysozyme-solubilized Micrococcus lysodeikticus cell wall fragments. The

Table 3

Amino acids and amino sugars in chromatographically separated fractions of Micrococcus lysodeikticus cell wall digested with the lytic enzyme

	mmoles of amino acids and amino sugars per g substance of		
	peak A	peak B	peak C
Glucoseamine	1.95	2.41	0.42
Muramic acid	2.20	2.25	0.45
Alanine	0	0.55	3.50
Glutamic acid	0	0.22	1.75
Lysine	0	0.27	1.60
Glycine	0	0.17	1.65

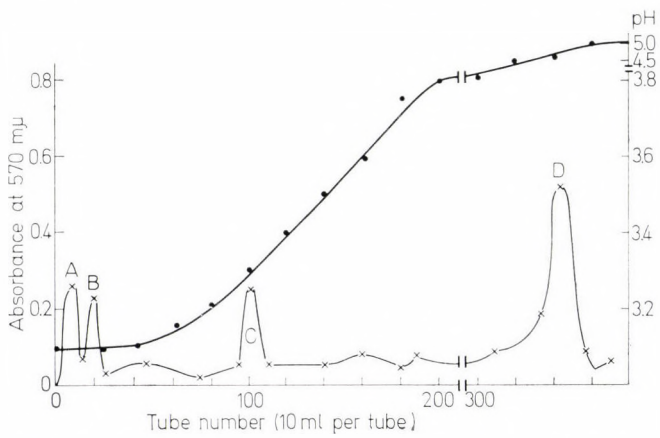

Fig. 7. Chromatography of soluble glucoseaminopeptides after incubation with the lytic enzyme. 10 mg/ml of Micrococcus lysodeikticus cell wall in 0.01 M phosphate buffer at pH 6.6 was digested by 20 μg/ml lysozyme for 6 hours at room temperature. Insoluble products were centrifuged off, the supernatant lyophilized. 220 mg of these lyophilized soluble glucoseaminopeptides were dissolved in 22 ml of 0.01 M phosphate buffer, pH 7.8, containing 100 μg/ml lytic enzyme and incubated for 48 hours in the presence of a few drops of toluene. The mixture was acidified to pH 3.1, centrifuged, and the supernatant containing 190 mg soluble fragments was chromatographed. pH = ●—●—●; A_{570} mμ of ninhydrin-reactive substances = x—x—x

soluble glucoseaminopeptides obtained through lysozyme action were incubated with our lytic enzyme and the mixture was chromatographed. Peak C (Fig. 7) appears in a relatively small quantity, compared with the amount of the same fraction (peak C in Fig. 4) when the lytic enzyme acted on the intact cell wall. Moreover, the lysozyme-solubilized cell wall fragments gave the same chromatographic pattern before and after treatment with our lytic enzyme. An inhibitory effect of the solubilized substances on the lytic enzyme was ruled out in the experiment, where the B. cereus 130 cell wall was suspended in the reaction mixture containing solubilized cell walls and the enzyme had the same activity as in the usual reaction mixture.

On the basis of these findings only the insoluble and intact cell wall seems to be a suitable substrate for the enzyme.

Discussion

The lytic enzyme isolated from B. cereus was supposed to split the cell wall to saccharide, peptide and glucoseaminopeptide. According to Weidel's model (Weidel, Pelzer, 1964) the separation of a saccharide free from peptide (peak A in Fig. 2) and that of a pure peptide (peaks E and F) suggest the disruption of muraminyl.alanine bonds. In addition, the isolation of saccharide and glucoseaminopeptide from Micrococcus lysodeikticus cell wall treated with lytic enzyme also points to the splitting of muraminyl.alanine bonds, the muramic acids being attached to peptides in this cell wall only. With respect to the increase in alanine N terminal groups in cell walls treated with the lytic enzyme, the splitting of peptide bonds by intrapeptidase may also be supposed. If this enzyme is involved in our lytic enzyme preparation, then an ala.glu.DAP.ala$-$CO would be split

$$\text{ala.glu.DAP}-\text{NH}$$

between DAP.ala amino acids. The molar ratio of 1:1:1 of ala, glu and DAP (peak F in Table 2) is in good agreement with the composition of the tripeptide suggested above. The other half of the peptide with a molar ratio of 2:1:1 of ala, glu and DAP amino acids was not isolated. The data presented have shown an enzyme activity belonging to the muraminyl.alanine amidase group as described by Ghuysen (1960, 1961, 1962). No intrapeptidase seems to occur in our lytic enzyme preparations.

From our data presented in Fig. 2 and Table 2 some insight may be obtained into the cell wall structure of B. cereus. The substance represented by peak A consisting of equimolar quantities of glucoseamine and muramic acid seems to be a polysaccharide chain split off from glucoseaminopeptide. Peak E has a composition similar to the characteristic cell wall peptide of E. coli (Pelzer, 1962; Primosigh et al., 1961) and of B. megatherium (Van Heichwort et al., 1967) with a ratio of 3:2:2 of ala, glu and DAP amino acids. These data suggest the occurrence of ala.glu.DAP.ala$-$CO in the cell wall of B. cereus. Peak F

$$\text{ala.glu.DAP}-\text{NH}$$

contains the same amino acids with a molar ratio of 1:1:1 which is compatible with a shorter ala.glu.DAP peptide. Peaks B and C represent mixed substances inasmuch as they consist of both amino acids and amino sugars. In peak B the molar ratio of glucoseamine and glutamic acid is 4:1, representing a cell wall glucoseaminopeptide disrupted almost completely if related to their molar ratio found in the intact cell wall (cf. Table 1). The substance of peak C contains the same amino acids in the same ratio as in peak E, but its glutamic acid is twice that of the glucoseamine and muramic acid, suggesting a less degraded structure than the former one. Peak D does not fit into the generally accepted model of Weidel, as it has an extraordinary ratio of amino acids. Further data are needed to establish its relation and site in the cell wall of B. cereus.

The author wishes to thank Prof. F. B. Straub for his interest and helpful discussion and to express his gratitude to the late Dr L. Beney for his collaboration in chromatographic analyses.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (4), pp. 385–397 (1970)

Factors Affecting the Reactivity of the Activated SH-Group of D-Glyceraldehyde 3-Phosphate Dehydrogenase

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(Received April 10, 1970)

The pH-dependence of the light absorption of the GAPD-NAD complex in solutions containing chloride or pyrophosphate ions can be described by single acidic dissociation curves with pK 4.6 and 6.8, respectively. The apparent acidic dissociation, determined by means of carboxymethylation, of the Cys-149 thiol group of GAPD containing firmly bound coenzyme coincides with the pH-dependence of the absorption of the enzyme-NAD complex in the presence of the above anions. It is assumed that this SH-group of the enzyme is activated by a functional group that is in the deprotonated state, and that the observed pH-dependence reflects the dissociation of this functional group. The various anions may exert their effect by influencing the dissociation of this activating group when bound in its neighbourhood.

The NAD bound to the enzyme accelerates the reaction with iodoacetate probably because the binding of the carboxylate ion of the reagent to the pyridinium ion of the coenzyme increases the probability of favourably oriented collisions. On the other hand, alkylation with iodoacetamide is inhibited by the coenzyme. This is explained by steric hindrance. In fact, the binding of NADH to the enzyme also reduces the rate of carboxymethylation as compared to the NAD-free enzyme.

Introduction

The characteristic light absorption of the GAPD–NAD complex has been known for a long time, but the origin of this absorption is still unknown. Racker and Krimsky (1952) postulated a bond between the "active" SH-group of the enzyme and one of the carbon atoms of the pyridinium ring of NAD, whereas Kosower (1956) raised the possibility of a charge transfer interaction. Cilento and Tedeschi (1961) claim that a tryptophyl side chain of the enzyme serves as electron-donor in the postulated charge transfer interaction, and Shifrin's (1964) experiments support this notion. However, the tryptophan-NAD charge transfer spectrum does not resemble that of the GAPD–NAD complex (Boross, Cseke, 1967). Van Eys and Kaplan (1957) have provided evidence that mercaptide ions are also capable of forming complexes with pyridinium compounds and the spectra of these complexes are more closely related to that of the enzyme-NAD complex. Thus the existence of a thiol-pyridinium charge transfer interaction in the GAPD–NAD complex can be easily conceived, since the enzyme

contains one activated, markedly nucleophilic SH-group per subunit (Cys-149). Our earlier experiments also support this hypothesis, as it was found that the apparent acidic dissociation curve of this SH-group in 5 per cent ammonium sulfate coincided with the pH-dependence of the characteristic light absorption of the enzyme-NAD complex (Boross, Cseke, 1967).

Later we have found that sulfate ions affect both the rate of carboxymethylation of the activated SH-group and the pH-dependence of light absorption of the GAPD–NAD complex (Boross et al., 1968, 1969). Trentham (1968) has described the effect of phosphate ion on the pH-dependence of light absorption. Thus the effect of anions provided a means for the further investigation of the relationship between the reactivity of the activated SH-group and the light absorption of the enzyme-NAD complex: if a clear-cut parallelism could be detected between the apparent dissociation of the SH-group and the pH-dependence of light absorption of the enzyme-NAD complex, that would corroborate the hypothesis according to which the activated thiol group participates in the charge transfer interaction.

It seemed advisable to determine the apparent dissociation of the activated SH-group of the enzyme also with iodoacetamide as reagent, since Racker and Krimsky (1958) have reported that in the GAPD–NAD complex iodoacetate reacts much more rapidly with the activated SH-group than in the NAD-free enzyme, whereas there is no increased reactivity towards iodoacetamide.

Our experimental data detailed in this paper were presented at Madrid FEBS Meeting (Boross et al., 1969) and summarized in a preliminary report (Boross et al., 1969a).

Materials and Methods

GAPD was prepared according to the method of Elődi and Szörényi (1956) from pig muscle and was recrystallized four times. NAD firmly bound to the enzyme was removed by charcoal treatment (Velick, 1953) as described in our previous paper (Cseke, Boross, 1967). Enzyme concentration was determined spectrophotometrically by using the molar extinction coefficients 1.4×10^5 and 1.27×10^5 for the GAPD–NAD complex and NAD-free enzyme, respectively. The molecular weight of the tetrameric enzyme was taken to be 140 000 (Elődi, 1958). Enzyme solutions were routinely gel-filtrated on a Sephadex G–100 column equilibrated with 0.05 ionic strength Tris-HCl buffer, pH 7.

Commercial iodoacetate and iodoacetamide were four times recrystallized from carbon tetrachloride before use. The titer of their solutions was checked spectrophotometrically ($\varepsilon_{265} = 400$) (Finkle, Smith, 1958). The alkylating agents were always used in equimolar amounts with the activated SH-group of GAPD. Alkylation was carried out at 5 °C. The pH of the reaction mixtures was measured at the same temperature.

The progress of alkylation was followed by different methods:

A) Decrease of absorption at 365 nm

This method was used when GAPD containing bound NAD was carboxymethylated, since the characteristic absorption band of the enzyme-coenzyme complex at 365 nm disappears on this treatment (Racker, Krimsky, 1952). The reaction mixture contained 3×10^{-5} M enzyme. The measurements were carried out in cells of 4 cm light path. The end-point of the reaction was checked by the addition of 8 moles of p-mercuribenzoate per mole of GAPD, as this reagent instantaneously abolishes the absorption at 365 nm of the enzyme-NAD complex (Velick, 1953).

B) Determination of unreacted Cys-149 SH-groups in the form of Cu-complex

The activated SH-group of the enzyme forms a complex with Cu^{2+} ions, which has an absorption maximum at 370 nm (Boross et al., 1969a). This Cu-complex is instantaneously formed in phosphate buffer, pH 7.5, containing 5 per cent ammonium sulphate. This method was adopted when NAD-free enzyme was alkylated or when the NAD-containing enzyme was treated with iodoacetamide. Alkylation was performed at 10^{-4} M enzyme concentration. The concentration of the Cu-complex was measured in the following reaction mixture:

3.33×10^{-5} M alkylated enzyme solution
1.33×10^{-4} M $CuSO_4$, in 5 per cent ammonium sulphate, 0.5 M phosphate buffer, pH 7.5.

C) Determination of unreacted SH-groups with Ellman-reagent (Ellman, 1959)

In Tris-HCl buffer, pH 7.5, containing 2 M NaCl, another SH-group (SH_{II}) also reacts very rapidly with 5,5'-dithio-bis(2-nitrobenzoate), in addition to the instantaneously reacting nucleophilic SH-group, whereas the two buried SH-groups react very slowly (Boross, 1969). Thus the amount of $SH_I + SH_{II}$ can be measured. This method was adopted when NADH-containing enzyme was used. The composition of the reaction mixture was as follows: 10^{-5} M alkylated enzyme, 8×10^{-5} M DTNB, 2 M NaCl, in 0.05 ionic strength Tris-HCl buffer, pH 7.5. Because of the high absorption of this mixture, the reference cell contained a methyl red stock solution of known optical density ($E_{412} = 0.400$)

D) Decrease of light absorption at 265 nm

Iodoacetate and iodoacetamide exhibit an absorption maximum at 265 nm and this band disappears on alkylation (Finkle, Smith, 1958). The alkylation of cysteine and mercaptoethanol, which only slightly absorb at 265 nm, was followed in this way. The reaction mixture contained 10^{-3} M alkylating reagent, 0.04 M cysteine and 0.04 M EDTA or 0.08 M mercaptoethanol and 0.08 M EDTA.

The measurements were carried out in thermostated Unicam SP 500 and SP 700 A spectrophotometers.

NAD and NADH (Reanal) were 90 per cent pure, all other chemicals were commercial preparations of reagent grade.

Results

Effect of various anions on the absorption of the GAPD–NAD complex

The absorption of the GAPD–NAD complex was measured in the near ultraviolet region in solutions of various ionic composition and pH. It was found that the shape of the spectrum was independent of the ionic composition of the medium, i.e. the maximum of the characteristic difference spectrum (the self-absorption of enzyme and NAD subtracted from the absorption of the complex,

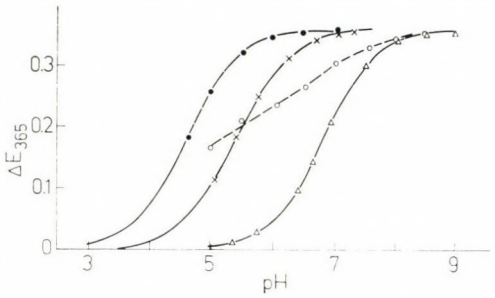


Fig. 1. pH dependence of the difference spectrum of the GAPD–NAD complex in the presence of various anions. The ordinate shows the values obtained at the maximum of the difference spectrum. The enzyme was gel-filtered in Tris/HCl buffer of 0.05 ionic strength. The solutions contained 10^{-4} M enzyme in Tris/HCl buffer (● — ●),to which 0.1 M ammonium sulfate (x — x), 0.1 M sodium pyrophosphate (△ — △), or 0.1 M sodium-potassium phosphate (○ — ○) was also added. Measurements were done at 5 °C. pH was measured at the same temperature. The continuous lines are theoretical dissociation curves with pK-s of 4.6, 5.4, and 6.8, respectively

all measured under identical conditions) was at 365 nm. At the same time the absorption at 365 nm is differently affected by pH in the presence of chloride (or acetate) sulfate, phosphate, or pyrophosphate ions (Fig. 1). These pH-profiles reasonably coincide with acidic dissociation curves which can be characterized by pK values 4.6, 5.4, and 6.8 in acetate or chloride, sulfate, and pyrophosphate, respectively. According to Trentham (1968), the pH-dependence of the light absorption of the lobster GAPD–NAD complex in phosphate + pyrophosphate buffer can be described by a dissociation curve with pK 6.86. We found that in phosphate buffer the pH profile of light absorption of the complex did not coincide with a simple dissociation curve. Thus the pH-dependence measured in phosphate + pyrophosphate buffer is to be attributed to the effect of pyrophosphate ion. Changing of the cation (sodium sulfate instead of ammonium sulfate, potassium chloride instead of sodium chloride) did not influence either the difference spectrum or the pH profile.

When increasing amounts of ammonium sulfate were added to the enzyme solution gel-filtered in 0.05 ionic strength Tris-HCl buffer, light absorption gradually decreased in the acidic range up to about 0.1 M sulfate concentration.

In the alkaline region, i.e. at the plateau of pH profiles shown in Fig. 1, light absorption did not change on the addition of ammonium sulfate. The same phenomenon was observed also with increasing phosphate or pyrophosphate concentrations.

Effect of various anions on the rate of carboxymethylation of Cys-149

As shown previously (Boross, Cseke, 1967), in ammonium sulfate solution the second order rate constant of carboxymethylation of the activated SH-group changes as a function of pH in a similar manner as the light absorption of the enzyme-coenzyme complex, and the pH profile corresponds to an acidic dissociation curve with pK 5.4 (Fig. 2). We examined whether these two pa-

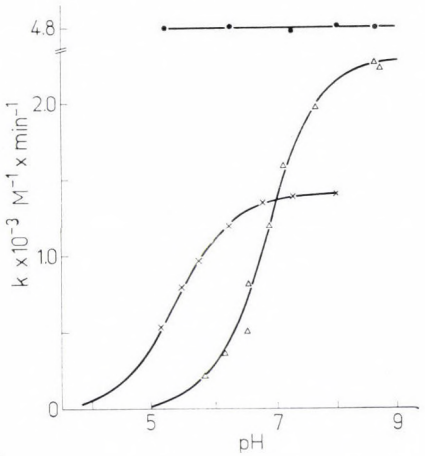


Fig. 2. Effect of anions on the pH-dependence of the rate of carboxymethylation of Cys-149 in the presence of strongly bound coenzyme. The reaction was followed spectrophotometrically at 365 nm. The calculated second order rate constants are plotted as a function of pH. The reaction mixtures contained 3×10^{-5} M enzyme and 1.2×10^{-4} M iodoacetate. For the explanation of symbols see legend to Fig. 1. The continuous lines represent theoretical dissociation curves

rameters changed in a parallel way also in media of other ionic compositions. In pyrophosphate buffer the pH-dependence of the apparent second order rate constant of carboxymethylation coincides with an acidic dissociation curve of pK 6.8 (Fig. 2). This pH-dependence agrees well with that of the Racker-band in pyrophosphate (Fig. 1). In the presence of acetate or chloride the rate constant of carboxymethylation was found to be independent of pH above pH 5.5, where the absorption of the enzyme-NAD complex is also constant (Fig. 2). Around pH 4.6 the reactivity of the activated SH-group could not be estimated by the carboxymethylation method, as experiments with model compounds have shown that in such acidic media iodoacetate cannot be

used to determine the dissociation of a thiol group. This is probably due to the protonation of the carboxylate ion in the reagent.

We may draw the conclusion that the apparent acidic dissociation of the activated SH-group, as determined by carboxymethylation, agrees well with the pH-dependence of the Racker-band in the presence of the examined anions. However, the effect of various anions on the reactivity of the activated SH-group is not confined to the displacement of the apparent acidic dissociation curve.

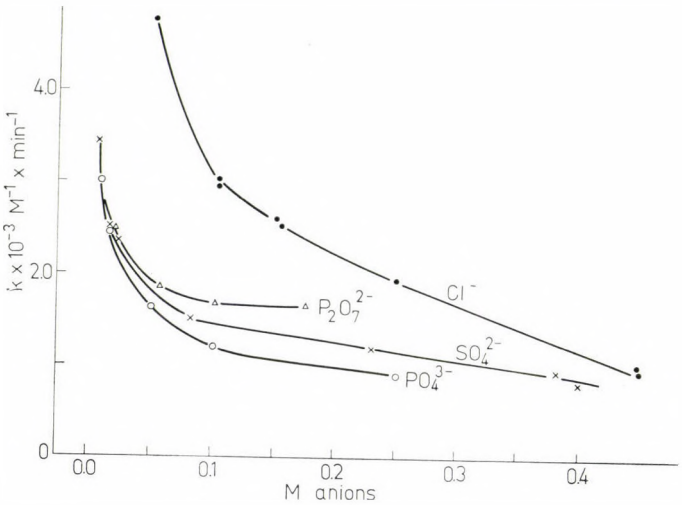


Fig. 3. Effect of anion concentration on the rate of carboxymethylation of Cys 149 at pH 7.5, in the presence of strongly bound coenzyme. The solutions contained Tris/HCl buffer with an ionic strength of 0.05, plus increasing concentrations of the following salts: sodium chloride (● — ●), ammonium sulfate (x — x), sodium pyrophosphate (△ — △), sodium-potassium phosphate (○ — ○). All solutions contained 3×10^{-5} M iodoacetate. The reaction was measured spectrophotometrically in 4-cm cells, and at 5 °C

An increase in the concentration of anions results in a decrease of the rate of alkylation with iodoacetate throughout the whole pH-range, i.e. also in a reduction of the maximal (plateau) values on the alkaline side (Fig. 3). The rate-decreasing effect of anions should be regarded specific for the protein, since the rate of alkylation of aliphatic mercaptanes increases with increasing ion concentrations (Table 1).

Effect of NAD and NADH on the rate of carboxymethylation of the activated SH-group

The above experiments indicate that the enzyme-coenzyme interaction and the reactivity of Cys-149 towards iodoacetate are related phenomena. Racker and Krimsky (1958) claimed that the rapid carboxymethylation was effected by bound NAD. On the other hand, the NAD-free enzyme possesses ester-hydro-

Table 1

Effect of various anions on the rate of carboxymethylation of the activated thiol group of GAPD, and effect of model mercaptanes

k corresponds to the apparent rate constant of the alkylation at 5 °C. The reaction was measured at pH 7.5 in Tris/HCl buffer containing additional salts (sodium chloride, ammonium sulfate, sodium pyrophosphate or sodium-potassium phosphate). The error of the determination of k is about ±10 per cent.

Enzyme	Anion added, 0.1 M	k $M^{-1} \times min^{-1}$	Apparent pK of thiol group
GAPD—NAD	—	4800	< 5
GAPD—NAD	chloride	2600	< 5
GAPD—NAD	sulfate	1500	5.4
GAPD—NAD	phosphate	1200	
GAPD—NAD	pyrophosphate	1700	6.8
GAPD—NADH	—	50	
GAPD	—	300	< 8
GAPD	chloride	270	
GAPD	sulfate	150	
GAPD	phosphate	50	
Cysteine	—	2.17	8.5
Cysteine	0.4 M sulfate	4.3	
Cysteine	0.4 M phosphate	5.6	
Mercaptoethanol	—	0.262	9.8
Mercaptoethanol	0.1 M chloride	0.30	
Mercaptoethanol	0.4 M chloride	0.37	
Mercaptoethanol	0.1 M sulfate	0.334	
Mercaptoethanol	0.4 M sulfate	0.57	
Mercaptoethanol	0.1 M phospate	0.428	

lyzing activity (Park et al., 1961). Therefore it should be assumed that the SH-group in Cys-149 is activated also in the absence of NAD.

In our experiments at pH 7.5 the rate constant of carboxymethylation of the NAD-free enzyme was one order of magnitude less than that of the enzyme containing NAD, but still two orders of magnitude higher than that of cysteine (Table 1). Consequently, the SH-group of Cys-149 of the NAD-free enzyme was indeed activated. The rate of carboxymethylation was less with the enzyme containing NADH, as compared to the free enzyme, but still higher than with cysteine. The thiol group of Cys-149 is to be regarded activated even in the enzyme-NADH complex (Table 1).

In the case of the free enzyme sulfate, and especially phosphate, ions markedly decreased the rate of carboxymethylation, whereas chloride ion exerted no significant effect (Table 1).

The removal of bound NAD also changed to pH-dependence of the rate constant of alkylation with iodoacetate (Table 1). The pK of apparent acidic dissociation in the absence of NAD fell between pH 7.5 and 8.

Reactivity of Cys-149 towards iodoacetamide

According to Racker and Krimsky (1958) the great reactivity of the activated SH-group towards iodoacetate is due to the carboxylate ion of the reagent, which presumably binds to the substrate-binding site of the enzyme. We have assumed that the various anions decrease the rate of carboxymethylation of the active SH by interfering with the binding of the carboxylate ion. To test this assumption we examined whether anions hindered alkylation with a neutral reagent, iodoacetamide.

In our experiments with simple aliphatic mercaptanes the same apparent acidic dissociation curve was obtained whether iodoacetate or iodoacetamide was used for alkylation. On the other hand, the apparent pK of the active thiol

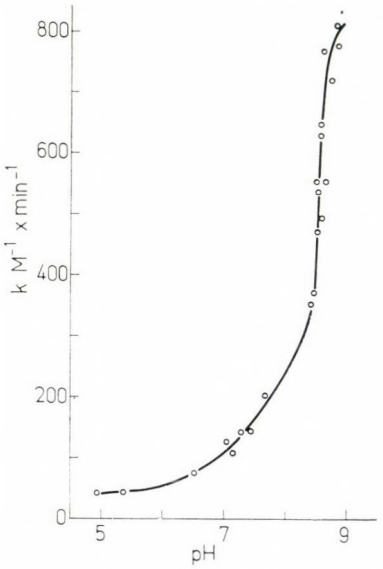


Fig. 4. pH dependence of the rate of reaction of Cys-149 with iodoacetamide in Tris/HCl buffer of 0.05 ionic strength. Alkylation was carried out at 5 °C. The reaction mixture contained 10^{-4} M enzyme and 4×10^{-4} M iodoacetamide. The reaction was followed by determining the unreacted thiol groups in the cupric complex form

group of Cys-149 in GAPD proved to lie above pH 8 when reacted with iodoacetamide (Fig. 4), whereas it fell below 5.5 with iodoacetate. Since the pK of cysteine SH-group is 8.5 (Benesch, Benesch, 1955), we examined, whether the thiol of Cys-149 of GAPD was activated as compared to cysteine when measured with iodoacetamide. As shown in Table 2, at pH 8.5 the rate constant of alkylation with iodoacetamide of Cys-149 was one order of magnitude higher than that of cysteine, consequently this thiol group of the enzyme is to be regarded activated also with respect to iodoacetamide.

The effect of anions on alkylation with iodoacetamide was examined at pH 8.5. An increase in the concentration of anions also resulted in the reduction

Table 2

Effect of various anions on the rate of alkylation of Cys-149 and model mercaptanes with iodoacetamide

The measurements were carried out at 5 °C in Tris/HCl buffer of 0.05 ionic strength containing the same additional salts as in Table 1.

Enzyme	Anion added 0.1 M	pH	k $M^{-1} \times min^{-1}$	Apparent pK of thiol group
GAPD	—	7.5	1075	>8
GAPD	—	8.5	4000	
GAPD	sulfate	7.5	660	
GAPD	chloride	7.5	530	
GAPD	chloride	8.5	3500	
GAPD	phosphate	7.5	84	
GAPD	phosphate	8.5	650	
GAPD – NADH	—	7.5	30	
GAPD – NADH	—	8.5	145	
GAPD – NAD	—	7.4	180	>8
GAPD – NAD	—	8.5	600	
GAPD – NAD	sulfate	8.5	250	
GAPD – NAD	chloride	8.5	525	
GAPD – NAD	phosphate	8.5	190	
Cysteine	—	7.5	16.3	8.5
	—	8.5	105.0	
	0.1 M sulfate	7.5	17.0	
	0.4 M sulfate	8.5	117.0	
Mercaptoethanol	—	7.5	2.53	9.8
	—	8.5	19.5	
	0.4 M sulfate	7.5	3.24	
	0.4 M sulfate	8.5	24.3	

of the rate of alkylation with iodoacetamide (Table 2). In 0.05 ionic strength Tris – HCl buffer and in the same buffer containing 0.1 M NaCl or 0.1 M phosphate the apparent rate constants were related to each other in the same way as in the case of alkylation with iodoacetate. As to the magnitude of rate constants, alkylation with iodoacetate was faster, even at high ion concentration, than with iodoacetamide. Accordingly, the carboxylate ion of iodoacetate is able to ensure the favourable, oriented binding of the reagent at neutral or slightly alkaline pH even at high ion concentration, i.e. the anions do not abolish the effect of the carboxylate ion. The significance of oriented binding near the SH-group of GAPD, affected by the negative charge of the reagent, is especially conspicuous if we consider that cysteine and mercaptoethanol are alkylated faster with iodoacetamide than with iodoacetate.

The removal of bound NAD increased the rate of alkylation with iodoacetamide, in contrast to alkylation with iodoacetate (Table 2). Thus in the case of NAD-free enzyme, just as with aliphatic mercaptanes, alkylation was faster with iodoacetamide than with iodoacetate. This finding suggests that binding

of NAD to the enzyme sterically hinders the approach of the electrophilic carbon atom of uncharged iodoacetamide to the activated SH-group.

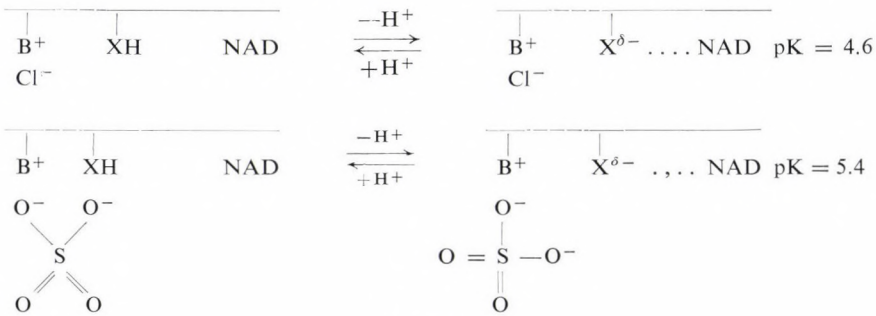
The inhibitory effect of anions was also observed after the removal of bound NAD (Table 2).

Similarly to NAD, the binding of NADH to the enzyme also hampered the alkylation of activated SH-group with iodoacetamide, and to an even greater extent (Table 2).

Discussion

One of the aims of our experiments was to determine which functional group of the enzyme took part in the charge-transfer interaction responsible for the light absorption of the GAPD–NAD complex. In the presence of various anions the pH-dependence of light absorption of the GAPD–NAD complex corresponds to acidic dissociation curves and depends on the ionic composition of the solution. The pK is 4.6 in chloride, 5.4 in sulfate (Cseke, Boross, 1967), and 6.8 in pyrophosphate. Trentham (1968) found a pK of 6.86 for the light absorption of lobster GAPD–NAD complex in phosphate-pyrophosphate buffer. According to our experiments with pig muscle GAPD, the pH-dependence in phosphate buffer cannot be described by a simple dissociation curve, thus the pK of 6.86 is to be attributed to the effect of pyrophosphate ion.

The pH-dependence of the absorption of the enzyme-NAD complex suggests that a dissociable functional group of the enzyme plays a role in light absorption. Since no absorption was found in the acidic range, the release of a proton from the functional group is necessary for the development of the characteristic spectrum. The effect of anions on the pH-dependence of absorption can be explained by assuming that the binding of anions at a B^+ basic group of the enzyme alters the dissociation of the functional group (XH). If group B^+ is sterically near group XH, then it seems conceivable that the binding of polyvalent anions (such as sulfate and pyrophosphate) will cause the dissociation of a proton from functional group XH to occur at a higher pH than with monovalent ions (such as chloride or acetate). In other words, binding of polyvalent anions in the neighbourhood of group B^+, by creating an electron-rich region, increases the pK of dissociation of group XH (scheme I).



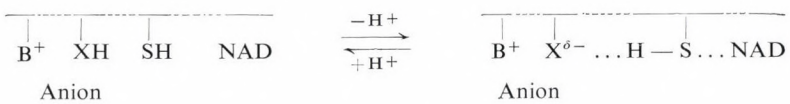
Scheme I

$X^{\delta-}$ designates a group rich in electrons and thus acting as electron donor, but not necessarily carrying a net negative charge, whereas B^+ denotes a nearby located anion-binding group. It has been shown by Polgár (1964) and Harris and Perham (1968) that Lys-183 of the amino acid sequence is sterically near the active centre of the enzyme. The ε-amino group of this residue may act as group B^+.

There is no experimental evidence that the deprotonated $X^{\delta-}$ group would play a direct role in the light absorption of the complex, i.e. it would serve as electron donor in the postulated charge-transfer complex. According to an alternative explanation the XH group in its dissociated form can activate another group, which in this activated state may fulfill the role of an electron donor. In this case the dotted line between the $X^{\delta-}$ and NAD indicates an indirect interaction (cf. scheme II).

It has not been clarified yet which functional group of the enzyme is involved in the charge-transfer interaction with the coenzyme. We have found earlier that the pH-dependence of the light absorption of the GAPD–NAD complex coincided with the apparent acidic dissociation curve of the activated SH-group of the enzyme. The latter was determined in an indirect way, by measuring the pH-dependence of the rate of carboxymethylation (Boross, Cseke, 1967). Our present investigations indicate that this coincidence also holds in the presence of pyrophosphate or chloride ions. If we accept that alkylation with iodoacetate reflects the true acidic dissociation of the activated SH-group, then the assumption readily follows that the active thiol group in the form of a mercaptide ion would act as electron donor in the charge-transfer interaction. This would mean that in scheme I $X^{\delta-} = S^-$.

However, the very low pK values suggest that by means of carboxymethylation we do not measure true acidic dissociation, but rather the pH-dependence of an activation process, an interaction between functional groups in the active centre of the enzyme (scheme II). The SH-group can be activated through H-bridge formation (Rabin et al., 1960, 1962; Polgár, Bender, 1966), as a result of which the sulfur atom will be relatively more rich in electrons. It is an obvious possibility that the thiol group thus activated may serve as electron donor, similarly to mercaptide ions.



Scheme II

The activated state of the thiol group of Cys-149 is proved by the fact that it can be alkylated with both iodoacetate and iodoacetamide faster than the SH-group of cysteine or mercaptoethanol. However, in the presence of bound NAD the alkylation of Cys-149 with iodoacetate is faster than with iodoacetamide, in contrast with aliphatic mercaptanes. In addition, the pH-dependences

of the two alkylations are also different (Figs 2 and 4). At first glance it seems logical to assume that reactivity towards the neutral iodoacetamide and the pH-dependence of the reaction reflects the actual activation of the SH-group, whereas in the case of iodoacetate the binding of carboxylate ion would play the decisive role in both the rate of alkylation and its pH-dependence.

However, this line of reasoning seems to be invalidated by the following consideration. If a weak basic group of the protein were responsible for the binding of carboxylate ion, the reaction with iodoacetate would be faster than with iodoacetamide in acidic solution only, where the weak basic group becomes protonated. This is not supported by our experimental findings. It seems therefore probable that a strong basic group attracts the carboxylate ion near the active SH-group and this effect is constant over the whole pH-range studied. Accordingly, the pH-dependence of carboxymethylation reflects the change in the activation of the Cys-149 thiol group with pH. The role of activated SH-group as electron donor thus appears to be corroborated.

Since the rate of alkylation with iodoacetate is greatly diminished after the removal of bound NAD it is reasonable to assume that the pyridinium ring of the coenzyme is the basic group that is responsible for the binding of carboxylate ion. Considering the mechanism of action of the reaction catalyzed by the enzyme, it seems very probable that the pyridinium ring is in the vicinity of activated SH-group. The possibility cannot be ruled out that the rate of carboxymethylation is a function of the distance between the pyridinium ion and the activated SH-group. However, it is more difficult to account for a minute displacement of the pyridine ring from the activated SH-group by the dissociation of a functional group, than the activation of an SH-group.

The difference between the pH-dependences observed with iodoacetate and iodoacetamide is probably due to the fact that the curve obtained with iodoacetamide (Fig. 4) is related to a structural change in the course of which the SH-group becomes accessible to the reagent rather than to the activation of Cys-149 SH-group proper. We have shown that NAD strongly inhibits the reaction with iodoacetamide. The suppression of this inhibition at higher pH-values may result in the observed phenomenon.

A similar steric hindrance is imposed by the binding of NADH. With iodoacetate the steric hindrance of NAD is presumably masked by the effect of oriented binding of the reagent to the pyridinium ion. In fact, by adding NADH instead of NAD to the apoenzyme the rate of alkylation is markedly reduced, indeed it is smaller than with the apoenzyme.

Our hypothesis does not account for the fact that the removal of NAD alters the pH-dependence of the rate of carboxymethylation (Table 1). NAD cannot play a major part in the activation of the SH-group, since this side chain remains activated even after the removal of NAD. It is known that the removal of bound coenzyme largely changes the structure of GAPD (Előd i, Szabolcsi, 1959). It is tempting to assume that in the neutral and slightly acidic pH-range Cys-149 and the activator functional group are separated from each other as a result of this structural change.

According to our hypothesis described above anions exert a two-fold effect on the rate of carboxymethylation: a) as shown in scheme I, they bind to group B^+ and thereby influence the dissociation of the functional group (XH) that has activated the SH-group and thus the pH-dependence of carboxymethylation, and b) they bind to the strongly basic group (pyridinium ion) that attracts carboxylate ion and thereby decrease the probability of favourably oriented collisions. This second effect is presumably constant over a wide pH-range and depends only on the concentration of anions. In accordance with this, our experiments have shown that an increase in anion concentration not only resulted in an alteration of the pH-dependence of carboxymethylation but also in a decrease of the rate of this reaction.

The authors wish to express their thanks to Professor F. B. Straub for his helpful discussion.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (4), pp. 399–408 (1970)

Structure and Function of Erythrocytes

IV. The Role of Nucleotides and Bivalent Cations in Determining the Shape of Normal and Trypsin Treated Erythrocytes

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(Received April 27, 1970)

The effects on the ATP*-membrane interactions of agents influencing the disk-sphere transformation of erythrocytes were studied. NaF was found to influence the ATP-membrane interactions in two different ways: 1. It induced a prompt detachment of ATP from the membrane, 2. It inhibited membrane ATPase activity. In contrast, trypsin treatment 1. enhanced greatly and permanently the binding of ATP to the membrane, 2. brought about a slight and transitory activation of ATPase. These two effects of trypsin did not manifest themselves in Ca^{2+} or Cd^{2+} pretreated cells. Disk-sphere transformation was influenced by trypsin treatment and NaF in concentrations up to 30 mM in concordance with their effects on membrane ATP binding. A further side effect is to be supposed at high NaF concentrations in the presence of ATP. This involves probably Ca^{2+} detachment from the membrane.

No ATPase activity, but only Ca^{2+}-chelation proved to be necessary for the regeneration of the biconcave shape of ghosts prepared from fresh, ATP depleted erythrocytes. The disadvantageous relation between ATP concentration and morphological index developing in the course of cold storage, however, as well as the superiority of ATP to EGTA in its ability to regenerate the shape of preserved erythrocytes refer to the eventual role of an ATPase in maintaining the "energized" state of the erythrocyte membrane.

Disk-sphere transformation is proposed to be divided into three processes: A) Loss of biconcavity, B) Loss of the smooth contour and C) Decrease in cell diameter. The deficiency of an ATPase activity might play a role in process A, the decreased binding of ATP to the membrane in process B, and the failure of the Ca^{2+} chelating activity of ATP in process C.

Little is known about the biochemical basis of the maintenance of the biconcave shape of erythrocytes. The limited number of data available can be classified into three groups: 1. Differences in composition (in cholesterol distribution, "energization", and fibrillar structure) between the dimple and the rim regions of the erythrocyte membrane (Murphy, 1965; Penniston, 1969; Shrivastav, Burton, 1969). 2. Role of ATP. The results of Nakao et al. (1961) have suggested

* Abbreviations: ADP, adenosine-5'-diphosphate; AMP, adenosine-5'-monophosphate; ATP, adenosine-5'-triphosphate; ATPase, ATP phosphohydrolase, EC 3.6.1.3; 2,3-DPG, 2,3-diphosphoglycerate; EDTA, ethylene diaminetetraacetate; EGTA, ethyleneglycol bis-(β-aminoethylether)-N,N'-tetraacetate; I_m, morphological index; P_i, inorganic phosphate; RBC, red blood cell; TCA, trichloroacetic acid.

that a contractile ATPase system plays a role in the maintenance of the biconcave shape. 3. Role of Ca^{2+}. Ca^{2+} being a determining factor is suggested by the works of Weed et al. (1969). These authors have found that the loss of the biconcave shape, the so-called disk-sphere transformation shows a parallelism with the Ca^{2+} influx following ATP depletion. Ca^{2+} deposition into the membrane seems to induce contraction (Wins, Schoffeniels, 1967; Palek et al., 1969), while the detachment of Ca^{2+} from the membrane results in an opposite effect, causing the expansion of the membrane (Deuticke, Gerlach, 1967; Kwant, Seeman, 1969). The second and third topics involve several problems. Nakao et al. (1960) themselves have found in ghost experiments that ADP restored the biconcave shape just like ATP. This was inconsistent with the ATPase theory. Pribor and Repka (1969) reported similar results, the shape of sphaeroid ghosts could be restored by the addition of ATP even if the ATPase activity was abolished by EDTA. Our own experiments with ATPase inhibitors have also led to contradictory results. While Cu^{2+} or Cd^{2+} caused on the one hand a complete ATPase inhibition and on the other hand a regular disk-sphere transformation in cells containing normal ATP levels, Salyrgan inhibited ATPase, but had no deleterious effect on the shape of the cell. The morphological changes brought about by another ATPase inhibitor, NaF, are also still to be explained: its effect of inducing disk-sphere transformation varied with the NaF concentration according to a maximum curve in ATP rich ghosts (ATP enrichment was necessary due to ATP breakdown caused by the simultaneous inhibition of glycolysis by NaF). The shape transformation was the most definite at 30 mM, at higher concentrations it became gradually insignificant (Szász et al., 1968a). Further problems are raised by the morphological effects of trypsin known to activate erythrocyte membrane ATPase at very small concentrations and inhibit it at higher concentrations (Masiak, Green, 1967). Proteolytic treatment namely protected the cell – within a broad range of trypsin concentration – against the disk-sphere transformation in both cases, when cells were treated with Cu^{2+} or Cd^{2+}, or with glycolytic inhibitors (Szász et al., 1967). This effect also involved the action of ATP, as a prerequisite for its manifestation was that the cells contain ATP at least in traces (Szász et al., 1969). Based on the available data it cannot yet be decided whether it is the enzymatic utilization of the high-energy stored in the ATP molecule, or the potent Ca^{2+} chelating ability of this nucleotide, which plays a role in the maintenance of the biconcave shape. In addition to the task of distinguishing between these two functions the study of a third problem, the effect of binding of ATP to the erythrocyte membrane on the membrane structure seemed necessary. This topic has already been investigated in connection with the regulation of the structure and function of different membranes (Hackenbrock, 1966; Gasik, Stewart, 1968; Karjalainen, 1968; Matsuda et al., 1968; Löwe, Jung, 1969). In our present work the role of 1. the binding of ATP to the membrane, 2. the enzymatic breakdown of ATP and 3. the Ca^{2+} chelating ability of ATP in the maintenance of the biconcave shape of erythrocytes has been studied.

Methods

Intact human erythrocytes from fresh defibrinated blood and from preserved blood stored at +4 °C in acid-citrate-dextrose anticoagulant were used.

Part of the experiments was performed on erythrocyte ghosts enriched with nucleotides (ATP, ADP, AMP) and Ca^{2+}-chelating agents (EDTA, EGTA) by reversible haemolysis. The compounds to be introduced into the erythrocytes were added to the red blood cells in a tenfold volume, in neutralized solutions of approximately 0.3 isosmolarity. Osmolarity was determined by freezing-point depression measurement and the amount of hypertonic saline necessary to restore isotonicity was calculated on the basis of these values. ATP free ghosts were prepared from erythrocytes pretreated with 1 mM iodoacetate and 15 mM Na bisulfite. The latter agent was applied in order to eliminate 2,3-DPG, a potential source of ATP (Mányai, Várady, 1956).

Isolated membranes were prepared according to Parker and Hoffman (1964).

ATPase activity of intact erythrocytes was determined according to the indirect method of Whittam and Ager (1964). The procedure is based on measuring the changes in the inorganic phosphate and lactate levels. Inorganic phosphate was determined by Fiske and SubbaRow's method (1925), lactic acid by the method of Barker and Summerson (1941).

$^{32}P-ATP$ production in erythrocytes was induced according to Yoshikawa et al. (1960) and Ågren and Ronquist (1964). In order to distinguish between the effects of $^{32}P-ATP$ and $^{32}P_i$ erythrocytes were treated in two different ways. 1. 250 μCi $Na_2H^{32}PO_4$ was added to each ml of erythrocytes. The final concentration of inorganic phosphate was adjusted to 3 mM by 0.1 M Na phosphate buffer, pH 7.0, and the system was supplemented by 10 mM inosine and 1 mM adenine. 2. $^{32}P_i$ was applied in the same amount, but, to prevent the synthesis of $^{32}P-ATP$, inosine and adenine were omitted and in addition to the 3 mM inorganic phosphate, 6 mM Na_2HAsO_4 and 8 mM NaF were applied. Both systems were incubated at 37 °C for 120′, then washed 5 times with 10 volumes of physiological saline supplemented by 2.5 mM $MgCl_2$ and 5 mM glucose. In the first system the specific radioactivity of the adenine nucleotides was high (20 000 – 40 000 cpm/μmole), and 90% of the nucleotides was represented by ATP. In the second system only AMP and IMP could be detected from among the nucleotides by spectrophotometry following a separation on Dowex-1-chloride ion exchange column (Deutsch, Nilsson, 1953). The radioactivity of these nucleotides was significantly lower (4 000 – 8 000 cpm/μmole).

The phosphate fractions of the erythrocytes were separated by using the Schneider and Schmidt – Thannhäuser methods as follows: 1. Acid soluble phosphate: 1 ml erythrocyte suspension was deproteinized by 1 ml 20% TCA, and the sediment was washed twice with 8 ml 10% TCA. The combined supernatants contain the acid soluble phosphates. 2. Acid insoluble phosphate: *A*. Lipid. The TCA-washed sediment was extracted 3 times by 8 ml of a 3:1 mixture of alcohol-ether, and twice by 8 ml of ether. *B*. Acid-insoluble-nonlipid (= protein).

Ether was evaporated at 37 °C and the sediment dissolved in 33% KOH. 0.2 ml aliquots were dried on aluminium plates and counted in a Geiger–Müller end window apparatus (Frieseke–Hoepfner, West-Germany).

The binding of ^{14}C–ATP (Radiochemical Centre, Amersham, England) to isolated erythrocyte membranes was checked in two ways: 1. by measuring the radioactivity of the supernatant following centrifugation at 8 000 *g* for 10 minutes and 2. by measuring radioactivity of the supernatant after deproteinization by TCA applied in a final concentration of 10%. The error caused by the "trapped fluid" was corrected according to Garby et al. (1968) by using $Na^{36}Cl$. The simultaneous determination of the two isotopes was carried out by liquid scintillation spectrometry in a Packard Tricarb apparatus.

The morphology of erythrocytes and ghosts was evaluated under a phase-contrast microscope. Six successive forms characteristic of the disk-sphere transformation process (smooth biconcave disk, crenated disk, crenated discoid, crenated sphaeroid, crenated sphere, smooth microsphere) were differentiated and the "morphological index" (I_m) was calculated as described earlier (Gárdos et al., 1966). $I_m = 100$ represents a cell population consisting of only smooth biconcave disks, whereas $I_m = 0$ a cell population consisting of only smooth microspheres. If transitory forms are present the I_m value varies between 100 and 0.

Results

The radioactivity of the protein fraction of erythrocytes, in which ^{32}P–ATP had been synthetized (see system 1) was influenced by the addition of NaF in two ways: 1. It was promptly diminished, parallel with the NaF concentration. 2. The gradual decrease of the radioactivity was slowed down or stopped depending on the NaF concentration (Fig. 1). On the other hand, trypsin treatment enhanced very rapidly and to a great extent the radioactivity of the protein fraction of the

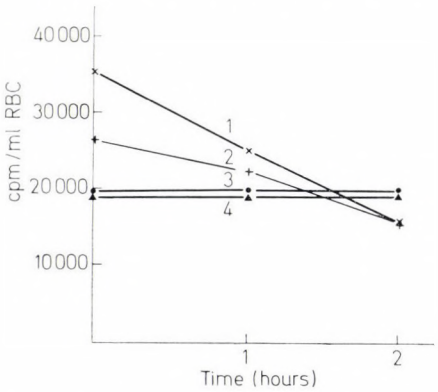

Fig. 1. Effect of different NaF concentrations on the radioactivity of the protein fraction of erythrocytes containing ^{32}P–ATP. NaF concentration: 1, ø; 2, 8 mM; 3, 32 mM; 4, 80 mM

erythrocytes containing $^{32}P-ATP$ (system 1), while in ATP-free, $^{32}P_i$ containing erythrocytes (system 2) no change could be observed (Fig. 2). In erythrocytes that had been pretreated with Cd^{2+} or Cu^{2+} during the period of $^{32}P-ATP$ synthesis, the decrease of the ^{32}P-label of the protein fraction—found in the control cells, in which $^{32}P-ATP$ had been synthetized in the absence of these heavy

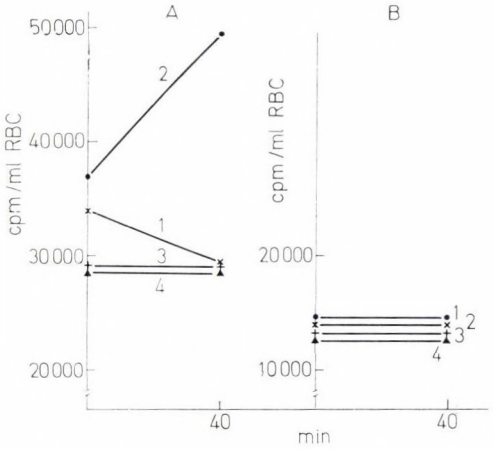

Fig. 2. Effect of trypsin on the radioactivity of the protein fraction of erythrocytes preincubated for 120 min in a Cd^{2+}-free medium or in the presence of 1.5 mM Cd^{2+}. A. Cells containing ^{32}P–ATP. B. Cells containing $^{32}P_i$. 1, Control RBC; 2, 0.1 mg trypsin/ml control RBC; 3, Cd^{2+} pretreated RBC; 4, 0.1 mg trypsin/ml Cd^{2+} pretreated RBC

metals—ceased and the trypsin treatment did not increase the radioactivity of the protein fraction of these erythrocytes (Fig. 2).

The prompt effect of NaF and the rapid and extensive effects of trypsin in the erythrocytes containing $^{32}P-ATP$ refer to changes of the binding of ATP to the membrane rather than to alterations in ATPase activity. The activation of ATPase by trypsin in intact cells was found to be only small and transitory (115–120% for 15–20 minutes), while the increased label incorporated into the protein fraction surpassed 200% in every case (sometimes even reaching 400%) and remained constant quite similarly to the previously found shifts in morphology (Szász et al., 1967, 1969). The parallelism of the two latter phenomena supports the view that an enhanced binding of membrane-ATP is closely connected with the morphological effect of trypsin.

The question, whether the nucleotide molecule became bound as a whole or only its terminal phosphate group was cleft and incorporated in the membrane, however, cannot be decided by using $^{32}P-ATP$. Therefore the question was studied by using $^{14}C-ATP$, too. The influence of trypsin and NaF, respectively, on the binding of $^{14}C-ATP$ to the isolated erythrocyte membranes was examined. Very small concentrations of trypsin increased the binding of $^{14}C-ATP$ to the membrane in all cases, while higher concentrations of trypsin only when the

membrane was protected by unlabelled ATP. On the other hand, ATP binding was diminished by NaF (Fig. 3).

To distinguish between the possible enzymatic and non-enzymatic ATP-requiring processes ghost experiments were carried out. In such experiments, by using reversible haemolysis ATP in itself can be introduced in the inside of

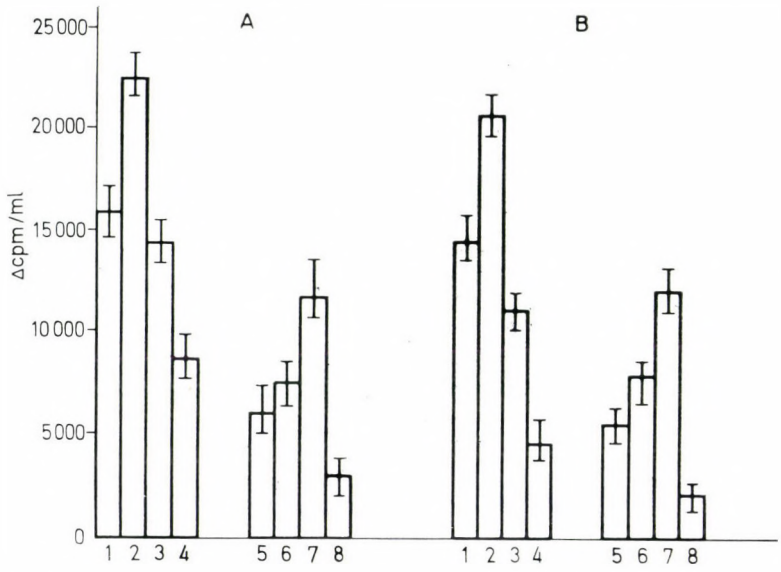

Fig. 3. Binding of ^{14}C-ATP to erythrocyte membrane (n = 3) A. Without deproteinization. B. Deproteinization by 10% TCA. 1–4, 0.05 μCi ^{14}C-ATP/ml; 5–8, 0.05 μCi ^{14}C-ATP/ml + + 0.3 μmole ATP/ml; 1 and 5, control; 2 and 6, 0.005 mg trypsin/ml; 3 and 7, 0.05 mg trypsin/ml; 4 and 8, 8 mM NaF; protein concentration: 1–8 = 2.5 mg/ml

the erythrocyte ghosts as well as together with the potent Mg^{2+} chelator EDTA. In the latter case, due to the chelation of the activator Mg^{2+} ion, ATPase activity is perfectly abolished. Thus only non-enzymatic reactions take place, while in the former case all kinds of processes involving ATP are undisturbed. At first the morphology of reconstituted ghosts prepared of fresh, artificially ATP-depleted cells was studied. Unexpectedly NaEDTA, applied in itself as a control, improved morphology significantly even without any supplementation. Whereas 1 mM $MgCl_2$ in itself proved to be favourable for some other Ca^{2+} dependent functions (Weed et al., 1969) it did not prove to exert any advantageous effect on the morphology of ghosts unless ATP was also present (Fig. 4). EGTA was as effective as EDTA or ATP.

The ghosts prepared from preserved erythrocytes behaved similarly to the ghosts prepared from fresh cells for about three weeks of storage. Thereafter some differences developed: 1. The diameter of the reconstituted ghosts decreased. 2. A microsphaerocyte population appeared, which could not be regenerated

in the afore-mentioned ways. 3. After a 3–6-week-storage the smooth biconcave disk shape could be restored in the majority of cells by both ATP and EGTA, but the advantageous morphological effects of EGTA proved to be transitory within hours. 4. After a 6–8-week-storage even ATP proved to be insufficient for a perfect morphological regeneration (cf. Szász et al., 1968b).

All these differences indicate that the microsphaerocytes developed during blood storage, are differently related to the energy metabolism of the cell than the

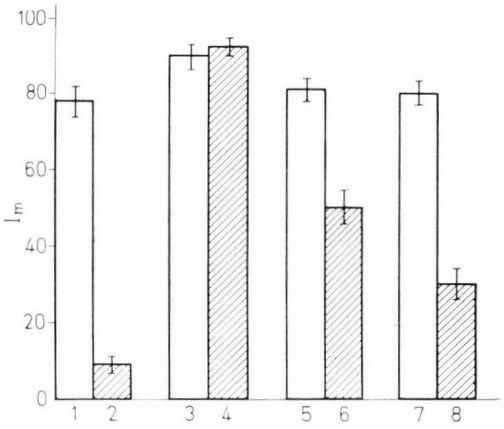

Fig. 4. Effect of adenine nucleotides and EDTA on the morphology of ghosts prepared from microspherocytes. Final concentrations of the applied reagents in the ghosts: 1, 1 mM NaEDTA; 2, 1 mM $MgCl_2$; 3, 1 mM NaATP + 1 mM NaEDTA; 4, 1 mM MgATP; 5, 1 mM NaADP + 1 mM NaEDTA; 6, 1 mM MgADP; 7, 1 mM NaAMP + 1 mM NaEDTA; 8, 1 mM MgAMP

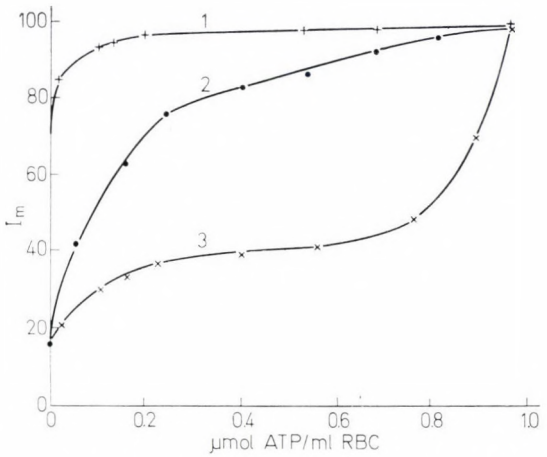

Fig. 5. Relation of morphological index to ATP concentration under different conditions. 1, Defibrinated blood incubated at 37 °C in the presence of 1 mM iodoacetate; 2, Defibrinated blood incubated at 37 °C in the presence of 16 mM NaF; 3, ACD-blood stored at +4 °C

microsphaerocytes produced by artificial ATP depletion. For the sake of comparison the morphological index was plotted against the actual ATP concentration during ATP depletion at 37 °C and cold storage, respectively (Fig. 5). Obviously the shape of the cells may be very different at the same ATP level. Even in short-term experiments at 37 °C striking differences can be observed if 16 mM NaF is used as glycolysis inhibitor instead of 1 mM iodoacetate. This shift in the relation of morphological index to ATP concentration proved to be even more conspicuous in the case of erythrocytes stored at +4 °C.

Discussion

We suggest that there are three different steps in the disk-sphere transformation process: 1. Loss of biconcavity, 2. loss of smooth contour, and 3. decrease of cell diameter (contraction). ad 1. In our own experiments a relation between the "energization" and the maintenance of biconcavity is indicated only by the results obtained with preserved erythrocytes. If an unequal "energization" of the membrane plays a role in the maintenance of the biconcavity at all, the "deenergization" on ATP breakdown must be a slow process. This has been proved by our finding showing that the biconcavity of the ATP depleted fresh erythrocytes can be perfectly regenerated by Ca^{2+} chelating agents (EGTA, EDTA) alone, or by ATP under circumstances when its enzymatic breakdown is prevented. Deenergization, however, might develop in the course of cold storage and may be responsible in part for the disadvantageous relation of the morphological index to the ATP concentration found under these circumstances. ad 2. The majority of the morphological changes induced by trypsin or NaF are likely to affect the maintenance of the smooth contour. Our results suggest that there is a connection between this morphological change and the disturbance of the normal ratio of the membrane-bound ATP and Ca^{2+}. Trypsinization favours the binding of ATP, while NaF promotes the detachment of ATP from the membrane. This latter effect may be due to the formation of an ATP-alkaline earth metal–fluoride complex. At lower NaF concentrations this could result in a detachment of the membrane-bound ATP, the nucleotide being involved in a complex including NaF and intracellular Mg^{2+}. At higher NaF concentrations in addition to the detachment of ATP, even Ca^{2+} could be mobilized from the membrane and bound in a complex form. The decrease of the membrane-bound Ca^{2+} in turn counteracts the contractile tendencies of the membrane. ad 3. Contraction may be induced by bivalent cations (Ca^{2+}, Cu^{2+}, Cd^{2+}). The process includes the "mechanical" overcoming of the resistance of the biconcavity, if the "energized" state has been spared. The process of contraction caused by Ca^{2+} develops in ATP depleted cells, that caused by Cu^{2+} and Cd^{2+} in cells containing normal ATP levels, but being in the state of perfect ATPase inhibition. When the Ca^{2+} dependent morphological changes were induced by 32 mM NaF ATPase activity was perfectly inhibited, too. These facts exclude the contraction in point being a function of a contractile ATPase. The mentioned bivalent cations when bound

to the membrane would rather induce a conformational change of the membrane proteins. A rather well characterized structural protein of the erythrocyte membrane, spectrin, is similar in a number of characteristics to the muscle protein, actin (Steers, Marchesi, 1969; Marchesi et al., 1969; Tillack et al., 1970). The peculiarities of the cation binding of G-actin has been thoroughly studied, and it has been established that the binding of bivalent cations was highly dependent on their ionic radius. Thus Cd^{2+} behaved very similarly to Ca^{2+}, Cu^{2+} differed from the former ions by its binding being irreversible (Strzelecka-Golaszewska, Drabikowski, 1968). The same results were obtained by us with erythrocyte ghosts: the morphological effects of Ca^{2+} could be reversed by Ca^{2+} chelators, those of Cd^{2+} by SH-compounds (e.g. cysteamine) while the effect of Cu^{2+} proved to be irreversible (Szász, unpublished results).

Our observation that trypsin hinders the contraction induced by Ca^{2+} only in the presence of trace amounts of ATP indicates that trypsin protects the membrane against the development of abnormal Ca^{2+} ligands by enhancing the binding of ATP to the membrane. As Cu^{2+} and Cd^{2+} get bound to the membrane even in the presence of ATP, the protecting effect of trypsin treatment in this case is to be sought probably in a modification of the membrane structure, involving the decrease of the number or affinity of heavy metal binding sites. When Cu^{2+} or Cd^{2+} were already bound to the membrane, trypsin treatment had no advantageous effect any more, neither on morphology, nor on ATP-binding.

Thanks are due to Dr G. Gárdos for his continuous support in accomplishing this work and Mrs Eva Irmai and Miss Anna Thaly for their skilful and precise technical assistance.

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Structure and Function of Erythrocytes

V. Differences in the Ca^{2+}-Dependence of the ATP Requiring Functions of Erythrocytes

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(Received April 27, 1970)

The Ca^{2+}-dependence of three processes induced by the breakdown of ATP was studied. The collapse of the biconcave shape, the so-called disk-sphere transformation was brought about mainly by the rearrangement of the intrinsic Ca^{2+}. The process could not be prevented by externally applied EGTA. Rapid K^+-outflow occurred only when Ca^{2+} influx took place and it could be suspended whenever the Ca^{2+} taken up was eliminated again from the membrane by supplementing the medium with EGTA. Plasticity changes developed in both cases, the alterations caused by the rearrangement of intrinsic Ca^{2+} were highly accelerated by Ca^{2+} influx. If non-physiological Ca^{2+} ligands were present in the cell, non-covalent (urea sensitive) bonds also developed and brought about a significant decrease of plasticity.

The Ca^{2+}-dependence of the rapid K^+ outflow from ATP depleted erythrocytes was reported by Gárdos (1958) and confirmed by Lepke and Passow (1960). A possible connection between the collapse of the biconvave shape and the deterioration of plasticity on the one hand and Ca^{2+} influx on the other was suggested by Weed (Weed, 1967; Weed et al., 1969). In order to get more information concerning these mechanisms and to establish the site of action of Ca^{2+}, the effect of the rearrangement of intrinsic Ca^{2+} and of the uptake of extrinsic Ca^{2+} in the course of ATP depletion on these processes has been studied.

Methods

Fresh, defibrinated human blood was used. Erythrocytes were washed three times in physiological saline supplemented with 5 mM glucose, 2.5 mM $MgCl_2$ and 2.5 mM $CaCl_2$. The hematocrit value of the incubation mixture was around 30%.

16 mM NaF or 1 mM iodoacetate + 10 mM adenosine were used for inducing rapid ATP breakdown at 37 °C.

The morphology of erythrocytes was investigated under a phase-contrast microscope. The six forms characteristic of the disk-sphere transformation process (smooth disk, crenated disk, crenated discoid, crenated sphaeroid, crenated

sphere, smooth sphere) were differentiated and the morphological index (I_m) was calculated as described earlier (Gárdos et al., 1966). $I_m = 100$ represents a population consisting of smooth biconcave disks, $I_m = 0$ a population of smooth spheres.

Potassium was determined by flame photometry (EEL Model A, England).

Plasticity of the erythrocyte sediments was examined by the filterability test (Teitel, 1967). Plasticity index (pT) was calculated by the method of the least squares. Its normal range lies between 0.6 – 1.0, if cells lose their filterability pT is infinite.

Results

The deterioration of the biconcave shape, the so-called disk-sphere transformation on rapid ATP breakdown brought about by the action of NaF or iodoacetate + adenosine, could be reduced only to a slight extent by applying the impermeable Ca^{2+} chelating agent EGTA (ethyleneglycol bis-(β-aminoethylether)-N,N'-tetraacetate). Likewise disk-sphere transformation was hardly influenced by an increase in the external free Ca^{2+} concentration (Fig. 1). In ATP

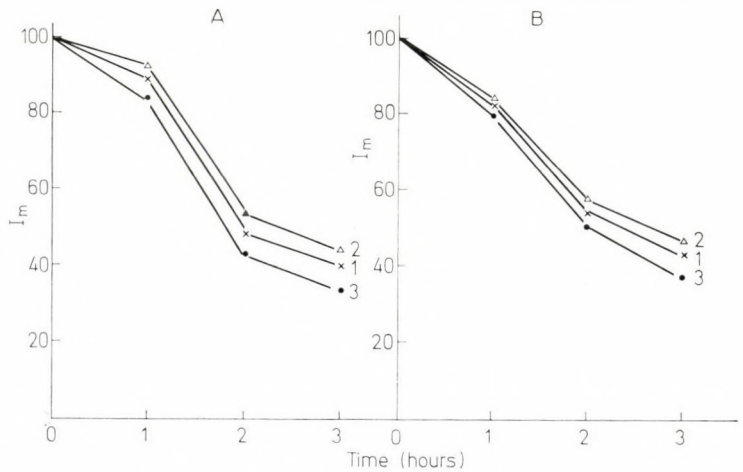

Fig. 1. Effect of EGTA and Ca^{2+} on ATP-depletion induced disk-sphere transformation of human erythrocytes. A. 1: 16 mM NaF; 2: 16 mM NaF + 2.5 mM EGTA; 3: 16 mM NaF + 2.5 mM $CaCl_2$. B. 1: 1 mM iodoacetate + 10 mM adenosine; 2: 1 mM iodoacetate + + 10 mM adenosine + 2.5 mM EGTA; 3: 1 mM iodoacetate + 10 mM adenosine + 2.5 mM $CaCl_2$. Incubation was carried out at 37 °C. Morphological index (I_m) was determined as described earlier (Gárdos et al., 1966)

depleted reconstituted ghosts Mg^{2+} at a concentration of 1 – 10 mM did not protect the cell from the effect of Ca^{2+}, in contrast, at higher concentrations Mg^{2+} itself promoted the development of the sphaeroid shape.

The results were quite different in the case of rapid K^+-outflow. An increase in the external Ca^{2+} concentration enhanced significantly, EGTA completely

abolished the process (Fig. 2). The rapid K^+-outflow could be suspended at any time by adding EGTA to the system. By increasing the external Mg^{2+} concentration the process of K^+-outflow could not be abolished.

In the behaviour of cell plasticity an early and a later stage could be distinguished following the breakdown of ATP. While in the early stage EGTA prevented the decay of plasticity, after 24 hours the plasticity of EGTA-containing

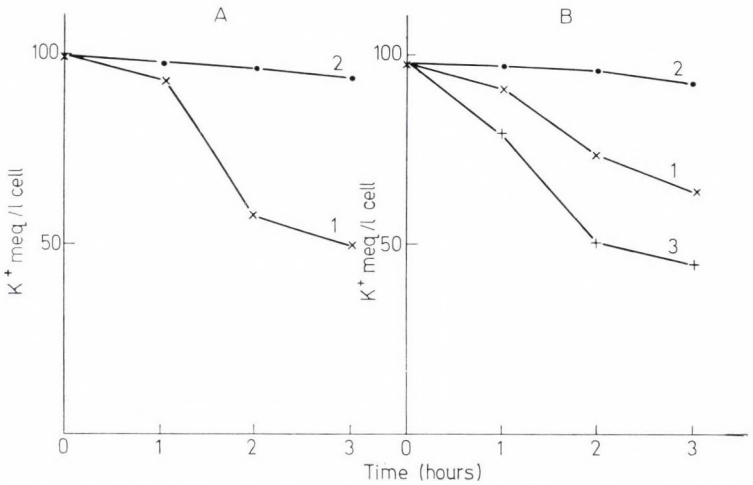

Fig. 2. Effect of EGTA and Ca^{2+} on rapid K^+-outflow, induced by ATP depletion, from human erythrocytes at 37 °C. A. 1: 16 mM NaF; 2: 16 mM NaF + 2.5 mM EGTA. B. 1: 1 mM iodoacetate + 10 mM adenosine; 2: 1 mM iodoacetate + 10 mM adenosine + 2.5 mM EGTA; 3: 1 mM iodoacetate + 10 mM adenosine + 2.5 mM $CaCl_2$. Note: Results of the NaF-treated systems supplemented by Ca^{2+} are not presented due to the disturbing effect of concomitant haemolysis

systems was totally lost quite similarly to that of EGTA-free systems. In the EGTA-protected systems, however, the plasticity after 24 hours could be regenerated by urea. This regeneration was nearly complete in NaF-containing systems. In iodoacetate-containing systems urea was also effective in improving plasticity after a 24 hour treatment with the glycolytic inhibitor. However, only that level of plasticity can be approached this way, which is characteristic of the fresh cells treated with the SH-reagent in the presence of urea, an agent known to increase the number of the reactive SH-groups in the cell (Teitel et al., 1968). In systems containing either NaF or iodoacetate the regeneration of plasticity by urea was only partial in the absence of EGTA (Fig. 3). The following results are remarkable concerning the connection between the different processes: 1. In the early stage plasticity changes precede permeability changes in the EGTA-free systems. 2. By regenerating the intracellular ATP, all three non-physiological processes can be suspended and, the physiological equilibrium of the cell is restored.

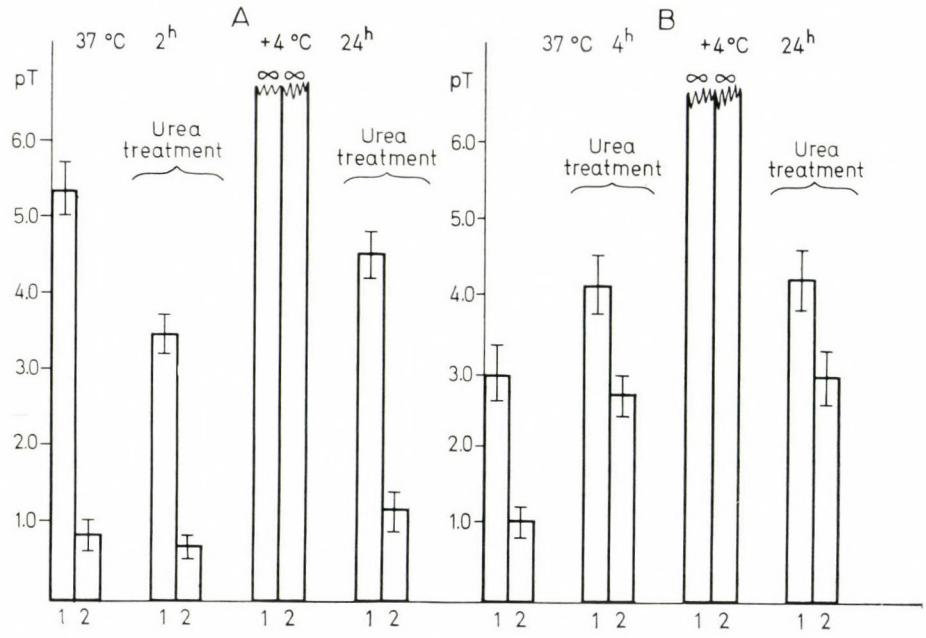

Fig. 3. Effect of EGTA and urea on the plasticity changes induced by ATP depletion (n=4). A. 1: 16 mM NaF; 2: 16 mM NaF + 2.5 mM EGTA; B. 1: 1 mM iodoacetate + 10 mM adenosine; 2: 1 mM iodoacetate + 10 mM adenosine + 2.5 mM EGTA; Urea treatment: 0.1 g urea/ml RBC. Plasticity index (pT) was calculated as described by Teitel (1967)

Discussion

From the experiments the conclusion can be drawn that the three processes are based upon different mechanisms. A rearrangement of the intrinsic Ca^{2+} is to be supposed on the breakdown of the potent Ca^{2+} chelator ATP (Haradin et al., 1969). The biconcave shape proved to be most sensitive to the rearrangement of the intrinsic Ca^{2+}, so that trace amounts of intracellular Ca^{2+} are sufficient to induce the process. The site of action of Ca^{2+} inducing morphological changes is not accessible to the externally applied EGTA, so it can possibly be localized on the inner surface of the membrane.

The rapid K^{+}-outflow represents the other extreme: it does not develop on the rearrangement of the intrinsic Ca^{2+}, the influx of extrinsic Ca^{2+} is a prerequisite. The site of action of Ca^{2+} is located superficially in the membrane (probably in the wall of the pores), as it is accessible to the externally applied EGTA.

In the most complex plasticity changes both reaction types are involved. During longer times even the rearrangement of the intrinsic Ca^{2+} destroys plasticity, but the process is significantly accelerated if Ca^{2+} influx is possible. Plasticity reacts to Ca^{2+} influx even more sensitively than the passive K^{+} perme-

ability. The reactions of loci in the inner surface of the membrane seem to be responsible for the loss of plasticity, but for the rapid or complete evolvement of the process more Ca^{2+} is needed than for the disk-sphere transformation. The effectivity of urea in regenerating plasticity after 24 hours in the EGTA-protected systems indicates that the deposition of intrinsic Ca^{2+} represents only a condition which promotes the development of non-covalent (urea-sensitive) bonds. Most probably these conditions are responsible for the perfect loss of plasticity. Weed et al. (1969) described that the Ca^{2+}-induced plasticity changes could be antagonized by Mg^{2+}. No similar phenomenon was observed in the case of disk-sphere transformation and Ca^{2+}-dependent rapid K^{+}-outflow.

For the skilful technical assistance thanks are due to Mrs Eva Irmai and Miss Anna Thaly.

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Base Composition of Cytoplasmic Ribonucleic Acids Isolated from the Organs of Normal and Brown-Pearce Tumour Bearing Rabbits*

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(Received June 3, 1970)

The base composition of cytoplasmic RNA*** extracted by means of guanidine hydrochloride from the liver and kidneys of normal rabbits was compared to that of similar nucleic acid preparations obtained from Brown–Pearce tumour bearing rabbits. The RNA was hydrolyzed in alkali and the resulting mononucleotides separated by paper electrophoresis. No clear-cut difference in base composition was found between the RNA obtained from the livers of control and tumorous animals, respectively. In the case of the RNA isolated from the kidneys, however, the adenine and uracil content was somewhat lower and the cytosine and guanine content slightly higher in the preparations obtained from tumorous tissues than in those obtained from the control. The results seem to indicate that the difference in the behaviour of normal and tumorous tissues towards guanidine hydrochloride observed in our earlier experiments cannot be explained by any significant difference in the base composition of their RNA.

Introduction

It has been found earlier that when RNA is isolated from rabbit liver cytoplasmic extract by the method of Grinnan and Mosher (1951) using GuHCl the success of preparation is greatly influenced by the appropriate choice of the concentration of the reagent (Zsindely et al., 1959). Subsequent experiments have shown that with livers of Brown–Pearce tumour bearing animals a lower GuHCl concentration is sufficient to keep the proteins in solution than in the case of the control. The same phenomenon was later observed with kidneys as well (Zsindely et al., 1961; Tankó, 1962). In view of the latest results concerning ribosome structure (Cox, 1969) and the interaction between RNA and protein within the ribosome (Cox and Bonanou, 1969) it has been suggested that in tumorous liver and kidney tissues the strength of the bond between ribosomal protein and RNA differs from that in the ribosomes of healthy tissues (Zsindely and Tankó, 1970).

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

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*** *Abbreviations:* RNA, ribonucleic acid; tRNA, transfer ribonucleic acid; GuHCl, guanidine hydrochloride; Pu, purine; Py, pyrimidine; Cp, cytidine-2'(3')-monophosphate; Ap, adenosine-2'(3')-monophosphate; Gp, guanosine-2'(3')-monophosphate; Up, uridine-2'(3')-monophosphate; C, A, G and U are the corresponding bases.

Consequently, a comparison of the properties of RNA prepared under identical conditions from the livers and kidneys of tumorous and healthy animals, respectively, seemed to be warranted. With respect to the UV absorption spectra and to the hyperchromicity (increase of ε(P)) caused by alkaline hydrolysis or ribonuclease digestion of the RNA, no significant difference was found between the preparations from normal and tumorous animals, respectively (Zsindely, 1962). The base composition of the RNA preparations was also determined, since this may reflect differences in the primary structure, and primary structure is an important factor in the maintenance of the steric configuration of nucleic acids and their binding to proteins. These experiments were further justified by the findings of Kuzin and Davidova (1954). They have observed that the thymine content of DNA was higher in organs of Brown–Pearce tumour bearing rabbits than in the control. This observation, however, could not be confirmed in our Institute by Rosdy and Tankó (1958).

Materials and Methods

RNA was prepared from the cytoplasmic extracts of liver and kidneys by using 3 M GuHCl as described earlier (Zsindely et al., 1959). Usually male rabbits of identical age (7 to 10 months) and origin were used, in some experiments, however, the animals were not so uniform. One animal was inoculated with Brown–Pearce tumour and the other one served as a control. The transplantation of Brown–Pearce tumour was performed by the method described in our earlier communication (Zsindely and Tankó, 1970).

Alkaline hydrolysis: 5 mg of the RNA preparation was dissolved in 0.3 N KOH and incubated at 37 °C for 20 hours (Marian et al., 1951). After cooling to 0 °C the pH of the hydrolyzate was adjusted to 3.5 with N $HClO_4$. After removal of the precipitated $KClO_4$ crystals 25 μl aliquots of the hydrolyzate (corresponding to 400 μg RNA) were applied to Whatman–1 filter paper strips and subjected to electrophoresis. The hydrolyzate was always used fresh after preparation, since on standing at 0 °C guanylic acid tends to precipitate.

For paper electrophoresis a moist chamber apparatus built in our Institute was used. The samples were run in 0.02 M citrate buffer at pH 3.5 (Davidson, Smellie, 1952) at 10–11 V/cm and 0.2–0.25 mA/cm for 7 hours. The hydrolyzate of each preparation was run in three parallels. Each time unloaded filter paper strips were also run as blank for quantitative evaluation. The apparatus and the methods are described in detail in a review by Tankó (1959b).

For quantitative evaluation the components were located under UV light according to the method of Holiday and Johnson (1949), eluted with 10 ml of 0.1 N $HClO_4$ and their UV absorption measured in the Beckman DU spectrophotometer at wavelengths corresponding to their absorption maxima (279 mμ for Cp, 257 mμ for Ap and Gp, and 262 mμ for Up) in silica cells of 1 cm light path. The results are expressed in mole per cent values (Tables 1 and 2). The

recovery of the nucleotides after electrophoresis was 95–98% with respect to the phosphorus content measured according to the method described in our earlier communication (Tankó et al., 1967).

Table 1

Base composition of RNA isolated by the GuHCl method from cytoplasmic extract of liver

The RNA was hydrolyzed with 0.3 N KOH at 37 °C and the products separated by paper electrophoresis at pH 3.5. After elution of the components with 10 ml of 0.1 N perchloric acid the amounts of the individual nucleotides were determined spectrophotometrically by using the following molar extinction coefficients at the given wavelengths: Cp, ε_{279}: 13 000 $M^{-1}\,cm^{-1}$; Gp, ε_{257}: 12 200 $M^{-1}\,cm^{-1}$; Ap, ε_{257}: 14 400 $M^{-1}\,cm^{-1}$; Up, ε_{262}: 9 900 $M^{-1}\,cm^{-1}$

Preparation	GuHCl concentration M	Number of experimental animals	Cytosine mole %	Guanine mole %	Adenine mole %	Uracil mole %	$\frac{A+U}{G+C}$	$\frac{Pu}{Py}$
Control tissue*	3	6	29.0±0.2	34.4±0.5	18.5±0.3	18.1±0.3	0.58	1.12
Tumorous tissue* (Brown-Pearce tumour)	3	6	29.2±0.1	34.6±0.3	18.2±0.2	18.0±0.2	0.57	1.12
Control tissue**	3	3	28.7±0.2	34.4±0.5	18.4±0.4	18.5±0.4	0.58	1.12
Tumorous tissue** (Brown-Pearce tumour)	3	3	28.9±0.4	34.7±0.2	17.9±0.5	18.5±0.2	0.57	1.11
Control tissue***	2	1	29.0	34.5	17.9	18.6	0.58	1.10
	3		29.3	34.2	18.0	18.5	0.58	1.09
	4		28.9	34.5	18.0	18.6	0.58	1.10
Tumorous tissue*** (Brown-Pearce tumour)	2	1	28.9	34.6	17.8	18.7	0.58	1.10
	3		29.0	34.5	17.9	18.5	0.57	1.10
	4		28.9	34.6	18.0	18.5	0.58	1.11
Volkin and Carter****	2		29.5	35.2	17.9	17.4	0.55	1.13
Davidson and Smellie****	---		28.2	32.6	19.3	19.9	0.65	1.08

* The livers of two rabbits of the same age and origin were used in parallel experiments.

** The experimental animals were not of the same age and origin and their organs were not used in parallel experiments.

*** Portions of the cytoplasmic extract of the liver of the same animal were used in parallel experiments with the given GuHCl concentration. The data are the means of parallel runs.

**** Converted literary data for the sake of comparison.

Results

Several methods are known by which RNA can be hydrolyzed and the products separated (cf. Tankó, 1959a; Zsindely, 1969b). We have applied alkaline hydrolysis and separated the resulting mononucleotides by paper electrophoresis. To check the reliability of the method some RNA preparations were hydrolyzed also with 70% perchloric acid (Marshak, Vogel, 1950) and the bases separated by paper chromatography (Wyatt, 1951). The results obtained by the two methods were in a satisfactory agreement.

In the majority of the experiments animals of the same age and origin were used under identical conditions. In some experiments these aspects were disregarded. As shown in Table 1 there was no significant and consistent difference in the base composition of the RNA preparations obtained from the cytoplasmic extracts of the livers of Brown–Pearce tumorous and normal animals, respectively. In certain experiments instead of 3 M GuHCl, 2 M (Volkin, Carter, 1951) or 4 M (Grinnan, Mosher, 1951) GuHCl was used. The base composition of the RNA preparations did not depend on the concentration of GuHCl used in nucleic acid extraction.

The base composition of RNA preparations obtained from kidneys by using 3 M GuHCl is shown in Table 2. Although no considerable differences were found here either, it is nevertheless remarkable that in the tumorous organs the adenine content was about 1 mole per cent lower and the guanine content was about 1 mole per cent higher than in the control. The difference in the amount of the pyrimidines was even lower: the uracil content was 0.6 mole per cent lower, while the cytosine content was 0.4 mole per cent higher in the RNA of tumorous organs than in the control. The cytoplasmic RNA extracted from the kidneys

Table 2

Base composition of RNA isolated by the GuHCl method from cytoplasmic extracts of kidney

The RNA was hydrolyzed with 0.3 N KOH at 37 °C and the products separated by paper electrophoresis at pH 3.5. The components were eluted and their amounts determined as described in the legend to Table 1.

Preparation	GuHCl concentration M	Number of experimental animals	Cytosine mole %	Guanine mole %	Adenine mole %	Uracil mole %	$\frac{A+U}{G+C}$	$\frac{Pu}{Py}$
Control tissue*	3	4	28.8±0.5	34.1±0.5	18.9±0.3	18.2±0.2	0.59	1.15
Tumorous tissue (Brown-Pearce tumour)	3	4	29.2±0.2	35.2±0.4	18.0±0.4	17.6±0.2	0.55	1.14

* The kidneys of two rabbits of the same age and origin were used in parallel experiments.

of animals bearing Brown—Pearce tumours seems to have a higher G—C content and a lower A—U content than the RNA of the kidneys of normal animals. Correspondingly, the A+U/G+C ratio is somewhat lower in the first group than in the second, while the Pu/Py ratio is the same for both. A comparison of the base compositions of the ribonucleic acids prepared from the cytoplasm of kidney and liver tissues reveals almost identical average values for the various bases; a difference higher than 0.5 mole per cent was observed only for the guanine content of the RNA prepared from the kidneys and livers of tumour bearing animals.

Discussion

Although as a result of the pioneering work of Holley et al. (1965) the primary structure of several tRNAs is known, and recently the sequence of some ribosomal 5 S RNAs has also been determined (Brownlee et al., 1968; Forget, Weissman, 1969), base analysis has not lost of its importance. It is primarily used for the characterization of various nuclear RNAs (Busch et al., 1968; Moriyama et al., 1969), because no satisfactory separation of these RNA fractions is yet possible, and for sequence analyses homogeneous fractions are needed.

Methods for separating the two main types of ribosomal RNAs have already been worked out. However, the determination of their primary structure is, mainly because of their large molecular size, still based on end group determination (Halloin et al., 1969; Fellner, Ebel, 1970), and on the analysis of the digestion products arising after ribonuclease action (Fellner et al., 1970; Beck et al., 1970). Our preparations obtained from cytoplasmic extracts most probably consisted mainly of ribosomal RNAs, since we have observed that with 3 M GuHCl no tRNA was precipitated. Accordingly, our preparations gave, in agreement with the findings of Grinnan and Mosher (1951), a single sedimentation peak in the ultracentrifuge. The peak was, however, broad (Szabolcs, Zsindely, 1963). In the polyacrylamide gels the nucleic acid migrated as a single, though somewhat diffuse band (Zsindely, Hutai, 1969; unpublished data). Therefore no attempt was made to carry out sequence analyses with our preparations. We had to content ourselves with the determination and comparison of base compositions.

The base composition of our RNA preparations obtained from liver is in a fairly good agreement with that reported by Volkin and Carter (1951) for RNA prepared with 2 M GuHCl but for a somewhat lower G—C and a slightly higher A—U content of our preparations. Davidson and Smellie (1952) extracted RNA from the dry powder of liver homogenizates with 10% NaCl and found lower cytosine and guanine and somewhat higher adenine and uracil values than we did. It should be noted that in the electrophoretograms in addition to the four clearly identified spots no other spots were observed in the hydrolyzates of either the liver or the kidney RNAs indicating that only negligible amounts of alkali resistant bonds were present in our preparations, if any (Lane, 1965; Tamakoi, Lane, 1967). No attempt was made to detect minor components (see

the review by Zsindely, 1969a). In earlier experiments a fairly high proportion of pseudo-uridylic acid was detected in the RNA prepared from liver (Zsindely, Berencsi, 1966).

No data were found in the literature on the base composition of RNAs prepared from the organs of Brown–Pearce tumour bearing rabbits. We were unable to establish clear-cut differences in the composition of RNA preparations obtained from the livers of tumorous and control animals, respectively. No difference could be observed either when instead of 3 M GuHCl 2 M or 4 M GuHCl was used during the isolation of RNA. On the other hand, in the RNA preparations isolated from the kidneys of the tumour bearing animals the guanine and cytosine content was somewhat higher and the adenine and uracil content somewhat lower, correspondingly the value of the A+U/G+C quotient was somewhat lower than in the controls. The differences exceeded only occasionally 1 mole per cent but were reproducible. The question arises whether such small differences are real. It should be noted that even very slight differences in the base composition of nucleic acids may reflect significant differences in primary structure. This question can be settled only by the preparation of homogeneous fractions.

The results reported in the present paper show that there is no significant difference in base composition between the RNA isolated from organs of Brown–Pearce tumour bearing animals and that obtained from organs of healthy animals. Kuzin and Davidova (1954) have determined the adenine to guanine+xanthine ratio in the RNA fraction of the organs of Brown–Pearce tumorous rabbits, but they were also unable to detect any unambiguous difference in the above ratio between the RNA isolated from tumorous animals and that extracted from the control. Thus, our earlier finding that ribonucleoproteins from normal and Brown–Pearce tumour-bearing animals differ in their behaviour towards GuHCl (Zsindely, Tankó, 1970) cannot be explained by any significant differences in their base composition. In other words the supposed looser bonds between ribosomal proteins and ribosomal RNA found in tumorous tissues cannot be attributed to any significant difference in base composition.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (4), pp. 423–434 (1970)

The Use of Diethyl Pyrocarbonate as a Nuclease Inhibitor in the Extraction of Ribonucleic Acids from Animal Tissues*

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(Received July 17, 1970)

Diethyl pyrocarbonate (DEP)** was used as a nuclease inhibitor in combination with guanidine hydrochloride (GuHCl) or phenol for the extraction of intact ribonucleic acids from rabbit livers and kidneys. Neither protein nor DNA was found in the preparations obtained from the cytoplasm, though the RNA preparations obtained with the phenol method were contaminated with glycogen. The nucleic acids extracted from tissue homogenates by the use of phenol contained also DNA in addition to RNA. The use of DEP had no significant influence on the UV absorption and other characteristics of the nucleic acid preparations. Polyacrylamide gel electrophoresis of the preparations revealed in addition to well-known RNA species several minor components. The RNA isolated by means of GuHCl contained no tRNA. The predominance of heavy rRNA was most marked when GuHCl and DEP were used together. The polyacrylamide gel electrophoretic patterns of nucleic acids prepared without the use of DEP appeared somewhat diffuse. The components moving behind the heavy rRNA, which had also been observed in cytoplasmic RNA preparations, were most clearly visible with preparations isolated directly from tissue homogenates.

Introduction

Of the many methods known for the isolation of RNA from animal tissues methods using phenol have been most widely used (Westphal et al., 1952; Kirby, 1956), though following the work of Cox and Arnstein (1963) the application of GuHCl has again found wide acceptance. Despite many favourable properties both reagents have the common drawback of not inhibiting completely nucleases in the course of preparation. To make up for this deficiency of both phenol and GuHCl the use of several additional reagents has been suggested (see review by Zsindely, 1969). Although these reagents are known to inhibit nuclease activity, their application in nucleic acid extraction has not led to a final solution of the problem. Recently, based on the observation of Rosen and Fedorcsák

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

** *Abbreviations:* DEP, diethyl pyrocarbonate; DNA, deoxyribonucleic acid; GuHCl, guanidine hydrochloride; RNA, ribonucleic acid; rRNA, ribosomal RNA; tRNA, transfer RNA; RNase, ribonuclease; SDS, sodium dodecyl sulphate; TEMED, N,N,N',N'-tetramethyl ethylenediamine.

(1966), Solymosy et al. (1968) have described a method in which in addition to sodium dodecyl sulphate DEP was used as a nuclease inhibitor in the extraction of undegraded nucleic acids from plant tissues. In the present paper we report the use of DEP in combination with GuHCl or phenol in the extraction of RNA from animal tissues.

Materials and Methods

Preparation of cytoplasmic extracts: Rabbits were bled after two days starvation. Their livers were removed (occasionally also their kidneys) and cooled in ice. From the organs cytoplasmic extracts were prepared by means of 3 volumes of extraction medium (0.14 M NaCl – 0.01 M sodium citrate – 0.002 M EDTA-Na_2) using the method described in an earlier communication (Zsindely et al., 1959) except that the tissues were homogenized in a Waring Blendor and a refrigerated centrifuge was used (3000 *g*, 30 minutes). Wherever indicated 1 ml DEP (Baycovin, Bayer Ltd., Leverkusen, Germany) was added to 100 ml of the extraction medium prior to use. Immediately before homogenization DEP was added again at a rate of 1 ml DEP per 100 ml extraction medium and before the slow agitation of the mixture at a rate of 0.5 ml per 100 ml slurry. If not otherwise indicated all operations were performed at a temperature between 0 and 5 °C.

Isolation of RNA from cytoplasmic extracts by using GuHCl (GuHCl + DEP method): With or without the addition of DEP (0.5 ml per 100 ml) in each portion of the cytoplasmic extract GuHCl (BDH, Great Britain) was dissolved to a final concentration of 4 M and the pH of the mixture was adjusted to 5.0 with 1 N HCl (Cox, 1966). The mixture was allowed to stand for one hour at −5 °C. After centrifugation at 5000 *g* for 45 minutes the gelatinous precipitate was washed twice with 3 M GuHCl followed by two washings with 0.14 M NaCl – 0.002 M EDTA. Both salt solutions contained freshly dissolved DEP in a concentration of 0.5 ml per 100 ml and from each a volume of 10 ml per 10 g pulp was used. The sediment was then suspended in the earlier NaCl – EDTA – DEP solution (10 ml per 10 g pulp) and the pH carefully raised to 6.5–7.0 by the addition of 0.5 M NaOH. At this pH most of the precipitate went into solution. In each case (Experiments 1 and 2) the suspension was divided into two parts. One portion was incubated after adding again 0.5 ml DEP/100 ml at 38 °C for 10 minutes to promote dissolution and then cooled. DEP was added again (0.5 ml per 100 ml) and the suspensions freed from proteins by shaking with an equal volume of a 4:1 chloroform:butanol mixture for 30 minutes. After centrifugation at 4000 *g* for 10 minutes the aqueous phase was treated in the same way. RNA was precipitated from the clear aqueous solution with 2.5 volumes of ice-cold ethanol and the precipitate allowed to stand overnight at −10 °C. The precipitate was washed with 75% and 95% ethanol and then twice with ether and dried in a vacuum desiccator.

Isolation of RNA from cytoplasmic extracts by the use of phenol (phenol + DEP method): To another portion of the cytoplasmic extract, after the addition

of DEP (0.5 ml per 100 ml), an equal volume of freshly distilled 90% phenol was added at 0 °C and the mixture was shaken for 30 minutes. After centrifugation for 45 minutes at 5000 *g* and the draining of the aqueous phase the extraction was repeated by adding to the combined interphase and phenol phase a fresh extraction medium containing DEP at a rate of 0.5 ml per 100 ml. After a repeated adding of DEP (0.5 ml per 100 ml) the removal of proteins and the recovery of RNA from the combined aqueous phases was performed as above.

Extraction of RNA from tissue homogenates by the use of phenol (phenol + DEP method): The fresh organ was homogenized in a 5-fold volume of extraction medium containing 1% of freshly dissolved DEP. DEP was added to the suspension at a rate of 0.5 ml per 100 ml prior to mixing it with phenol. The suspension was then shaken with phenol at 0 °C as described above. After re-extraction of RNA from the mixture of the interphase and phenol phase an equal volume of fresh extraction medium (NaCl – sodium citrate – EDTA – DEP) containing 0.5 per cent SDS was added to the mixture of the interphase and phenol phase and the mixture was rapidly heated to 65 °C in a water-bath, then stirred for 10 minutes at 65 °C, cooled rapidly and centrifuged. Removal of proteins and recovery of the ribonucleic acids from the extracts obtained at 0° and 65 °C from the homogenates were performed after repeated adding of DEP (0.5 ml per 100 ml) as above.

Determination of $\varepsilon(P)$ and measurement of hyperchromicity after alkaline hydrolysis were carried out in the same way as described in an earlier communication (Zsindely, 1962). UV absorption was measured in 1 cm quartz cells in the Beckman DU spectrophotometer.

Phosphorus was determined according to a modified version of the method of Martland and Robison (Tankó et al., 1967).

Total nucleic acid content of the pulp and of the cytoplasmic extract was determined according to the method of Schmidt and Thannhauser modified by Shibko et al. (1967). RNA and DNA were measured, after their separation, by UV absorption and by their colour reactions with orcinol (Brown, 1946) and diphenylamine (Burton, 1956).

Protein contamination in the RNA preparations was determined by two independent methods (turbidimetric method and Folin reaction) according to Solymosy et al. (1968): 4 mg of the RNA sample was hydrolyzed in 4 ml of 5% TCA for 15 minutes at 90 °C, and after cooling to 20 °C turbidity was measured in the Beckman DU spectrophotometer at 500 nm. The centrifuged precipitate was used to estimate protein content by the Folin reaction according to Lowry et al. (1951).

Determination of glycogen contamination in RNA preparations: After the precipitation of the RNA from a 4 mg sample at 0 °C with 0.5 N perchloric acid the precipitate, obtained by adding 1.1 volume of ethanol to the supernatant, was hydrolyzed in 1 N hydrochloric acid at 100 °C for 3 hours and the glucose content determined in 1 cm cells at 665 nm in aliquot samples by means of the colour reaction with o-toluidine (Hyvärinen, Nikkula, 1962). As a standard glucogen (Merck, Germany) hydrolyzed under identical conditions was used.

Determination of DNA contamination: The precipitate obtained from the 4 mg RNA sample at 0 °C with perchloric acid (see the determination of glycogen contamination) was hydrolyzed with 0.3 N NaOH at 60 °C for 1 hour, the solution cooled to 0 °C and the DNA precipitated with perchloric acid. After 1 hour standing at 0 °C and centrifugation, the precipitate was washed twice with 0.3 N perchloric acid at 0 °C, hydrolyzed with 3.0 ml of 0.3 N perchloric acid at 100 °C for 15 minutes, cooled and the deoxyribose measured in an aliquot portion with the diphenylamine test (Burton, 1956). Thymus DNA was used as standard.

Polyacrylamide gel electrophoresis: 2.4% gels were prepared according to Loening (1967). To 5 ml of the stock solution (15% acrylamide + 0.75% bis-acrylamide) 6.25 ml of the buffer stock solution (0.18 M Tris – 0.15 M NaH_2PO_4 – 5 mM EDTA · Na_2; pH 7.6–7.7) and 19.7 ml of water were added, the mixture was freed of absorbed gases under vacuum for 15–30 sec and 25 μl TEMED and 0.25 ml of freshly dissolved 10% $(NH_4)_2S_2O_8$ were added. From this solution 4.6 ml portions were pipetted into the tubes (0.8 × 9.4 cm) made of Plexiglas and closed at their base with a Millipore filter disc. The tubes were then inserted to a stand. The solution was overlayered with the above buffer in a five-fold dilution and polymerization allowed to proceed for 15 minutes at room temperature and for 30 minutes at 4 °C. The tubes were then placed in the vertical electrophoresis apparatus and filled with the five times diluted buffer. A preliminary run was performed at 4 °C for 30 minutes (12 V/cm, 6 mA/tube). Thereafter 0.06 to 0.1 mg RNA in not more than 60 μl of the same buffer containing 5% of sucrose was layered over the gel. The run lasted 160 minutes at 4 °C. To eliminate interference by an eventual ribonuclease contamination in the sucrose, the 5% sucrose solution was treated according to Solymosy et al. (1968) with DEP. The gels blown out of the tubes were fixed for 2.5 hours in a 1:1 mixture of 96% alcohol and 2% acetic acid, stained for 5 minutes with toluidine blue (1.25 g toluidine blue per 400 ml of 1% acetic acid), and finally differentiated for about 20 hours in a solution containing 2% acetic acid and 10% glycerol. At the beginning the differentiating solution was changed rather frequently.

Results

RNA was prepared from rabbit livers and occasionally also from kidneys by applying GuHCl and phenol in the presence of DEP, and as a control sometimes without DEP. The RNA preparations obtained in this way were compared with respect to their UV absorption and their purity, as well as their electrophoretic behaviour in polyacrylamide gels.

The spectra of some RNA samples obtained from liver are shown in Fig. 1, the ε(P) values and the extinction ratios are illustrated in Table 1. The UV spectra of the cytoplasmic RNA were the same whether isolated by the GuHCl or the GuHCl+DEP method at 0 °C (Figs 1a and 1b) as indicated also by the almost identical extinction ratios. The spectrum of the cytoplasmic RNA obtained by the phenol+DEP method (Fig. 1c) was the same as that of the preparations

obtained by the GuHCl method, neither was there any significant difference between their extinction ratios. The UV spectrum of the RNA isolated from the homogenate by the phenol+DEP method was also similar to the above (Fig. 1d), but the extinction ratios were somewhat lower than those of the other RNA preparations. The ε(P) values and hyperchromicity after hydrolysis (Table 1)

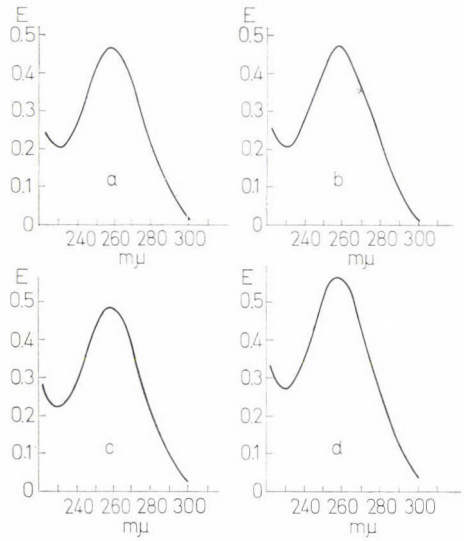

Fig. 1. UV spectra of RNAs (in 0.05 M potassium acetate) extracted from liver by different methods. a) RNA prepared from cytoplasmic extract with GuHCl without DEP; b) RNA prepared from cytoplasmic extract by the GuHCl + DEP method; c) RNA prepared from cytoplasmic extract by the phenol + DEP method; d) RNA prepared from tissue homogenate by the phenol + DEP method at 0 °C.

were almost the same with the preparations obtained by means of GuHCl. The ε(P) values of the RNA isolated by the phenol method were somewhat lower than those of the preparations obtained by the GuHCl method, but their increase after alkaline hydrolysis was the same. This is in agreement with our earlier findings (Szabolcs, Zsindely, 1963). Hyperchromicity after alkaline hydrolysis of the RNA preparations isolated from the homogenate by the phenol+DEP method at either 0 °C or 65 °C was lower than the above values, probably due to contamination by DNA (Table 2).

Nucleic acid preparations obtained from cytoplasmic extracts by means of GuHCl or phenol were practically free of proteins – as indicated by the high extinction ratios too – and contained no DNA (Table 2). The nucleic acids extracted by the phenol method were, however, more or less contaminated with glycogen in contrast to the preparations obtained by the GuHCl method. Accordingly the phosphorus content of RNA prepared by the phenol method was lower (7.2–8.2%) than that of the preparations obtained by the GuHCl

Table 1

UV absorption characteristics of ribonucleic acids prepared by different methods from rabbit liver

Experiment No.	Method	$\varepsilon(P)_{257}$ M^{-1} cm^{-1}	Per cent hyperchromicity after alkaline hydrolysis	$\frac{E_{260}}{E_{230}}$	$\frac{E_{260}}{E_{280}}$
	*From cytoplasmic extracts**				
1.	GuHCl + DEP	7870	41.5	2.32	2.18
	GuHCl + DEP, at 38 °C, 10 min	7890	41.0	2.29	2.17
	phenol + DEP	7320	41.2	2.32	2.14
2.	GuHCl without DEP	7760	40.3	2.22	2.16
	GuHCl + DEP	7780	41.3	2.29	2.17
	GuHCl + DEP, at 38° C, 10 min	7830	40.8	2.23	2.15
	phenol + DEP	7450	41.2	2.21	2.12
3.	GuHCl without DEP	7900	39.7	2.27	2.17
	GuHCl + DEP	7850	41.7	2.29	2.17
	phenol + DEP	7400	42.0	2.21	2.12
	*From tissue homogenates***				
	phenol + DEP, at 0°	7350	36.0	2.13	2.07
	phenol + DEP, at 65°	8000	38.0	2.11	2.09

* In an aliquot part of the cytoplasmic extract prepared from liver homogenates with 3 volumes of the extraction medium GuHCl was dissolved to give a final concentration of 4 M. Another aliquot was shaken with an equal volume of 90% phenol. Wherever indicated DEP was applied during the preparation of the cytoplasmic extract and in the course of further operations as described in Materials and Methods.

** Liver tissues were homogenized in a 5-fold volume of the extraction medium and treated with an equal volume of 90% phenol at 0 °C. To the mixture of the interphase + phenol phase fresh extraction medium containing SDS was added, and the mixture was heat-treated at 65 °C for 10 minutes. During extractions both at 0° and at 65 °C and in the further steps of preparation DEP was subsequently added as described in Materials and Methods.

method (8.5–9.0%). This is in agreement with our earlier results (Zsindely et al., 1959). The RNA preparations obtained from tissue homogenates were also free of proteins, but contained about 4–10% DNA and 5–10% glycogen.

The total nucleic acid content of liver homogenates was 7.5 mg per 1 g fresh weight; the RNA content was 5.5 mg per 1 g fresh weight. From this amount about 4.2–4.6 mg per 1 g tissue went into the cytoplasmic extract. This is about 80% of the total RNA content. The quantity of RNA obtained with GuHCl was 3.5–4.1 mg per 1 g tissue representing an average yield of 83–88 per cent. The amount of RNA extracted with phenol was somewhat higher than that, corresponding to an average yield of about 93 per cent. This higher yield with the phenol method is due to the fact that with phenol tRNA is also extracted, in contrast to GuHCl. From the homogenate it was possible to extract with

Table 2

Purity and yields of RNA isolated from rabbit liver by different methods

Experiment No.	Method	Protein	DNA	Glycogen	Phosphorus	Total RNA content*** of the		RNA**** extracted
		content (μg/1 mg RNA)				Homogenate	Cytoplasmic extract	
						mg/g fresh weight		
	*From cytoplasmic extracts**							
1.	GuHCl + DEP	2	1	Ø	87	5.8	4.6	4.1
	GuHCl + DEP, at 38 °C, 10 min	2	Ø	Ø	87			4.1
	phenol + DEP	1	2	46	82			4.3
2.	GuHCl without DEP	2	Ø	Ø	89	5.2	4.2	3.5
	GuHCl + DEP	1	Ø	Ø	90			3.6
	GuHCl + DEP, at 38 °C, 10 min	2	Ø	Ø	89			3.6
	phenol + DEP	1	1	180	72			3.9
3.	GuHCl without DEP	2	1	Ø	85	5.6	4.5	3.7
	GuHCl + DEP	1	1	Ø	85			3.8
	phenol + DEP	2	3	100	76			4.1
	*From homogenate***							
	phenol + DEP, at 0 °C	2	43	116	74			4.2
	phenol + DEP, at 65 °C	3	96	50	80			0.95

* In aliquot parts of the cytoplasmic extract prepared from liver homogenate with 3 volumes of the extraction medium GuHCl was dissolved to a concentration of 4 M or an equal volume of 90% phenol was added. Wherever indicated DEP was applied during the preparation of the cytoplasmic extract and in the course of further operations as described in Materials and Methods.

** Liver tissue was homogenized in a 5-fold excess of the extraction medium and treated with an equal volume of 90% phenol at 0 °C. To the mixture of the interphase and the phenol phase fresh extraction medium containing SDS was added and the mixture was heat-treated for 10 minutes at 65 °C. During extraction both at 0° and 65 °C and in the further steps of preparation DEP was repeatedly added as described in Materials and Methods.

*** Total RNA content was determined according to the method of Schmidt and Thannhauser modified by Shibko et al. (1967).

**** The quantity of extracted RNA was calculated from the UV absorption of the solution and from its phosphorus content.

phenol at 0 °C about 75% of the total RNA and then at 65 °C a further 17%. These data indicate that the presence of DEP had no significant effect – at least in the GuHCl method – either on the yield or on the purity of the RNA preparations.

In the course of fractionation by means of polyacrylamide gel electrophoresis (Fig. 2) under the given experimental conditions heavy rRNA moved 1.4–1.5 cm, light rRNA 3.3–3.5 cm and the tRNAs 8.3–9.0 cm. Under identical experi-

mental conditions these distances could be satisfactorily reproduced. On the electrophoretograms of the cytoplasmic RNAs isolated by the GuHCl+DEP method and phenol+DEP method, respectively (Figs 2b and 2d) the location and number of bands were almost the same except the lack of the wider bands of tRNAs in the preparations obtained by the GuHCl method. It can be

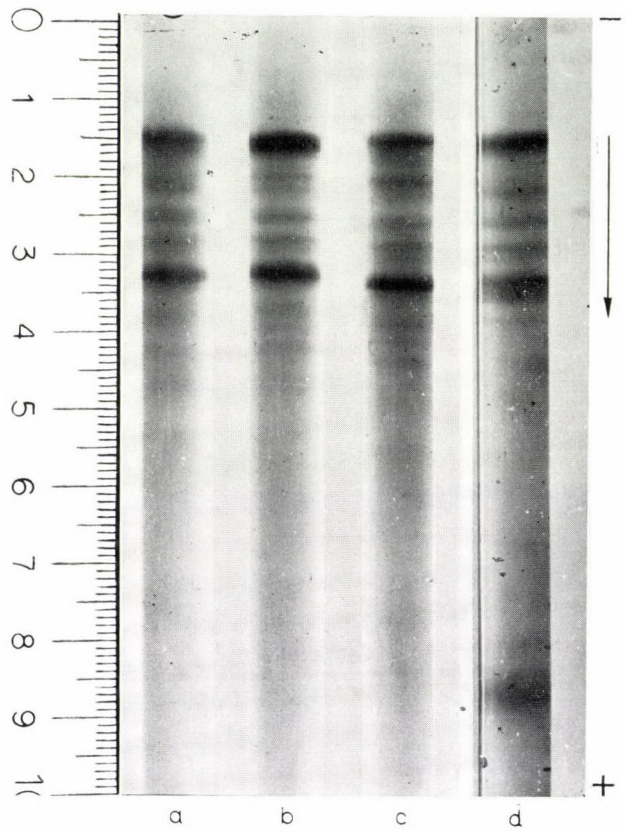

Fig. 2. Polyacrylamide gel electrophoresis patterns of liver cytoplasmic RNAs (Expt. No. 2). a) RNA prepared with GuHCl without DEP; b) RNA prepared by the GuHCl + DEP method; c) RNA prepared by the GuHCl + DEP method with heat treatment at 38 °C for 10 minutes; d) RNA prepared by the phenol + DEP method

observed for the preparations obtained by any of the two methods – though this is not sufficiently reflected in the diagrams – that there were two bands which moved slower than heavy rRNA and that there was a distinct, though faint, band of 5 S rRNA moving slower than the tRNAs (7.2–7.6 cm). As far as the distribution of the main rRNA components is concerned in the preparations obtained in the presence of DEP heavy rRNA predominated and in the preparations obtained by the phenol method the band of light rRNA was somewhat diffuse and appeared to be composed of two bands. When extraction was per-

formed by the GuHCl + DEP procedure, in which after suspension of the GuHCl precipitate and after addition of DEP again a heat treatment at 38 °C was applied for 10 minutes, the predominance of heavy rRNA was reduced and the component which run slower than heavy rRNA disappeared (Fig. 2c). When nucleic acids were isolated in the presence of GuHCl but without using DEP the predominance

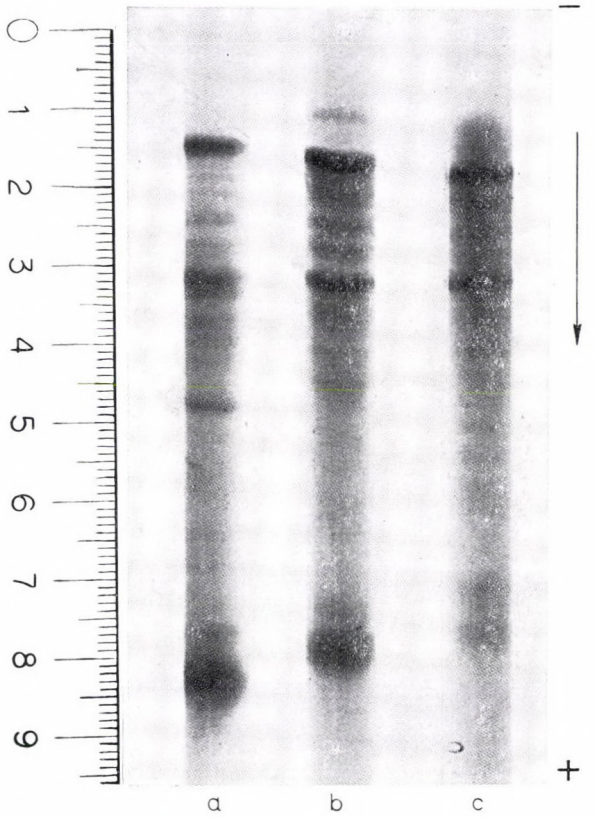

Fig. 3. Polyacrylamide gel electrophoresis patterns of RNAs obtained from liver homogenates and cytoplasmic extracts, respectively, by the phenol + DEP method (Expt. No. 3). a) RNA isolated from cytoplasmic extract at 0 °C; b) RNA extracted from tissue homogenate at 0 °C; c) RNA extracted from tissue homogenate at 65 °C

of heavy rRNA on the electrophoretogram was less clear-cut (Fig. 2a) than in the presence of DEP and the components moving more slowly than heavy rRNA were also less distinct and the entire pattern was more diffuse.

The main RNA fractions and the fainter bands appeared quite distinctly also on the electrophoretograms of RNA preparations extracted from liver homogenates by means of the phenol + DEP method at 0° or 65 °C (Figs 3b and 3c). The components moving behind heavy rRNA are best observed in these figures. They may have resulted from DNA contamination. It should be noted, however, that DNA prepared on another occasion from liver did not migrate

at all but remained on the top of the gel. For the sake of comparison the electrophoretogram of an RNA preparation obtained in the same experiment from the cytoplasmic extract by the phenol+DEP method is also shown.

RNA preparations of similar properties and with a similar electrophoretic behaviour in polyacrylamide gels were obtained from kidneys. The nucleic acid yields from the kidneys were lower than from the livers.

Discussion

A modified version of the DEP method originally described by Solymosy et al. (1968) for the extraction of undegraded nucleic acids from plant tissues has now been used for the isolation of RNA from animal tissues. The modification consisted in adding DEP to the extraction medium first at a low concentration and then replacing it several times during extraction. In this way, despite the high decomposition rate of DEP in aqueous media (cf. Öberg, 1970), saturation could be ensured in the period required for the preparation of the cytoplasmic extracts (about 90 to 120 minutes), during the extraction of nucleic acids from tissue homogenates (about 90 minutes), as well as during the various phases of preparation. Our aim was to avoid the concentration drop of DEP resulting from its decomposition on the one hand and its sedimentation during centrifugations on the other (the specific gravity of DEP is higher than 1).

DEP reacts with proteins and thereby irreversibly inactivates enzymes including nucleases (Rosen, Fedorcsák, 1966). Depending on the experimental conditions it reacts, however, to different degrees with RNA as well (Solymosy, personal communication), and inactivates the infectivity of TMV–RNA (Gulyás, Solymosy, 1970) and that of single-stranded poliovirus RNA (Öberg, 1970). The template activity (Fedorcsák et al., 1969) and the amino acid acceptor activity (Abadom, Elson, 1970) of RNA extracted by the DEP method (Solymosy et al., 1968) are, however, retained and the use of this method in analytical work remains warranted (Solymosy et al., 1970).

The application of DEP may be particularly significant in the extraction of undegraded nuclear RNAs (Steele et al., 1965; Busch et al., 1968) as well as in the isolation of rRNA precursors with high sedimentation coefficients (Attardi et al., 1966; Werner et al., 1966). According to a report by Hidvégi (1969) the isolation of e.g. 80 S precursors of rRNA is greatly impeded by the presence of traces of ribonuclease. Therefore these precursors could be prepared so far only from special objects lacking RNase activity.

Methods in which GuHCl is used in the isolation of RNAs (Volkin, Carter, 1951; Grinnan, Mosher, 1951) had been for some time fairly neglected and largely replaced by the phenol method, but are now again gaining importance (Cox, Arnstein, 1963; Cox, 1966) for the preparation of ribosomal RNAs. When 4 M GuHCl and a pH of 5.0 are used RNA is precipitated at a temperature below -5 °C. With cytoplasmic extracts this method was successfully combined with the use of DEP. A marked predominance of heavy rRNA was observed

primarily with the GuHCl + DEP method. After a heat treatment of the suspension of the GuHCl precipitate at 38 °C for 10 minutes this predominance of heavy rRNA dropped, but since DEP was again added to the suspension prior to heat treatment this reduction is to be attributed to a temperature effect rather than to the effect of ribonuclease. We have found that at a concentration of 4 M GuHCl tRNA did not precipitate, while 5 S RNA did. This phenomenon offers a new possibility for the preferential extraction of 5 S RNA from animal tissues. Experiments along these lines are in progress.

The purity of the RNA preparations obtained in our experiments, was not influenced by DEP, at least not when the GuHCl method was used. The RNA preparations obtained from cytoplasmic extracts were practically free of both protein and DNA with both the GuHCl and phenol methods, while in the nucleic acid preparations obtained from organ homogenates about 4 to 10% DNA was found with the phenol method. RNA preparations obtained by the phenol method always contained polysaccharide contamination despite the fact that the experimental animals had been starved before use. On the other hand, RNA preparations obtained by the GuHCl method were practically free of polysaccharide contamination. This may be explained by the essential difference between the two methods (see review by Tankó, 1959). With respect to the yields there was no significant difference between the two methods. Difficulties are encountered in the subsequent removal of the accompanying polysaccharides since additional manipulation may lead to an eventual degradation of nucleic acids. Hence, from this point of view the application of the GuHCl method is more favourable.

The results of polyacrylamide gel electrophoresis show that when nucleic acid extraction by the GuHCl method is performed at 0 °C a preparation of at least the same quality can be obtained as with the phenol method. In agreement with literary data a number of fainter bands appeared on the electrophoretograms in addition to the well-known main RNA components. Peacock and Dingman (1967) have found that cytoplasmic RNA from rat liver and kidneys contained about 10–12 minor components as revealed by polyacrylamide gel electrophoresis. These fractions were not considered as degradation products. We suggest that the minor bands in the polyacrylamide gel electrophoretic patterns obtained from our RNA preparations are not degradation products either.

Thanks are due to Dr F. Solymosy (Institute of Plant Physiology, Hungarian Academy of Sciences) for his help in polyacrylamide gel electrophoresis and for providing us with a sample of diethyl pyrocarbonate.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (4), pp. 435–440 (1970)

Thin Layer Ion Exchange Chromatography on Resin-Coated Chromatoplates

I. Separation of Aromatic and Basic Amino Acids (Phenylketonuria-Test)

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(Received July 22, 1970)

A simple method is described for the separation of aromatic and basic amino acids by thin layer ion exchange chromatography on plates coated with Dowex 50×8 resin. The method can be applied as a screening test of phenylketonuria.

Attempts to combine thin layer chromatography with ion exchange chromatography, with the aim of uniting the favourable properties of the two different methods date back almost to the beginning of the application of thin layer chromatographic methods. The primary objective is to combine the resolving capacity of ion exchange with the simplicity of thin layer chromatography (e.g. detection).

However, in the field of ion exchange thin layer chromatography only the weak anion and cation exchanger cellulose derivatives have so far found wider acceptance, since the fixation of thin layers of strong anion and cation exchange resins could not be solved in a satisfactory manner. Though by means of binders commonly used in thin layer chromatography it is possible to prepare apparently homogeneous layers of high mechanical strength. These, however, are liable to peel off the glass or other solid surfaces under the action of water or aqueous solutions.

We have worked out in our Laboratory a hydrophilic heterogeneous binder by means of which chromatoplates of high mechanical strength can be prepared from any synthetic ion exchange resin and which can be used similarly to ion exchange columns in aqueous solutions under the traditional conditions of thin layer chromatography (Dévényi, Zoltán, 1970).

In the present paper we wish to describe the separation of aromatic and basic amino acids on Dowex 50×8 resin-coated chromatoplates. The method is suitable among others for the detection of phenylketonuria by screening tests.

Materials and Methods

Dowex 50×8 resin-coated chromatoplates

Chromatoplates 20×20 cm were used in Na^+ cycle (Dévényi, Zoltán, 1970). The plates were washed for 48 hours with sodium citrate, pH 3.28, 0.02 N Na^+ to achieve equilibrium. This procedure was performed in the usual ascending

system of thin layer chromatography with the difference that to ensure the adequate flow of the solvent a filter paper strip was fixed by means of a rubber band to the upper edge of the layer. This paper strip extends to about 10 cm at the other side of the plate. After washing the plates were dried at room temperature. The plates can be stored at room temperature for several months.

Preparation of the eluting buffer solution (sodium citrate, pH 5.28, 0.35 N Na^+).

The eluting buffer is the same as used in amino acid analysis by the operation of the short column, but contains no additives (BRIJ, etc.):

citric acid monohydrate	24.6 g
sodium hydroxide	14.0 g
hydrochloric acid, 37% (sp. gr. 1.19)	6.5 ml

made up to 1000 ml with ion-free distilled water.

Ninhydrin spray reagent

Solution A: 1 g ninhydrin dissolved in 100 ml acetone

Solution B: 1 g cadmium acetate dissolved in a mixture of 50 ml glacial acetic acid and 100 ml distilled water.

Prior to use 100 ml of solution A is mixed with 20 ml of solution B. For development the plates are dried by hot air from a hair dryer, sprayed with the reagent and heated by blowing hot air on them. The plates should be dried at a temperature not higher than 60 °C.

Quantitative determination of amino acids

For the quantitative determination of amino acids an amino acid analyzer of the type BioCal BC 200 was used, with a single column procedure (Dévényi, 1969).

Results and Discussion

For the separation of aromatic and basic amino acids sodium citrate, pH 5.28 and 0.35 N Na^+, as used in the analysis of amino acids was found to be a suitable buffer. The sample was applied, run, dried and developed by the methods and with the tools usually applied in conventional thin layer chromatography. A 10–12 cm high run of the buffer is sufficient for optimum separation, for the time required is 60 to 120 minutes depending on the particle size of the (fixed) resin.

Figure 1 shows a chromatogram obtained on Dowex 50×8 resin-coated chromatoplate.

Fig. 1 shows that at pH 5.28 the elution sequence of amino acids is the same as usually found in amino acid analysis. At the applied pH and molarity amino acids which have higher R_f values than leucin do not interfere with the separation and identification of aromatic and basic amino acids even at very high concentrations.

The method described is suitable for the detection of changes in the concentrations of aromatic and basic amino acids. In this respect particularly those metabolic diseases are worth noting which are accompanied by changes in the concentrations of these amino acids in the blood and urine. Such metabolic diseases are e.g. phenylketonuria, lysinaemia, tyrosinosis, histidinaemia, etc. The most important of these metabolic disorders is phenylketonuria which is

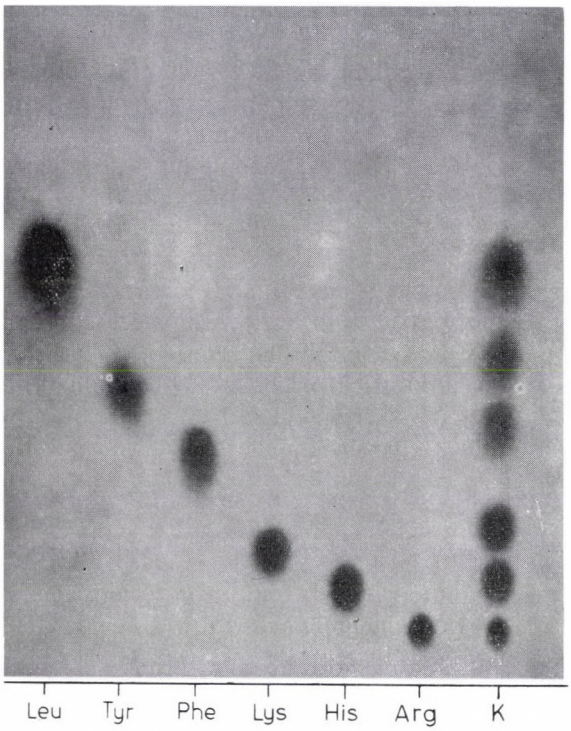

Fig. 1. Separation of aromatic and basic amino acids on a Dowex 50×8 resin-coated chromatoplate. Eluting buffer: sodium citrate, pH 5.28, 0.35 N Na^+

accompanied by the accumulation of phenylalanine in the blood and urine and whose timely diagnosis makes possible a successful therapy. In addition to the Guthrie test (Guthrie, Susi, 1963) which is based on a microbiological reaction and is not quite reliable, it is only the use of the automatic amino acid analyzer which may provide information of diagnostic value. This latter technique, however, cannot be applied as screening test for obvious technical reasons. Due to its very nature ion exchange thin layer chromatography may be developed for use in screening tests of the above-mentioned metabolic disorders, primarily phenylketonuria.

For the examination of blood plasma or serum the samples are freed from proteins by precipitation with trichloroacetic acid and applied to the plates

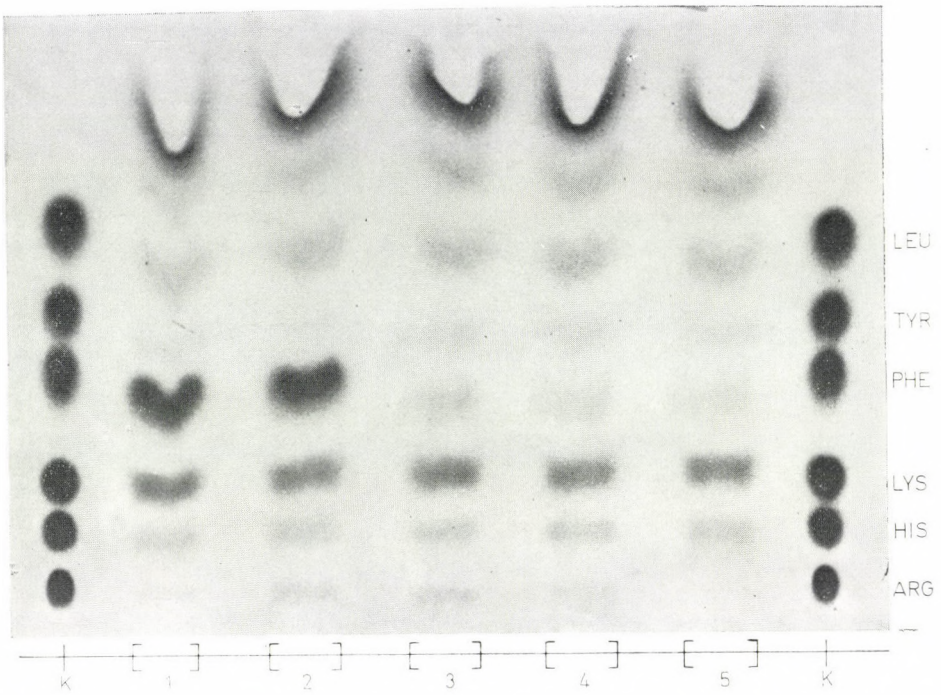

Fig. 2. Chromatogram of supernatants after precipitation with trichloroacetic acid of phenylketonuria and normal blood sera on a Dowex 50×8 resin-coated chromatoplate. K, control mixture (in the order of increasing R_f values Arg, His, Lys, Phe, Tyr, Leu); 1 and 2 phenylketonuria samples; 3 to 5 normal samples

(to the sample 20% trichloroacetic acid is added in a ratio of 2:1 (v/v)). After centrifugation the supernatant liquid is applied to the plate. Experience has shown that a 10 μl aliquot of the protein-free supernatant contains sufficient free amino acid for the unequivocal detection of any eventual (pathological) concentration differences. The plate is eluted in two steps: first with 0.01 N hydrochloric acid up to a height of 13 cm of the solvent. In this solution the trichloroacetic acid which greatly interferes with separation runs with the solvent front, while the aromatic and basic amino acids remain at the origin. The plate is dried at room temperature and then run again in sodium citrate buffer (pH 5.28, 0.35 N Na^+) up to a front height of 12 cm.

Figure 2 shows a typical chromatogram.

The detection of a high phenylalanine concentration characteristic of phenylketonuria makes in fact quantitative evaluation unnecessary, since the concentration increase compared to the normal value is so marked (generally 8 to 10 times higher than the normal) that it can be quite unambiguously detected by visual inspection. Detection is further facilitated by the fact that in the normal serum histidine, tyrosine and phenylalanine are present in almost

identical concentrations, so that on the chromatogram of a given sample the intensity of the histidine and tyrosine band may serve as an "internal standard" (Table 1).

Table 1

Concentration of free amino acid in phenylketonuria and normal plasma (in mM)

Amino acid	Phenylketonuria		Normal*	
	1	2	1	2
Lysine	0.22	0.20	0.10	0.21
Histidine	0.08	0.14	0.03	0.10
Arginine	0.05	0.06	0.04	0.14
Aspartic acid	0.04	0.05	trace	0.01
Threonine	0.23	0.23	0.08	0.20
Serine + glutamine	0.13	0.15	0.21	0.73
Proline	0.12	0.26	0.10	0.29
Glycine	0.19	0.20	0.18	0.32
Alanine	0.21	0.27	0.21	0.47
1/2 Cystine	trace	trace	0.07	0.11
Valine	0.10	0.11	0.17	0.32
Methionine	0.02	0.02	0.01	0.03
Isoleucine	0.04	0.07	0.04	0.10
Leucine	0.07	0.11	0.08	0.18
Tyrosine	0.02	0.04	0.02	0.08
Phenylalanine	*0.67*	*1.00*	*0.04*	*0.07*

* Soupart, 1962, partly own data

Urine samples can be chromatographed in exactly the same way as the trichloroacetic acid supernatant of the plasma (serum, blood), though the pH of the urine samples has to be adjusted first to 10 by the addition of alkali, the sample evaporated to dryness in a vacuum desiccator and the dry residue dissolved in 0.01 N hydrochloric acid of half the volume of the initial liquid. Chromatography is carried out again in two steps. The first run is effected with 0.01 N hydrochloric acid up to a 13 cm front height and then by the same sodium citrate buffer as in the case of blood samples up to 12 cm height. In the case of urine this is preferable since 0.01 N hydrochloric acid will remove the urea which greatly interferes with the separation and decomposes only partially in the course of evaporation in alkali, just as it will remove many other interfering components while the aromatic and basic amino acids to be determined remain at the origin.

Due to its simplicity the method appears to be suitable for routine determinations both in biochemistry and clinical practice.

I wish to thank Mrs Judit Báti for the many technical ideas in the elaboration of the method and for the performance of the amino acid analyses. I should also like to thank Mr S. Ferenczy for his contribution in the testing of the physiological fluids. I am further indebted to Dr Judit Kovács (Heim Pál Children's Hospital) for kindly putting at our disposal the serum samples of patients suffering from phenylketonuria.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (4), pp. 441–445 (1970)

Thin Layer Ion Exchange Chromatography on Resin-Coated Chromatoplates

II. Racemization Test in Peptide Synthesis

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(Received July 22, 1970)

A simple method is described for the separation of L-L and D-L Gly.Ala. Leu by thin layer ion exchange chromatography on plates coated with Dowex 50×8 resin. The method can be applied as a racemization test of reaction mixtures used in peptide synthesis.

The coupling of L Gly.Ala to L-leucine is a simple method for the detection of any eventual racemization which may occur in the course of peptide synthesis. The L-L Gly.Ala.Leu formed by the above process can be easily separated from the eventually formed D-L isomer by means of standard amino acid analyzer techniques (Izumiya, Muraoka, 1969). With sodium citrate buffer at pH 4.25 first the L-L and then the D-L isomer is eluted. A similarly satisfactory separation may be achieved by means of ion exchange thin layer chromatography. In this paper we shall describe the separation of the L-L and D-L Gly.Ala.Leu tripeptide pair on a Dowex 50×8 resin-coated chromatoplate.

Materials and Methods

Dowex 50×8 resin-coated chromatoplates

Plates (20×20 cm) were used in the Na^+ cycle (Dévényi, Zoltán, 1970). The plates were equilibrated by the method described in an earlier communication (Dévényi, 1970).

Preparation of the eluting buffer (sodium citrate, pH 4.25, 0.8 N Na^+):

Citric acid monohydrate	14.1 g
hydrochloric acid, 37% (sp. gr. 1.19)	8.4 ml
sodium hydroxide	8.0 g
sodium chloride	35.0 g

made up to 1000 ml with ion-free distilled water.

Preparation of the ninhydrin spray reagent

The reagent was prepared by the method described in an earlier communication (Dévényi, 1970).

Analyzer technique

For quantitative determinations an amino acid analyzer of the type BioCal BC 200 was used with sodium citrate, pH 4.25 (0.8 N Na^+) as buffer, the second buffer used in the single column three buffer technique (Dévényi, 1969).

Results and Discussion

The tripeptide preparations under investigation were dissolved in 0.01 N hydrochloric acid to give a final concentration of 10 mg/ml. For adequate detection it is sufficient to apply as little as 10 μl. The samples are applied in 1 cm bands to the plates and eluted up to 10 to 12 cm front height. The time required for the preparation of a chromatogram is 60 to 120 minutes depending

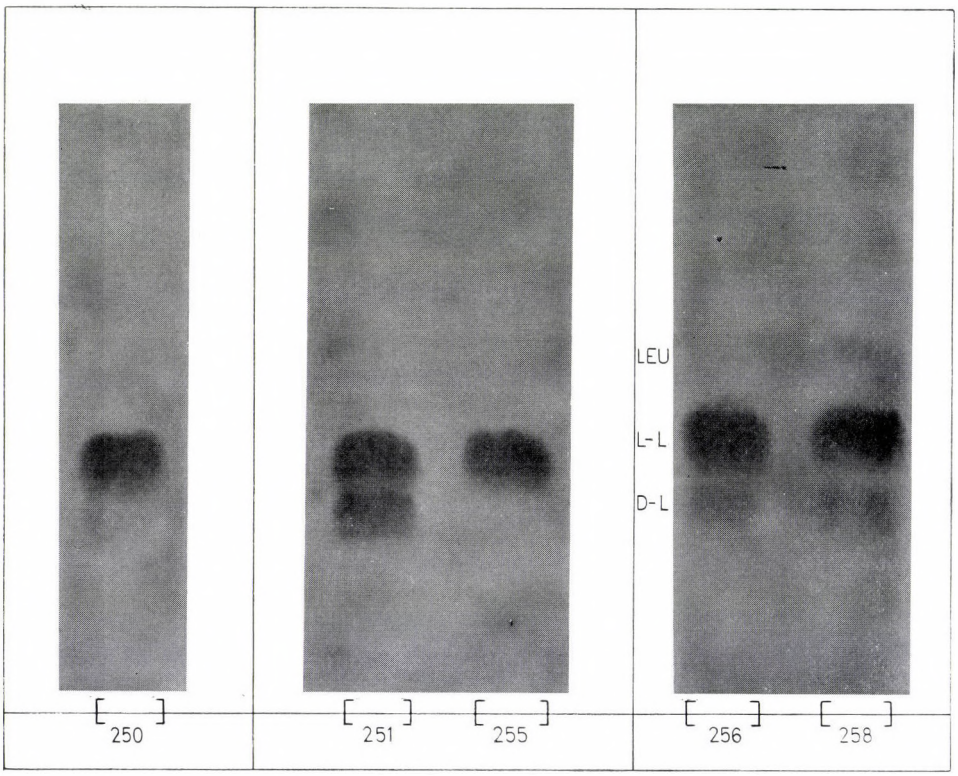

Fig. 1. Separation of L-L and D-L Gly.Ala.Leu on a Dowex 50×8 resin-coated chromatoplate. Elution: sodium citrate, pH 4.25, 0.8 N Na^+. Five preparations were tested on the plates (see Table 1)

on the particle size of the fixed resin. Fig. 1 shows typical chromatograms.

The separation of the L−L and D−L tripeptides on the Dowex 50×8 resin-coated chromatoplates is based on the same principle as other analyzer

techniques, namely, at the applied pH and molarity the R_f value of the L–L isomer is higher than that of the D–L isomer.

As shown in Fig. 1 the separation on the chromatoplate indicates to some degree the measure of racemization, e.g. it may be seen quite clearly that sample No. 251 contains almost identical amounts of the two isomers, while sample No. 255 is practically homogeneous and that in the other three preparations the extent of racemization is about 10 to 20%. This rough estimate was fully confirmed by quantitative measurements (Table 1).

Table 1

Determination of L–L and D–L Gly.Ala.Leu by means of the automatic amino acid analyzer

No. of preparation	Amount of	
	L–L μmole	D–L μmole
250	1.0	trace
251	0.50	0.40
255	1.35	—
256	1.30	0.15
258	1.40	0.15

Consequently ion exchange thin layer chromatography may be considered a suitable method to be applied to the above described racemization test when a large number of samples have to be compared.

Experiments are now in progress to apply this method for the solution of similar problems.

I wish to express my gratitude to Mrs Judit Báti for her help in carrying out the experiments and for performing the quantitative determinations. I also should like to thank Dr Lajos Kisfaludy (Gedeon Richter Pharmaceutical Factory, Budapest) for his courtesy in making the tripeptide samples available to us.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (4), pp. 445—448 (1970)

The N-Terminal Group of Pig Pancreas Amylase

(Short Communication)

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(Received July 22, 1970)

In amylase (α-1,4-glucane-4-glucanohydrolase, E. C. 3.2.11) isolated in the presence of diisopropylfluorophosphate from pig pancreas by the method of Hatfaludi et al. (1965) and recrystallized three times components corresponding to terminal groups were found only as contaminants when assayed by the 2,4-dinitrofluorobenzene method (Sanger, 1945). This observation is in good agreement with the results reported by Marchis-Mouren and Pasero (1967). These authors have shown that pig pancreas amylase possesses no α-amino group. This may have two causes: the N-terminal amino acid is either acylated or, due to ring closure, pyroglutamic acid (PCA = pyrrolid-2-one-5-carboxylic acid) is present. In the present work an attempt was made to identify the N-terminal by analyzing the ninhydrin negative fraction isolated from the enzymatic hydrolyzate of amylase.

To a solution containing 150 mg of amylase the same volume of a 1:1 (v/v) mixture of 1 N HCl : acetone was added and the solution centrifuged at 8 000 *g*. The precipitate was washed twice with acidic acetone and then three times with distilled water. The protein precipitate was then suspended in 0.1 M ammonium bicarbonate to give a protein concentration of about 10 mg/ml. To achieve complete digestion the precipitate was hydrolyzed successively with 1/20 (w/w) of trypsin (Calbiochem), chymotrypsin (Reanal, Budapest) and nagarse (Teikoku Chemical Industry, Japan) at pH 8 for two hours each time. The digest was then lyophilized.

The lyophilized digest was dissolved in 0.05 N ammonia and subjected to electrophoresis on Whatman 3 MM paper at pH 1.9. It was assumed that the amino acid formed during hydrolysis with a blocked alpha-amino group had no charge at the given pH and would therefore remain at the origin. After electrophoresis control strips were cut from the two edges of the paper, and were developed with ninhydrin. At the start line only a very weak reaction with ninhydrin occurred. This zone gave, however, a very intensive positive reaction when tested with the chlorine-starch-potassium iodide method of Rydon and Smith (1952). This strip was cut out, sewn to a Whatman 3 MM paper and subjected to electrophoresis in a pH 5 buffer, since at this pH acidic peptides are very well separated (Dévényi, 1963). The strips cut from the electrophoreto-

gram were developed with ninhydrin and chlorination as described above. In this case three zones appeared (Fig. 1/a) of which N1 and N2 were slightly ninhydrin positive. With chlorination all three zones gave a positive reaction.

For further separation strips N1 and N2 were sewn on Whatman 3 MM paper and subjected to chromatography by the ascending technique in a pyri-

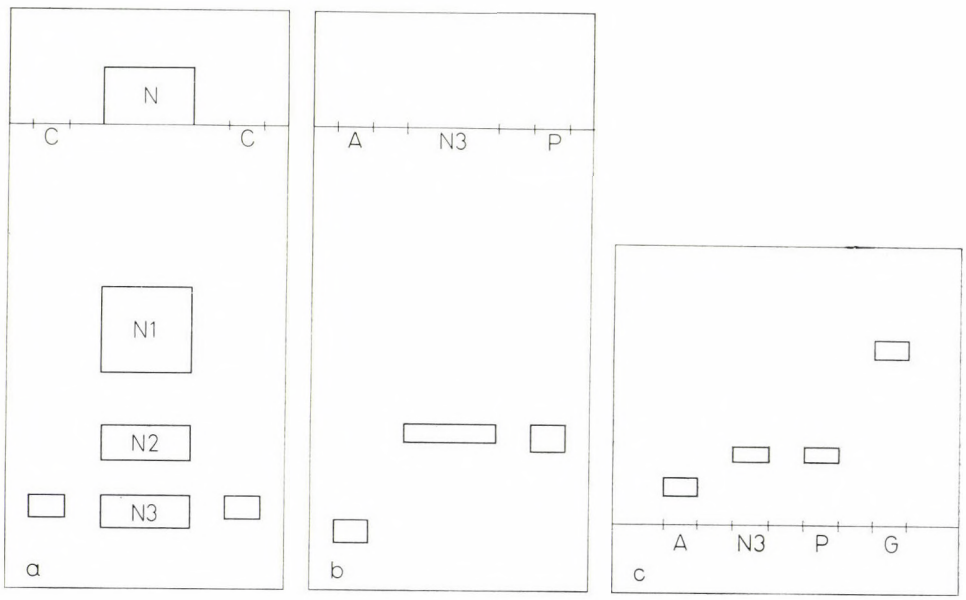

Fig. 1. Flow sheet of the isolation of PCA from the enzymatic hydrolyzate of amylase. 1/a Electrophoretogram of the zone cut out from the electrophoretogram obtained after a run at pH 1.9, in pH 5 buffer, (pyridine: glacial acetic acid: water 10:10:980 (v/v)), voltage gradient 30 V/cm, developed with ninhydrin and chlorination, duration of the run 1.5 hours. "C", cysteic acid; 1/b Electrophoretogram obtained after running zone N3 in Fig. 1/a in a pH 6.5 buffer (pyridine : glacial acetic acid : water 100:4:496 v/v), voltage gradient 30 V/cm, duration of the run 90 minutes, development by means of chlorination. "A", N-acetylglutamic acid; "P", PCA; 1/c Thin layer chromatography of the fraction marked N3 in Fig. 1/a on a 10×10 cm resin-coated chromatoplate. Resin: Dowex 2×8, ascending technique, pyridine : glacial acetic acid : water 10:100:890 v/v, pH 3 buffer, run 20 minutes, development with ninhydrin and chlorination. "A", N-acetylglutamic acid; "P", PCA; "G", glutamic acid

dine–isoamylalcohol–water 35:35:30 (v/v) system. Upon chromatography both fractions separated into several chlorine positive strips, but at the same time all were also ninhydrin positive. The amount of material in zones N1 and N2 was, however, so little that no further purification of the chromatographic fractions was possible.

The material from the strip marked N3 was eluted (Fig. 1/a). A control test was carried out on a small portion of the eluate by means of the "dansyl" method (Gray, Hartley, 1963). By this method no terminal group could be

detected. Another aliquot portion was hydrolyzed with 5.7 N HCl at 105 °C for 16 hours and then subjected to amino acid analysis in a BioCal BC 200 analyzer by using Dévényi's (1969) single column method. In the hydrolyzate of the eluted material only glutamic acid was detected.

A further portion of the substance eluted from strip N3 was electrophorezed on Whatman 3 MM paper at pH 6.5. At this pH – as indicated by our experiments with synthetic N-acetyl-glutamic acid and PCA – the charges of the two amino acid derivatives make their electrophoretic separation possible. Synthetic N-acetyl-glutamic acid and pyroglutamic acid were run as controls. As shown in Fig. 1/b the ninhydrin negative and chlorine positive substance isolated by us had the same mobility as pyroglutamic acid. Identification was carried out also on a Dowex 2×8 resin-coated chromatoplate. In this case the thin layer fixed onto the glass plate was washed for 24 hours with a pyridine–glacial acetic acid–water 10 : 100 : 890 (v/v) pH 3 buffer and air-dried. The same buffer was used for the run by the ascending technique similarly to the technique of conventional thin layer chromatography. The results of this experiment (Fig. 1/c) were the same as those of the electrophoresis at pH 6.5, that is the R_f value of component N3 was identical with that of pyroglutamic acid.

The quantity of isolated pyroglutamic acid calculated with reference to the original amount of amylase varied between 0.1 and 0.2 mole pyroglutamic acid per mole amylase. This yield does not differ significantly from values obtained by similar tests on other proteins (cf. Montgomery et al., 1970; Rüde, Givol, 1967; etc.).

It has been observed (see the review by Blombäck, 1967) that pyroglutamic acid may be formed under relatively mild conditions from glutamine. The possibility of pyroglutamic acid being formed in the course of the preparation can therefore not be rejected. This, however, is contradicted by the finding that not even traces of glutamic acid or glutamine terminal group could be detected in the native enzyme.

Consequently, according to our experiments pig pancreas amylase possesses a PCA N-terminal. Our experiments do not of course exclude the possibility of acetyllysine, acetylarginine or acetylhistidine being the N-terminal, since these amino acids migrate during electrophoresis at pH 1.9 and are bound to the cation exchanger used in some of our experiments. The possible existence of an acetyl terminal is, however, contradicted by the fact that in intact amylase acetyl groups were found in amounts of only about 0.1 mole/mole amylase by gas chromatography. This method is known to give almost 100 per cent yields.

Thanks are due to Dr Tibor Dévényi for making the Dowex 2×8 type resin-coated chromatoplate available.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (4), pp. 449—462 (1970)

Structural Investigations on Pancreatic α-Amylase

The Effect of Urea on the Ultraviolet Absorption

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(Received August 19, 1970)

1. The changes in light absorption of hog pancreatic amylase (α-1,4-glucan-4--glucanohydrolase, EC 3.2.1.1) observed in the 270 to 300 and 220 to 240 nm wavelength regions were studied under different conditions producing changes in the conformation.

2. In the native molecule about 9 of the total 17 tryptophan and 6 of the 16 tyrosine residues were found to be accessible to urea at neutral pH, as calculated from the perturbation red shift.

3. Denaturation of amylase at neutral pH occurred at urea concentrations higher than 5 M. The effect of urea was followed by analyzing the effect of both denaturation blue shift and perturbation red shift. The molar absorption changes characteristic of the transfer of chromophores at 293, 287 and 232 nm were calculated.

4. The rate of unfolding of amylase at neutral pH in the presence of 5 to 9.5 M urea fitted first order kinetics. Calcium strongly inhibited the denaturation of amylase by urea. The binding of one atom calcium per mole amylase was effective in preventing the unfolding of amylase and an apparent dissociation constant of $K_1 = 10^{-4}$ M was calculated for the binding of calcium.

5. Ethylenediamine tetraacetate greatly increased the effect of urea. The first order kinetics of the denaturation of amylase by urea changed to a more complex reaction in the presence of the chelating agent.

Pancreatic amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) exhibits remarkable resistance towards proteases in the presence of calcium ions (Fischer et al., 1958). Previous papers (Vallee et al. 1959; Hsiu et al. 1963; Stein et al. 1963) have shown that amylases from various sources contain at least one atom firmly bound calcium per mole enzyme and various amounts of less firmly bound calcium, which play an important role in the maintenance of the structure as well as in the stabilization of the enzyme function. Thus, for example, incubation of the protein in concentrated urea solution in the presence of calcium ions does not irreversibly alter the activity of the enzyme (Hatfaludi et al. 1966). Moreover, acidic pH or treatment with sodium dodecyl sulfate does not cause profound changes in the ultraviolet spectral properties of pancreatic amylase (Krysteva, Elődi, 1968). These facts suggest that the polypeptide chain of amylase is folded into a rigid three-dimensional structure.

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Amylase which contains a relatively large number of aromatic side chains (Caldwell et al. 1954; Straub et al. 1970; Cozzone et al. 1970) appeared to be a particularly interesting object for the study of the distribution of chromophoric groups. It has been found by difference spectrophotometry that the majority of tryptophyl residues are accessible to the solvent (e.g. water), whereas tyrosyl side chains occur mainly in the buried state (Krysteva, Előd, 1968).

The present paper is concerned with the denaturation of pancreatic amylase by urea. The inhibitory effect of calcium and the promoting effect of EDTA* on denaturation were also studied. The structural alterations of the protein were followed by ultraviolet difference spectrophotometry which seemed to be a sensitive indicator of the changes in the environment of the chromophoric side chains (Wetlaufer, 1962; Donovan, 1969). In the wavelength regions characteristic of proteins, i.e. between 270 to 300 and 220 to 240 nm, this method can be applied both to estimate the number of chromophores located in the non-polar interior and polar exterior parts of the protein as well as to follow the changes in their distribution due to various effects.

Literary data on the denaturation difference spectra of various proteins appeared to be incongruous, particularly with respect to the values of absorption differences calculated for the transfer of individual chromophoric groups from the hydrophobic interior to the polar solvent medium. This fact arose presumably from the effect of the specific protein matrices on the absorption rather than from the different experimental conditions used in different laboratories. Further difficulties may be encountered in determining the absorption changes of the partially buried groups, i.e. those located in the interfaces between polar and nonpolar regions. Therefore, by studying the denaturation difference spectra of amylase and the spectral properties of model compounds the determination of absorption changes due to the transfer of chromophores was also attempted.

Experimental

Materials

Hog pancreatic amylase was prepared and twice recrystallized according to Hatfaludi et al. (1966). Preparation of the solutions and determination of protein concentration were carried out as described earlier (Krysteva, Előd, 1968). Protein solutions contained 1 to 5×10^{-5} M calcium chloride, when not mentioned otherwise. Amylase solutions were pretreated with diisopropyl fluorophosphate to avoid the effect of probable proteolytic contaminations. Calculations were based on a molecular weight of 52 600 determined by sedimentation equilibrium and light scattering (Závodszky, Előd, 1970). This value agrees very well with that published recently by Cozzone et al. (1970).

Urea (A grade) was recrystallized twice from 70 per cent ethanol and dried *in vacuo* over P_2O_5. Fresh solutions were prepared daily. Analytical grade

* Abbreviation: EDTA, ethylenediamine tetraacetate.

N-acetyl-DL-tyrosine ethyl ester was purchased from Reanal (Budapest). N-acetyl-DL-tryptophan ethyl ester was a product of Calbiochem. All other chemicals were of reagent grade.

Methods

Difference spectra were recorded in a Unicam SP 700 or Cary Model 15 recording spectrophotometer in the 260 to 300, and 220 to 240 nm wavelength regions. To avoid any possible error due to dilution, the same solutions were measured in 10 and 1 nm silica cells in the near and far ultraviolet regions, respectively. Dynode voltages of 2 and 3 were selected for total absorbancies between 1 and 2 at 280 and 230 nm (0.4 to 0.8 mg protein per ml). The slit width never exceeded 0.15 and 0.25 mm in the measurements at longer and shorter wavelengths, respectively. Precautions were taken to avoid errors due to stray light and other artefacts (Mihályi, 1965). Measurements were carried out at 22 ± 0.5 °C. The reference cell compartment of the Cary 15 spectrophotometer was slightly modified to fit tandem cells.

Solvent perturbation difference spectra were recorded by the tandem cell method of Herskovits and Laskowski (1960, 1962). Tandem cell system was also applied for taking denaturation difference spectra in the presence of different urea concentrations.

Tryptophan content was determined in 6 M guanidine hydrochloride dissolved in 0.02 M phosphate buffer, pH 6.5, according to the method of Edelhoch (1967). These determinations yielded a value of 17.2 ± 0.4 tryptophan residues per 52 400 molecular weight. This value, in contrast to that used in our previous paper (Krysteva, Előd i, 1968) was in a fairly good agreement with 17 and 15 tryptophans per mole protein, as reported by Caldwell et al. (1954) and Cozzone et al. (1970), respectively. For the tyrosine content a value of 16 residues per mole was accepted (Straub et al., 1970).

Results and Discussion

Effect of urea on the absorption of amylase

Since the effect of concentrated urea on proteins may simultaneously bring about two phenomena of opposite sign, i.e. perturbation red shift and denaturation blue shift, first the absorption differences due to the perturbation of chromophores were investigated with model compounds. Solvent perturbation difference spectra of N-acetyl-DL-tyrosine ethyl ester and N-acetyl-DL-tryptophan ethyl ester were recorded in 0.01 M tris buffer, pH 7.4, containing 8 M urea, against references in the absence of urea in the wavelength ranges of 260 to 300 and 220 to 250 nm (Fig. 1). The maximum molar perturbation difference values calculated for 1 M urea concentration are listed in Table 1.

The ratios of absorption differences measured at shorter and longer wavelength maxima are 8.7 and 3.8 for tryptophan and tyrosine, respectively. The

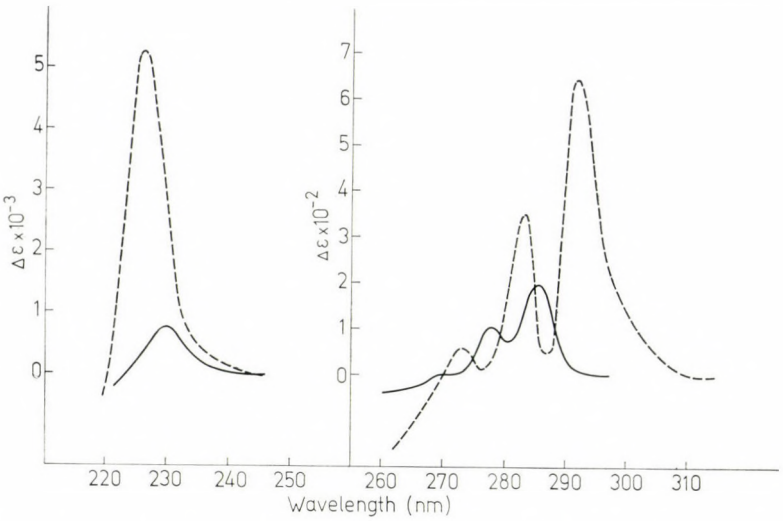

Fig. 1. Solvent perturbation difference spectra of amino acids. The spectra of N-acetyl-tryptophan ethyl ester (dotted lines) and N-acetyl-tyrosine ethyl ester (solid lines) were measured in 8 M urea, pH 7.4, against references in 0.01 M tris buffer, pH 7.4

ratio for tryptophan agrees with that found in the presence of 20 per cent ethylene glycol whereas the value for tyrosine is somewhat higher than 3.4, a value found previously (Móra, Elődi, 1968).

The changes in the absorption of amylase at three characteristic absorption maxima were measured in a urea concentration range of 1 to 9.5 M in the presence

Table 1

Molar absorption differences due to perturbation in 1 M urea as calculated from the data in Fig. 1

Compound	λ_{max}	$\Delta\varepsilon_{max}$	Ratio
N-acetyl-tryptophan ethyl ester	292	76	
	292	78, 80*	
	284	44	$\Delta\varepsilon_{292}/\Delta\varepsilon_{284} = 1.73$
	227	658	$\Delta\varepsilon_{227}/\Delta\varepsilon_{292} = 8.7$
N-acetyl-tyrosine ethyl ester	(292)	0	
	286	25	
	285	25*	$\Delta\varepsilon_{229}/\Delta\varepsilon_{286} = 3.8$
	229	94	$\Delta\varepsilon_{227}(\text{trp})/\Delta\varepsilon_{229}(\text{tyr}) = 7.0$

* cf. Donovan (1969)

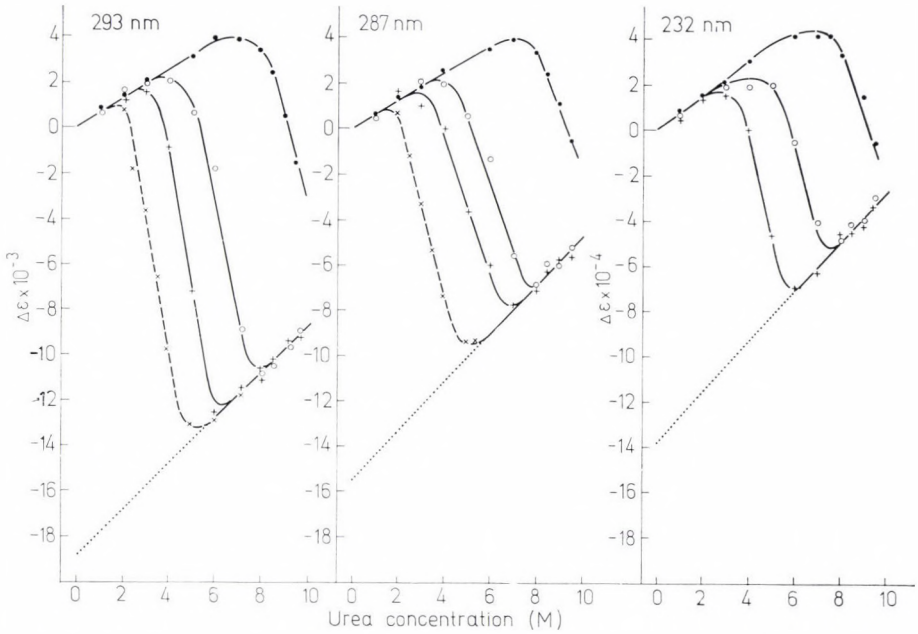

Fig. 2. Molar absorption differences produced by different urea concentrations in amylase solutions. Amylase solution contained 5×10^{-4} M calcium chloride. Experiments were carried out at pH 7.4 (solid lines) and pH 4.0 (dotted lines). Readings were taken without incubation (● and ×), after four hours (○) and twenty four hours (+) incubation

of 5×10^{-4} M calcium chloride, at pH 7.4 and pH 4.0. An instantaneous absorption change was observed at acidic pH at any urea concentrations studied (Fig. 2). The positive absorption differences at low urea concentrations did not change in time. These differences can be attributed to the perturbation red shift of chromophores accessible to urea without alteration of the native structure of the protein.

The blue shift spectral effect due to denaturation began to predominate in neutral solution over 4 to 5 M urea concentration. In these experiments incubation with urea lasted for hours. Under these conditions an inversion of the curve occurred, which indicates the unfolding of the protein (Fig. 2). A second inversion was observed, when the unfolding process was apparently completed. Above 6 M urea concentration the red shift due presumably to the perturbation of all tyrosines and tryptophans again partly compensated the effect of denaturation blue shift. The magnitude of the solvent induced red shift can be characterized by the final slope obtained by extrapolating the absorption differences measured at high urea concentrations to zero urea concentration (Bigelow, 1964; Martin, Bhatnagar, 1966). On the other hand, the initial slope of the perturbation effect at low urea concentrations makes it possible to estimate the number of chromophores accessible to urea in the native state (see below).

Table 2

Molar absorption differences characteristic of denatured amylase

Experimental conditions	Wavelength, nm		
	293	287	232
Extrapolation to 0 M urea concentration (see Fig. 2)	18 600± 500	16 000±400	138 000± 5 000
Measured in 8 M urea and in 5×10^{-5} M EDTA (corrected for solvent effect)	21 400± 800	16 500±500	—
Amylase digested with proteolytic enzymes	22 000±1200	16 700±900	—

The molar values of the maximum differences at 293, 287 and 232 nm corrected for solvent effect by extrapolation to zero urea concentration are shown in Table 2. The ratio of corrected maximum absorption differences found at 232 and 293 nm is 138 000/18 000 = 7.4.

Denaturation of amylase in urea at acidic pH

The unfolding of amylase occurred at lower urea concentrations at pH 4 than at neutral pH as judged from the absorption changes at 293 and 287 nm. The absorption differences of amylase in acidic urea solutions were measured against neutral reference solutions in the absence of urea. Denaturation blue shift was observed instantaneously above 2 to 2.5 M urea concentration and the denaturation was completed at about 5 M urea concentration as suggested by the inversion of the measured absorption changes (Fig. 2).

It is to be mentioned that at pH 5 and below, the data obtained were rather intriguing. When the protein was incubated at room temperature at pH 4 in the absence of urea its absorption at 240 or 280 nm sharply increased after 20 to 30 min. Light scattering measurements (unpublished data) indicated that aggregation of amylase occurs at acidic pH. This observation may explain why a poor denaturation difference spectrum was obtained earlier at pH 2.5 (Krysteva, Előd, 1968).

Perturbation of chromophores in native amylase

The number of tryptophans in native amylase accessible to urea can be calculated from the initial slope determined at 293 nm (Fig. 2), where the contribution of tyrosine is practically negligible (cf. Fig. 1). This calculation (Table 3) suggests that 8.6 tryptophans of the total 17 are accessible to urea without alteration of the native protein fabric.

Table 3

Absorption differences due to the perturbation of tryptophan and tyrosine residues in amylase

Values are given for 1 M urea concentration

Wave-length nm	Native amylase		Denatured amylase		
	$\Delta\varepsilon^*$	Number of chromophores accessible to urea	$\Delta\varepsilon^{**}$	Calculated	$\Delta\varepsilon_{calc} - \Delta\varepsilon_{obs}$
293	650	650 : 76 = 8.6 Trp	1 150	Trp 17× 76 = 1 292	142
287		376 : 44 = 8.6 Trp		Trp 17× 44 = 748	
		164 : 25 = 6.6 Tyr		Tyr 16× 25 = 400	
	540	540	1 050	1 148	98
232		5 660 : 658 = 8.6 Trp		Trp 17×658 = 11 190	
		620 : 94 = 6.6 Tyr		Tyr 17× 94 = 1 050	
	6400	6 280	12 000	12 690	690

* From the initial slopes in Fig. 2.
** From the final slopes in Fig. 2.

The estimation of the number of free tyrosines is based on the following considerations. The ratio of tryptophan perturbation at 292 nm and at 284 nm in 1 M urea (see Table 1) is 76:44 = 1.73 and the perturbation of 8.6 tryptophans in amylase results at 287 nm in a molar absorption difference of 650:1.73 = 376. Subtracting the contribution of tryptophan from the measured value we obtain 540 − 376 = 164. Thus 164:25 = 6.6 is obtained for the number of tyrosines accessible to urea in native amylase.

As far as the far ultraviolet perturbation difference spectrum is concerned similar calculations can be carried out for the perturbation differences at 232 nm (see Table 3). The calculated value is close to 6400, a value obtained from the initial slope. We may conclude from these calculations that the number of chromophoric side chains accessible to urea agrees well with that found with ethylene glycol as perturbant (Krysteva, Elődi, 1968).

Perturbation of chromophores in denatured amylase

The calculated and measured absorption differences due to the perturbation of tryptophans and tyrosines in denatured amylase are compared also in Table 3. The measured values are lower than the calculated ones. This discrepancy may be connected with the fact that amylase was still not completely unfolded at neutral pH even after incubation in 8 M urea for 24 hours. On the other hand, the decrease of absorption seemed to be completed under these conditions.

An attempt was made to obtain absorption differences by other methods which may be characteristic of completely denatured protein. Thus, for example amylase samples digested with proteolytic enzymes were also studied. Amylase

solutions of about 1 mg per ml protein concentration were treated with trypsin, chymotrypsin or papain in a 50:1 amylase to protease weight ratio for four hours at 37 °C in 0.01 M tris buffer, pH 7.4, containing 0.01 M EDTA. The absorption of digested protein samples was measured against that of untreated samples. As it can be seen in Table 2, the molar absorption differences found with proteolytically digested amylase were greater than those obtained by extrapolation to zero urea concentration.

A related phenomenon was observed when the molar absorption differences measured in 8 M urea in the presence and absence of 5×10^{-5} M EDTA were compared (Table 2). The absorption changes found with EDTA-treated protein appeared greater than those measured without the addition of the chelating agent. Similar differences were observed when the kinetics of urea denaturation was studied with and without addition of EDTA (see below).

The results obtained with digested and EDTA-treated proteins (Table 2) may indicate that 8 M urea at neutral pH did not cause a complete unfolding and a part of the polypeptide chain remained, at least, partially buried under these conditions. The urea resistant part of the polypeptide chain may contain about 1.5 tryptophan and at least 1 tyrosine residues, as derived from the measured and calculated perturbation differences listed in Table 3. Therefore, the following calculations are based on the assumption that about 7 tryptophan and 8 tyrosine residues are released from the hydrophobic interior of amylase upon denaturation with urea.

Absorption changes due to the transfer of chromophoric groups

The contribution of tyrosine and tryptophan to the denaturation difference spectra can be estimated from the corrected molar absorption differences found at the absorption maxima. If we assume that the value of 18 600 obtained at 293 nm reflects the transfer of about 7 tryptophan residues, the average value for the transfer of one residue is 2650. The absorption difference at 287 nm caused by the transfer of tryptophans is 10 750 = 18 600 : 1.73. Subtracting this value from the corrected molar absorption difference at 287 nm, 16 000 − 10 750 = 5250 is obtained. Since this figure arises from the transfer of about 8 tyrosines, the average value for the transfer of one tyrosine at 287 nm is 656. Accordingly, the transfer of one tryptophan at 287 nm results in an absorption difference of 1536.

Finally, the corrected absorption difference at 232 nm was 138 000 (Table 2). Since the extent of perturbation is proportional to the refractive index of the perturbant, we may presume that the ratio of perturbation of tyrosine to tryptophan due to the hydrophobic protein fabric will be similar to that measured in urea (see Table 1). As seen in Fig. 1, the molar absorption difference due to the perturbation of tryptophan at this wavelength is seven times greater than that of tyrosine. Thus, the following relations are valid:

$$138\,000 = 7 \times \Delta\varepsilon_{trp} + 8 \times \Delta\varepsilon_{tyr}$$

$$\Delta\varepsilon_{trp} = 7 \times \Delta\varepsilon_{tyr}$$

From these equations we obtain 2420 and 16 940 for the molar absorption differences at 232 nm, due to the transfer of one tyrosine and one tryptophan, respectively.

A relatively wide variation is observed when the average values of molar absorption differences, published from different laboratories for the transfer of chromophoric groups, are compared. For example, the absorption difference at 293 nm measured for the transfer of one tryptophan residue is 2800 (Oppenheimer et al., 1963) or 2500 (Martin, Bhatnagar, 1966) in chymotrypsin, 1870 in phosphoglucose isomerase (Dyson, Noltmann, 1969), 1600 in aldolase (Donovan, 1964, 1969a) and 2650 in pancreatic amylase, as calculated in this paper. The variation in molar absorption differences of tryptophan transfer can be only partially attributed to different experimental conditions applied in various laboratories. An alternative explanation for the different molar absorption difference values observed with various proteins upon transfer from nonpolar to polar environment may be based on differences in the three-dimensional structural pattern of the complex system that we call the hydrophobic interior of a protein.

Spectrophotometry, similarly to some other indirect techniques, e.g. other optical, hydrodynamic, etc. methods, may yield only average information on the protein investigated. Neither specific differences of individual protein molecules of the same biological function, nor the specific properties of individual side chains within a protein molecule can be distinguished by these procedures. Therefore, very important characteristics of the protein structure are necessarily neglected, when structural conclusions are based only on the denaturation difference spectra. For example, we obviously cannot take into account the contribution of partially buried residues to the difference spectra. Furthermore, specific interactions, which may occur in the local environment of chromophoric groups can also influence the optical properties of chromophores. For the time being, spectrophotometric investigations can hardly denote anything particular about these interactions. Since a specific three-dimensional structure may create very particular conditions for the amino acid side chains, these may also be reflected in the denaturation difference spectra. Due to the very specific nature of these interactions we cannot easily distinguish by means of difference spectrophotometry those chromophores which are located in microenvironments of various composition. To obtain specific information about the localization of certain chromophoric groups spectrophotometry can be successfully combined with specific chemical modifications (Williams, Laskowski, 1965; Libor, Előd, 1970).

It follows from the above considerations that uniform absorption differences are not necessarily expected when the transfer of aromatic chromophores in different proteins is investigated. Since these interactions are brought about by specific structural conditions, difference spectrophotometry of proteins seems to be a more suitable method for following the structural alterations of proteins, than for the quantitative determination of chromophoric groups located in different parts of a protein molecule. When the latter estimation is attempted,

various proteins should be treated individually and experiences with a certain protein should be generalized or transferred to others with great care.

Kinetics of urea denaturation

Amylase dissolved in 0.01 M tris buffer, pH 7.4, was first treated with diisopropyl fluorophosphate. The rate of denaturation was studied at 22 °C in a concentration range of 5 to 9.5 M urea. The solutions contained 8×10^{-6} M protein in the presence of 1×10^{-5} M calcium chloride. The changes in absorption were measured at 293, 287 and 232 nm. The decrease in absorption ($\Delta\varepsilon_t$) measured at time t was subtracted from the maximum decrease ($\Delta\varepsilon_{max}$) characteristic of each urea concentration. The plot of the logarithm of these differences *versus* time resulted in straight lines indicating that the unfolding of amylase in urea fitted first order kinetics. The rate constants of denaturation at different urea concentrations are listed in Table 4.

Table 4

Apparent first order rate constants of amylase unfolding in different urea concentrations in the presence of 1×10^{-5} M calcium chloride

Urea, M	k (min^{-1})		
	293 nm	287 nm	232 nm
5	0.011	0.010	0.011
6	0.046	0.048	0.048
7	0.18	0.18	0.16
8	0.32	0.38	0.35
9	1.1	1.3	1.2
9.5	1.8	1.9	1.9

The unfolding at neutral pH and low urea concentrations was very slow and different transitional equilibrium states were attained. The transition at pH 7.4 appeared in the concentration range of 4 to 7 M urea, as it is also seen in Fig. 2. At pH 4.0 the unfolding was very rapid and kinetic measurements were difficult to perform. The transition occurs at acidic pH in the concentration range of 2 to 5 M urea.

The equilibrium states in the transition range strongly depended upon urea concentration. According to the assumption of Martin and Bhatnagar (1966) the equilibrium at any urea concentration may be described as follows:

$$E_n + m\,U \underset{}{\overset{K_i}{\rightleftharpoons}} E_d\,U_m$$

where m equals the moles of urea, U, combining with E_n native protein and produces E_dU_m unfolded state. Thus, a straight line should be obtained by plotting log K_i *versus* log urea concentration, where the slope equals m, i.e. the

order of reaction with respect to urea concentration (Barnard, 1964). The apparent equilibrium constant, K_i for any urea concentration in the transition range is defined as

$$K_i = \frac{F}{1-F}$$

where F represents the fraction of protein in unfolded state. The F values can be calculated from the data shown in Fig. 2, corrected for the solvent effect (Martin, Bhatnagar, 1966).

As shown in Fig. 3 the plots of log K_i *versus* log urea concentration gave straight lines both at pH 7.4 and pH 4.0, and m values of 8.9 and 3.2 were obtained

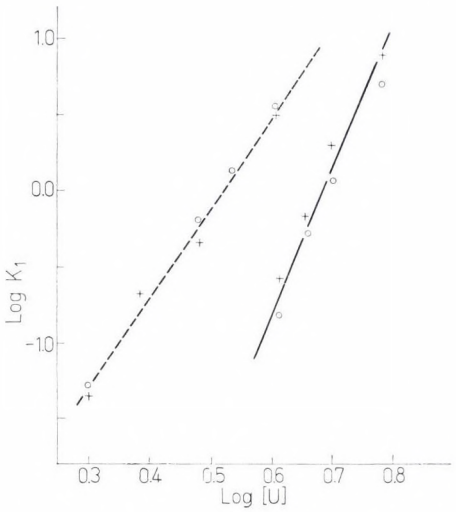

Fig. 3. Variation of the logarithm K_i apparent equilibrium constant of unfolding (see text) with the logarithm of urea concentration. Data were calculated on the basis of experiments shown in Fig. 2 from the readings taken at 293 nm (+) and 287 nm (o), corrected for solvent effect. Solid line: 24 hours experiment at pH 7.4; dotted line: experiment at pH 4.0, without incubation

at neutral and acidic pH, respectively. An m value of 15 was reported at neutral pH for DIP-chymotrypsin (Martin, Bhatnagar, 1966) and 16 for ribonuclease (Barnard, 1964).

Effect of calcium ions on urea denaturation

Amylase solution was gelfiltered on a Biogel P−2 column in 0.01 M tris buffer, pH 7.4, to remove excess calcium ions from the solution. The rate of denaturation caused by 8 M urea was measured with 8×10^{-6} M protein solution in the presence of 1×10^{-5} to 2×10^{-2} M calcium chloride added prior to urea treatment.

Addition of calcium ions markedly decreased the rate of unfolding and in the presence of 2×10^{-2} M calcium chloride practically no absorption change could be detected within 3 hours at 293 or 287 nm. At pH 4.0, on the other hand, calcium ions did not influence the denaturation of amylase in 8 M urea.

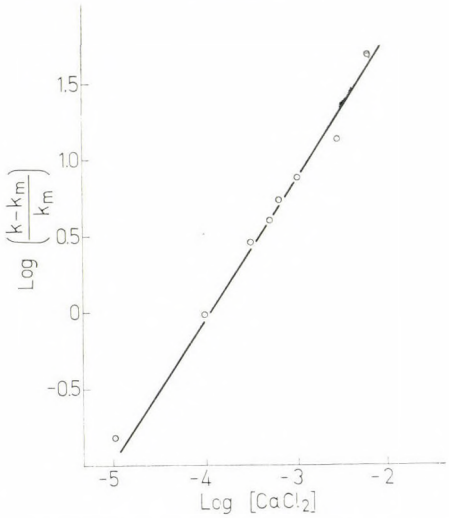

Fig. 4. Variation of log $(k_o - k_m)/k_m$ with the logarithm of calcium chloride concentration in 8 M urea, pH 7.4

The amount of bound calcium ions can be calculated from the protecting effect against urea, as suggested by Chervenka (1960). Accordingly, the denaturation of P protein in the presence of M metal ions is described as

$$PMn \overset{K_1}{\rightleftharpoons} P + nM$$

where PMn is the metal-protein complex containing n bound metal ions and K_1 is the apparent dissociation constant of the metal-protein complex. If k_{Ca}, the rate constant of denaturation of PMn complex is zero, the following relation holds:

$$\log \frac{k_0 - k_m}{k_m} = n \log M - \log K_1$$

where k_0 is the rate constant of denaturation of the free protein and k_m that measured in the presence of calcium ions. By plotting log $(k_o - k_m)/k_m$ *versus* log calcium chloride concentration a straight line was obtained (Fig. 4). The slope of this line is $n = 1$, and from the intercept $K_1 = 1 \times 10^{-4}$ M was calculated. Similar treatment of the data found with chymotrypsinogen and α-chymotrypsin also yielded n values near unity and K_1 values of 7.9×10^{-4} and 3.2×10^{-3} M, respectively (Chervenka, 1960; Martin, Frazier, 1963).

Effect of EDTA on the denaturation of amylase by urea

EDTA may react with the loosely bound calcium in amylase, but most probably it cannot remove the firmly bound calcium from the protein in the absence of urea (Hsiu et al., 1964; Stein et al., 1964). The influence of EDTA

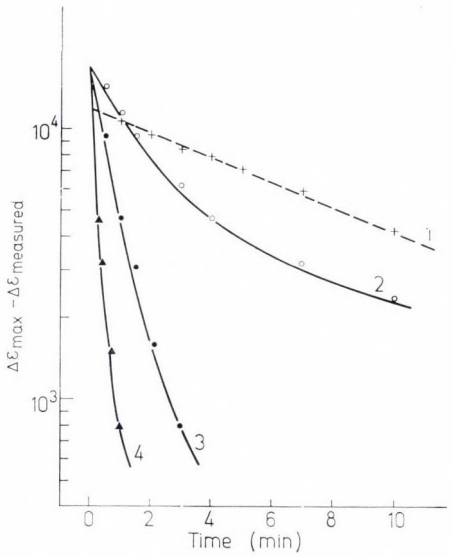

Fig. 5. Effect of EDTA on the rate of amylase denaturation in 8 M urea at pH 7.4, 22 °C. Amylase was treated with diisopropyl fluorophosphate and gelfiltered on Biogel P-2 column before addition of urea. Curve 1 — control, in the absence of EDTA; curves 2, 3 and 4 in the presence of 1×10^{-6} M, 1×10^{-5} M and 5×10^{-5} M EDTA, respectively

on the rate of the denaturation of amylase was studied with diisopropyl fluorophosphate-treated and gelfiltered protein solution in the presence of 8 M urea. EDTA was added in 10^{-7} to 5×10^{-5} M concentrations to a protein solution of 8×10^{-6} M final concentration. The decrease of absorption was followed at 293 nm. A pronounced increase was observed in the rate of denaturation already at 1×10^{-6} M EDTA concentration (Fig. 5). The unfolding reaction was completed within 4 minutes, when EDTA concentration reached 1×10^{-5} M, i.e. when the molar ratio of EDTA to amylase exceeded 1:1. The semilogarithmic plot of $\Delta\varepsilon_{max} - \Delta\varepsilon_t$ *versus* time (Fig. 5) failed to give straight lines indicating that the first order kinetics of amylase denaturation changed to a more complex reaction in the presence of EDTA.

This effect can be interpreted by assuming that the polypeptide chain of amylase is composed of segments which interact differently with urea in the presence and absence of EDTA, respectively. It can be supposed that EDTA in 8 M urea may have reacted with the firmly bound calcium and also that part of the polypeptide chain underwent a structural alteration which was resistant

to 8 M urea in the absence of EDTA. The rate of denaturation of the part which is involved in the binding of firmly bound calcium, may be different from those which are not stabilized by this relatively strong interaction. This may also be reflected in the complex reaction which was observed in the presence of EDTA. The characterization of the chemical nature of calcium binding site(s) is in progress in our laboratory.

The authors are indebted to Dr D. M. Kirschenbaum for his valuable comments. The skilful technical assistance of Miss A. Bertók is greatly acknowledged.

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Discrimination of Isotopes by Living Systems*

I. Frog Hearts in ^{39}K and ^{41}K

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(Received June 1, 1970)

The very important role that potassium plays in living systems was widely treated in the literature (e.g. Ernst, 1963, 1966; Ling, Cope, 1969); many of the papers described the effect of potassium on frog hearts. Thus, it was proved that Straub-hearts washed e.g. 20 times with a K-free Ringer's-solution and brought thereby to standstill started beating again when the Ringer solution contained again potassium (Ernst, 1966). Neglecting the isotope ^{40}K (Ernst et al., 1959) we raised the question whether importance should be attached to the isotope ^{41}K. Experiments performed with KCl containing 96 per cent ^{41}K (and 4 per cent ^{39}K) showed that Ringer's solutions prepared with ^{41}KCl were at $P = 0.01$ level significantly more effective than those containing normal KCl (93 per cent ^{39}K, 7 per cent ^{41}K).

Introduction

After the general statement (Ringer, 1882–3) about the indispensability of inorganic compounds in a "physiological" solution, the special importance of potassium for the activity of the heart was emphasized (Howell, Duke, 1908). The further specialization (Zwaardemaker, 1921) according to which the heart effect is to be ascribed to the radioactive isotope ^{40}K turned out to be (at least) quantitatively unacceptable (e.g. Ernst et al., 1959). Notwithstanding the following facts were demonstrated (Ernst, 1966): frog hearts washed with a K-free Ringer's solution at intervals of e.g. 1/2 *h* 10–20 times, stopped beating; the ashes of some of these hearts still contained 1/3 – 1/2 of the original potassium content, others restarted beating when repeatedly treated with normal Ringer's solution.

Considering this situation we raised the question: what is the difference between the residual potassium content of the hearts, which stopped beating due to the treatment with a K-free solution, and the potassium content of the normal Ringer solution restarting the hearts beating. Below experiments are described in which the effect of a changed ratio of isotopes $^{39}K : ^{41}K$ was investigated.

* Concerning this question the author expressed a positive opinion several times (e.g. Ernst, E. "Introduction to Biophysics". Publishing House, Hung. Acad. Sci. Budapest, 1967. p. 414–5)

Methods*

In the commercially available analytical grade KCl the ratio of $^{39}K : ^{41}K$ comes to 93:7 per cent, in the other KCl used in these experiments the ratio was nearly the inverse one amounting to 4:96 per cent. The mass-spectrogram gave the values 13.5 and 0.04 for the ratios of $^{39}K : ^{41}K$ in the ^{39}KCl and ^{41}KCl, respectively.

The effect of these two kinds of potassium were compared on frog hearts and pieces of the sinus venosus the activity of which had stopped due to a treatment with K-free Ringer's solution; (for details of this procedure see our previous paper, Ernst, 1966). The stopped hearts or sinus-pieces were afterwards repeatedly treated with new solutions containing ^{39}KCl or ^{41}KCl in the amounts of 5, 10, 20, 40, 60 mg KCl/l, resp., in such a manner that each heart (of a series) with an odd number was given a Ringer's solution containing normal KCl and each one with an even number a solution prepared with KCl enriched in ^{41}K. They were then repeatedly treated, checked e.g. three times a day, and it was recorded whether they had restarted beating (+) or not (−) as follows: e.g.

heart No	1	2	3	4	5	6	7	8
+ or −	−	+	+	+	−	+	−	+

Four to ten series of such experiments were performed with the solutions containing 5−60 mg KCl/l. The results were evaluated mathematically by the *t*- or *chi*-square tests and also graphically.

The sinus-pieces were previously put in a normal Ringer's solution and it was observed which of them were contracting normally; only these latters were put afterwards in a K-free solution where they stopped beating after a certain period of time. Thereafter half of this series was put in a Ringer's solution containing normal KCl and the other half in a solution prepared with KCl enriched in ^{41}K. The further investigation was similar as described above for the hearts.

The temperature of the cold room in which the preparations were kept during the intervals between the treatments was +2−0 °C.

Results

1. *Frog hearts.* Six series of experiments, with 8 hearts each, were performed; the hearts on Straub-cannulae were treated 10−20 times with K-free Ringer's solution (6.8 g NaCl, 0.2 $CaCl_2$, 0.2 $NaHCO_3$ in 1000 ml of bidistilled water). After they had stopped beating Ringer's solutions containing 5, 10, 20, 40, 60 mg KCl and $CaCl_2$ per 1 were given (7.0 NaCl, *x* g KCl and $CaCl_2$, 0.100 g $NaHCO_3$ in 1000 ml of bidistilled water). Every series was read off (+ or −) until the hearts were dead, the results are shown in Table 1.

* Co-workers: M. Hajnal-Papp and M. Fenyvesi.

Table 1

Effect on heart activity of Ringer's solutions containing 5—60 mg KCl/l; number of hearts beating (+), or not beating (—)

mg KCl per l	Total numbers			Number of hearts			
				^{39}KCl*		^{41}KCl*	
		+	—	+	—	+	—
5	816	222	594	97	311	125	283
10	864	279	585	115	317	164	268
20	960	491	469	223	257	268	212
40	1104	634	470	330	222	304	248
60	1152	698	454	344	232	354	222

* For brevity's sake ^{39}KCl marks common KCl with the ratio of $^{39}K : {}^{41}K = 93 : 7$ per cent, and ^{41}KCl marks KCl containing potassium enriched in ^{41}K to a ratio $^{39}K : {}^{41}K = 4 : 96$ per cent.

The numbers in Table 1 indicate that 5—60 mg KCl + $CaCl_2$ per one liter of Ringer's solution are in increasing degree able to restart the hearts stopped by K-free Ringer's solution. 48 hearts stopped and treated afterwards with a Ringer's solution containing 5 mg KCl + $CaCl_2$ per liter were checked on 17

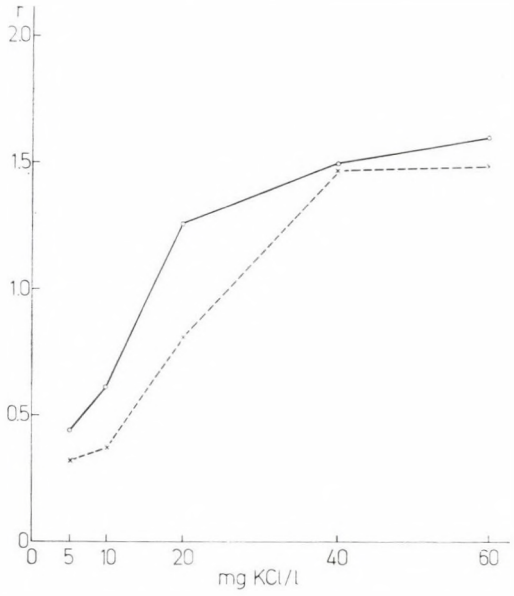

Fig. 1. Restarting effect of ^{39}KCl and ^{41}KCl; abscissa = mg KCl/l, ordinate: r = number of beating hearts (+) per number of not beating hearts (—) in a series of experiments performed with Ringer's solutions containing 5, 10, 20, 40 and 60 mg of KCl/l, resp.; ○—○ ^{41}KCl, x—x ^{39}KCl

occasions, i.e. in 816 cases, and were found beating in 222 cases, i.e. in 27 per cent. In contrast to this hearts treated with a Ringer's solution containing 60 mg KCl + $CaCl_2$ per liter were found beating in 61 per cent of the cases.

In addition, also the difference in the effectiveness of ^{39}KCl and ^{41}KCl is very conspicuous as shown in Fig. 1. At lower concentrations the solutions containing ^{41}KCl restart hearts beating in a greater proportion than do those made with ^{39}KCl. And, what is more important, in order *to exert equal restarting effects the Ringer's solutions need less ^{41}KCl than ^{39}KCl*, especially at lower concentrations.

2. *Pieces of the sinus venosus.* The following experiments were performed: in Ringer's solution containing

5 mg/l	^{39}KCl/l or	^{41}KCl/l	103
10 mg/l	^{39}KCl/l or	^{41}KCl/l	118
20 mgl/l	^{39}KCl/l or	^{41}KCl/l	139
40 mgl/l	^{39}KCl/l or	^{41}KCl/l	37
60 mgl/l	^{39}KCl/l or	^{41}KCl/l	58

pieces of sinus venosus previously stopped beating in the K-free solution were checked 5–10 times a day, and it was recorded whether they were contracting (+) or not (–). This reading-off lasted until no one single piece contracted any more. Table 2 contains all the pertaining data.

Table 2

Effect of Ringer's solutions containing different amounts of KCl/l; number of the sinus venosus contracting (+) or not (–)

KCl mg/l	^{39}KCl*		^{41}KCl*	
	+	–	+	–
5	109	247	130	226
10	182	449	234	397
20	572	685	730	518
40	227	205	308	124
60	299	207	296	210
Sum	1389	1793	1698	1475

* For brevity's sake ^{39}KCl marks common KCl with the ratio of $^{39}K : {}^{41}K = 93 : 7$ per cent, and ^{41}KCl marks KCl containing potassium enriched in ^{41}K to a ratio of $^{39}K : {}^{41}K = 4 : 96$ per cent.

A much deeper insight is obtained when a diagram is prepared from these results; that was accomplished by forming the ratio of

$$r = \frac{\text{number of } +}{\text{number of } -}$$

for the sinus-pieces contracting or not contracting in Ringer's solutions containing ^{39}KCl or ^{41}KCl. This diagram is shown by Fig. 2; it can be seen that, at very low KCl concentration, the restarting effect is also small and very similar for both ^{39}KCl and ^{41}KCl. On increasing the concentration of KCl also the

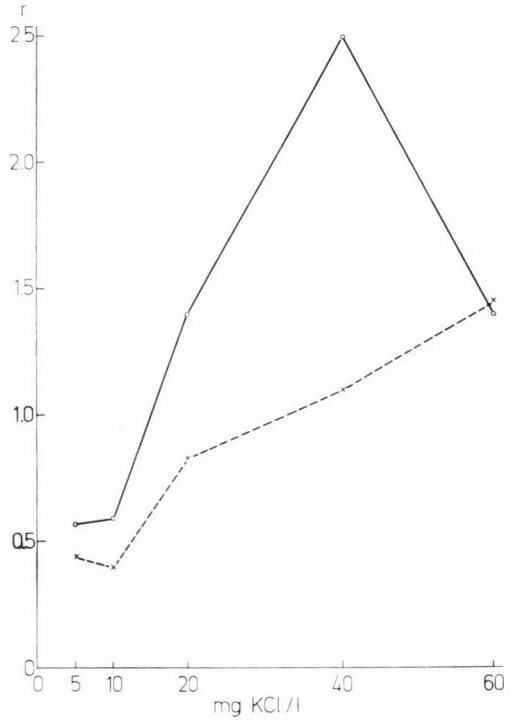

Fig. 2. Restarting effect of ^{39}KCl and ^{41}KCl; abscissa = mg KCl/l, ordinate r = number of contracting pieces/number of not contracting pieces in a series of experiments performed with Ringer's solutions containing 5, 10, 20, 40 and 60 mg of KCl/l, resp.; ○—○ ^{41}KCl, x—x ^{39}KCl

restarting effect increases, and *^{41}KCl proves to be more effective than ^{39}KCl.* Further, increase in the KCl concentration further increases the restarting effect, but *the effectiveness becomes nearly equal for ^{39}KCl and ^{41}KCl.*

Discussion

On the basis of the Tables and, especially, of the Figures one can easily establish the perhaps most important feature of these experiments: *equal restarting effects are exerted by less ^{41}KCl than by ^{39}KCl.*

At the present stage of our experimental research on these lines it would seem to be premature to seek theoretical explanations for the greater effectiveness of the heavier isotope of potassium. Instead of this, the fact should be considered

that among all metallic elements only In and Tl are capable of forming halogen compounds with the valency of 1 (similar to K) and that these occur in 2 isotope forms, the *heavier of which is enriched in the natural state* ($^{113}_{49}$In and $^{115}_{49}$In; $^{203}_{81}$Tl and $^{205}_{81}$Tl). Thus it seems advisable to perform similar experiments as described above with halogen compounds of both, though similar experiments with In^1Cl seem to be impracticable (perhaps InI in parallel with KI). Perspectives for a comparison of the effects of K and Tl seem more promising according to our knowledge concerning the *often* similar effects of Tl to that of K, as repeatedly described in the last decade (Mullins, Moore, 1959; Holló, Zlatarov, 1960; Naslund, Hultin, 1970; Pinkerton, Steinrauf, 1970).

Thanks are due to the Central Research Institute for Physics of the Hungarian Academy of Sciences for the mass-spectrography of our KCl compounds.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (4), pp. 469–476 (1970)

Discrimination of Isotopes by Living Systems

II. Barley Seedlings in D_2O and H_2O

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(Received June 1, 1970)

In accordance with the fact that "heavy" water is concentrated in biological materials to a very little degree only barely corns were germinated in the present experiments in an appropriate nutrient solution made with a) aqua bidestillata and b) bidistilled water containing 1 per cent D_2O. The germination of about 10 000 corns seemingly proceeded equally in both groups in which every single experiment was made with 50 corns. The 50 seedlings growing in a glass (with a dish-cover made of plexiglass) exerted force and performed work by lifting the plexi-cover loaded with 50 gr. *The seedlings in 1 per cent D_2O performed greater work than those in H_2O; the "Student" test showed statistical significance at a level of $P \leq 0.001$.*

Introduction

A survey of the influence of D_2O on biological systems shows very different results and views (Kritchevsky, 1960; London, 1961; Thomson, 1963). Viruses, bacteria, cells from plants and animals, tissues, organs and whole living organisms have been described to tolerate 0.5–99.9 per cent D_2O in a very unequal measure (e.g. Moses et al., 1958; Chorney et al., 1960). In contrast to the noxious effects of D_2O, especially at concentrations over 25–30 per cent, some data can be found in the literature about a certain "physiological" i.e. promoting effect (besides the preceding ones: Katz, 1960). In this series of experiments dealing with the more general problem if living organisms be able to distinguish isotopes the question was investigated whether any change takes place in material and force of barley seedlings growing in a nutrient solution made with D_2O of different percentages.

Methods*

In the experiments performed in 1968–1970 barley corns harvested in the preceding year were used; the nutrient solutions were as follows: The basic solutions: 1. 50 ml 10 per cent $Ca(NO_3)_2 . 4H_2O$, 2. 20 ml 10 per cent KNO_3, 3. 20 ml 10 per cent KCl, 4. 50 ml 2.5 per cent KH_2PO_4, 5. 20 ml 5 per cent $MgSO_4 . 7H_2O$, 6. 20 ml 5 per cent $FeCl_3 . 6H_2O$. From these solutions were

* Co-worker: G. Metzger–Török.

taken: *1)* 5 ml, *2)* 1.25 ml, *3)* 0.6 ml, *4)* 5 ml, *5)* 2.5 ml, *6)* 1 drop diluted a) in 485.5 ml aqua bidestillata and b) in the same amount of fluid containing D_2O of different percentages.

The germination of the corns took place in uniform glasses made of glass tubes the wall-thickness of which amounted to 2 mm and the internal diameter to 6 cm; the height of the glasses was 9 cm. The glasses had dish-covers (*c*) on their tops, made of sheets of plexi-glass (1 mm in thickness) and having rims

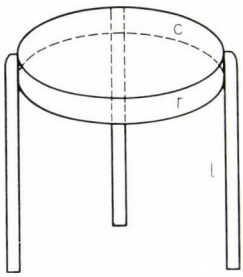

Fig. 1. Dish-cover; r = rim of it, l = leg of it

(made of the same sheet of plexi-glass) as shown in Fig. 1 (*r*). The growing seedlings lifted the dish-cover which was held in proper position by three legs (6 cm rodlets of plexiglass) fixed to the rims of the dish-cover at intervals of 120° (*l*).

The barley corns were germinated on filter paper placed on a low frame (made of glass rods 2 mm in diameter) standing on 4 (1 cm) legs at the bottom of the glasses as shown in Fig. 2. The ring of the frame was stiffened with two

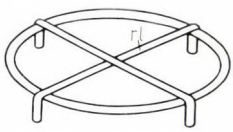

Fig. 2. The frame on which a sheet of filter paper bears the barley corns

rodlets of glass *(rl)* to ensure that the filter paper should be able to bear the weight of 50 corns or seedlings. The edges of the filter paper were bent down to the bottom to imbibe the nutrient solution poured on the bottom of the glass.

At the beginning of the experiments 15 ml of the nutrient solution were poured into each glass; later, when the nutrient solution decreased, further 5–15 ml were added repeatedly. In order to not disturb the whole system of the growing seedlings by newly supplying it with nutrient solution, an oblique hole was bored into the top sheet of the dish-cover, and the new portion of nutrient solution was injected through this narrow perforation. (The increase in evaporation due to this little perforation was neglected.)

A single experiment consisted of two parallels: 50 barley corns germinating in a nutrient solution a) with normal water, b) with water containing D_2O. The glasses containing the seedlings were put in natural light and irradiated during the day by sunshine. To ensure that the seedlings in both glasses should obtain equal amounts of light and sunshine, the glasses were alternately turned towards light and sunshine. This was arranged as follows: glass vessels 5 cm in height

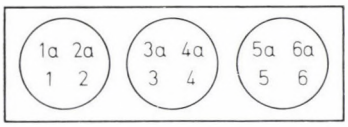

Fig. 3. The wooden tray with 3 large vessels in which the positions of the glasses containing 50 seedlings are marked

and 20 cm in diameter contained a plate of plexi-glass of 1 cm thickness, fitting into the vessels and resting on 1 cm high feet. The plate had 4 diagonally arranged holes bored for the glasses which, once having been put in these perforations, stood fixed in them so that the positions of the glasses were not influenced when the vessels were turned by 180°.

In every vessel 2 experiments took place (each with D_2O and the control); 3 vessels (with 4 glasses in each) were placed on a large wooden tray so that 6 experiments with controls were performed simultaneously situated as shown in Fig. 3. After turning the large wooden tray by 180°, the controls ($1a - 6a$) came in the same position as the D_2O-seedlings had been before, both getting equal illumination (and sunshine). The wooden tray was turned once a day or even three times on a summer* day, e.g. at the time intervals of $4^h - 9^h$, $9^h - 14^h$, $14^h - 19^h$. An experiment lasted 2–3 weeks.

Evaluation of the results was carried out in the following way: the heights to which the dish-covers loaded with 50 g had been lifted were measured every day and the same values in millimeter were recorded also for the controls. The differences between these values were computed according to the "Student" test. At the end of germination the dish-covers were unloaded and removed. Thereby, the seedlings straightened, they were counted in every glass, their plumules cut off and measured in fresh and dried state, then incinerated in an electric stove at 450 °C.

Results

1. The barley corns did not show any sign of germination in 99.8 per cent D_2O, in contrast to those in H_2O; this can be seen in Fig. 4 showing that the barley corns in 99.8 per cent D_2O (right) did not begin to germinate, in contrast to the controls in H_2O (left). Fig. 5 shows that the seedlings germinating in 50 per cent D_2O (right) grow much less than the controls in H_2O (left). Fig. 6 demonstrates that the barley seedlings germinating even in 10 per cent D_2O

* Hot summer days did not prove useful for such experiments (c.f. e.g. Thomson, 1963 p. 63).

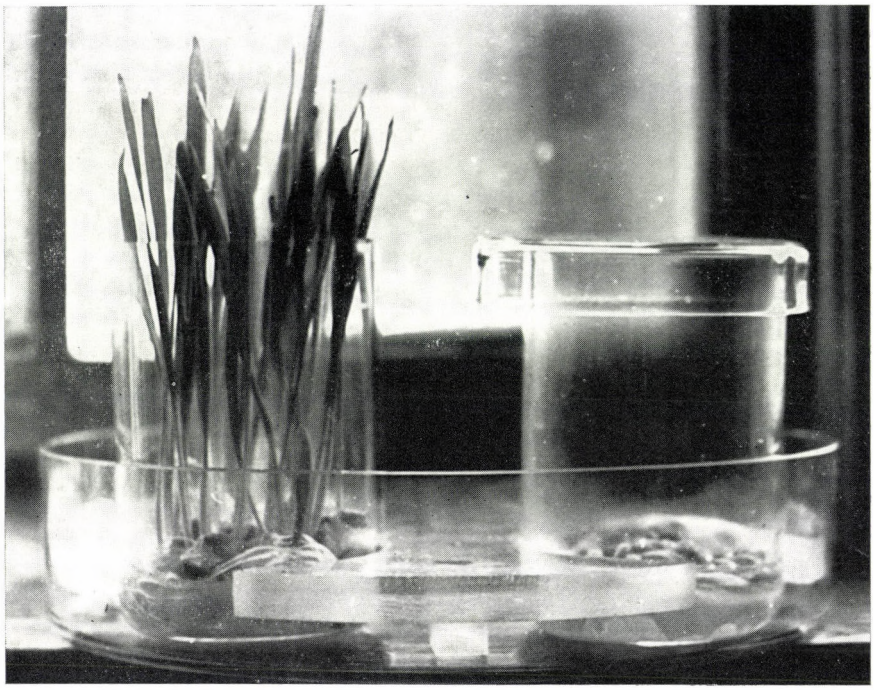

Fig. 4. Barley corns germinating in 99.8 per cent D_2O (right) and in H_2O (left)

(right) lag behind the controls growing in H_2O (left). Fig. 7 shows that barley seedlings grow seemingly equally in 1 per cent D_2O (right) and H_2O (left). See however item 3.

2. The germination in the nutrient solution made with 1 per cent D_2O was seemingly equal to that in H_2O. In 66 such experiments (D_2O- and H_2O-experiments) $2 \times 66 \times 50 = 6600$ barley corns were germinating; of the corns put in a single glass 43 ones germinated on the average (35–49). The summed results in Table 1 show that no significant weight differences were found between the barley seedlings germinated in nutrient solution made either with H_2O or with 1 per cent D_2O.

Table 1

Seedlings of barley corns

Solution made with	Number of plumules	Weight		
		fresh g	dried g	ashes mg
H_2O	2829	425.30	30.55	2.2372
1 per cent D_2O	2836	418.05	30.60	2.2126

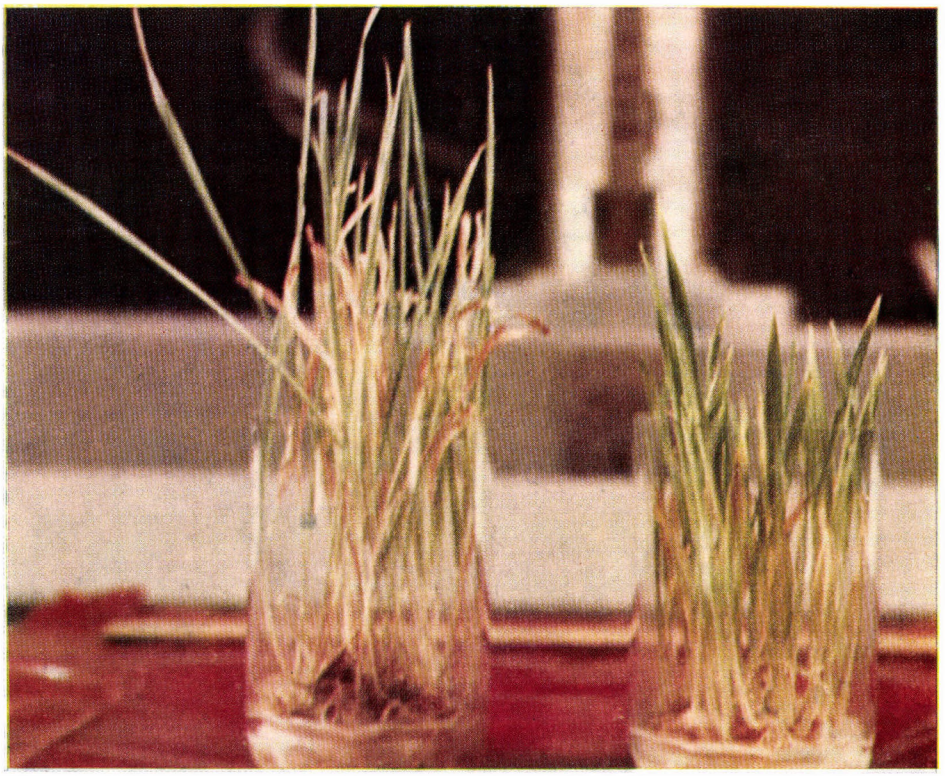

Fig. 5. Barley seedlings in 50 per cent D_2O (right) and in H_2O (left)

3. In contrast to what was described in items 1 and 2, Fig. 8 shows that the seedlings in 1 per cent D_2O (marked with even numbers) lifted the dish-covers higher than did the H_2O controls (marked with odd numbers).

90 experiments were performed in which the dish-covers of the glasses containing the growing seedlings were loaded with 50 g (the load consisting of lead shots put in a matchbox could be changed). The growing seedlings lifted the loaded dish-covers to different heights in the parallel experiments performed in nutrient solution made with 1 per cent D_2O or H_2O. The 180 numerical values marking the differences between the heights to which the dish-covers had been lifted were computed with the "Student" formula used for $n \gg 1$ in the reduced form of

$$t_n = \frac{\sum_{i=1}^{90} x_i}{\sqrt{\Sigma (x_i - \bar{x})^2}}$$

This formula gave the value $t_{90} = 3.83$ against the tabulated value of $t_{90} = 3.40$, which means that *the seedlings growing in D_2O performed greater work than those in H_2O, the numerical value representing significance at a level of $P < 0.001$.*

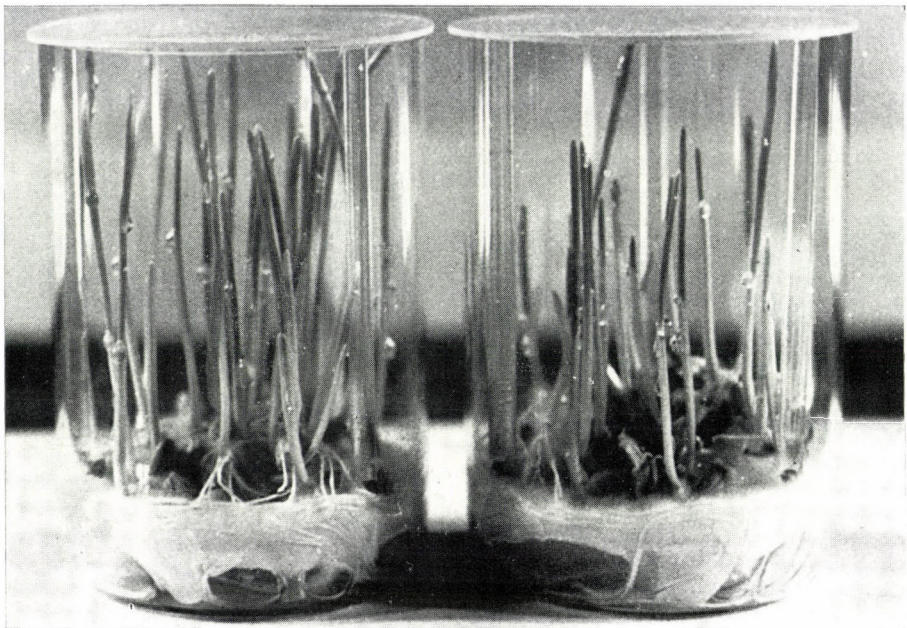

Fig. 6. Barley seedlings in 10 per cent D_2O (right) lagging behind those in H_2O (left)

Fig. 7. Barley seedlings in 1 per cent D_2O (right) and in H_2O (left)

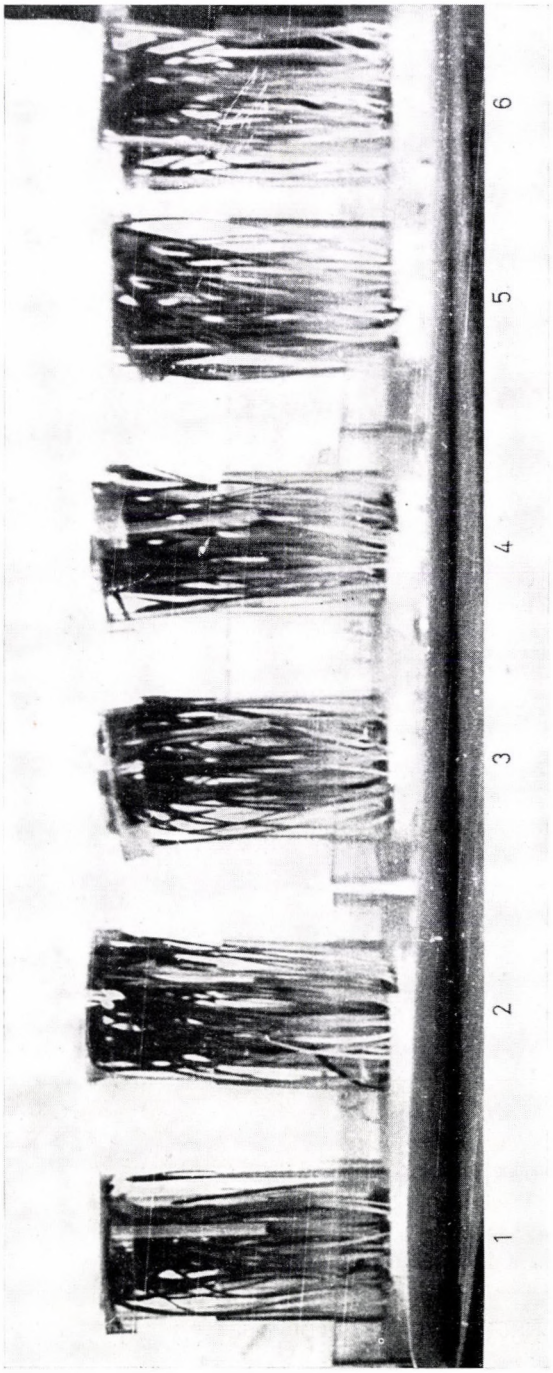

Fig. 8. Barley seedlings in 1 per cent D_2O marked with even numbers lift the dish-covers higher than the controls in H_2O (odd numbers)

Discussion

The results described above indicate that D_2O inhibits the germination of barley corns, when used at high concentration level but it does not in a low concentration. Moreover, barley seedlings growing in D_2O and lifting their covers higher, perform greater work than those in H_2O. These results seem, on the whole, to be in agreement with the data of the literature, though the greater work exerted by the 1 per cent D_2O seedlings requires explanation.

In this connection the book about "Hydrogen bonding" (Hadzi, Thompson, 1957) may be mentioned, which deals with the energy values of H-bonds from several points of view. In addition, the problem of the changes in the strength of the hydrogen bond at $H \rightarrow D$ substitution should be considered (besides the preceding ones: e.g. Calvin et al., 1959; Scheraga, 1960; Dahlgsen, Lang, 1960; Creswell, Allred, 1962; Benjamin, Benson, 1963). The conformational changes in biopolymers due to the substitution of D for H may also be taken into consideration, as emphasized by some papers (e.g. Katchalski, Steinberg, 1961; Ken-Ichi Tomita et al., 1962; Hvidt, 1964; Di Sabato, Ottesen, 1965).

These and other points will certainly play a role in the picture in which the problem discussed here would get a reasonable formulation. Though this will be drawn but in the future some parts can already be found in the literature, e.g. Scheraga's "Protein Structure" (cf. Ernst, 1970). On these lines this question will probably arrive even at the general problem of bioenergetics.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (4), pp. 477—484 (1970)

Discrimination of Isotopes by Living Systems

III. The Effect of Heavy Water on the Motility and Lifetime of Sperms

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(Received July 13, 1970)

The *in vitro* effect of different D_2O concentrations on the motility and lifetime of frog sperms was investigated in fluid media containing 25, 50, 75 and 100 per cent, respectively, of D_2O. It was demonstrated that the duration of the movement of sperms increases significantly ($P = 0.001$) in the case of a D_2O concentration of 50 per cent or more. The period of time up to the decrease of the number of moving sperms to its half increases with the increase of the concentration of D_2O; the relation between the two data can be described, as first approximation on the basis of a linear regression. Our data corroborate the view that the biological system does discriminate the isotopes of a given element.

Introduction

Some years after the discovery of the deuterium by Urey et al. in 1932 (Urey et al., 1932, 1932a) communications concerning the biological effect of heavy water have already appeared. Kinosita and Nakamura (1935) investigated the effect on the activity of isolated frog hearts of Ringer's solution containing heavy water in concentrations both lower and higher than the 0.02 per cent heavy water content of normal water. It was demonstrated that the working amplitude of the hearts decreased alike in the case of heavy water concentrations both lower (0.004 per cent) and higher (0.08 and 1.0 per cent) than the normal (0.02 per cent). Their results contradict the results of Brandt's (1935) similar experiments who observed a complete inefficiency up to a heavy water concentration of 10 per cent. The fact that heavy water concentrations higher or lower than the normal have an inhibitory effect on the increase was demonstrated also by Kinosita and Nakamura (1935) on chicken embryo tissue-pieces. Itoh et al. (1935) described that heavy water concentrations of 0.03 to 2.2 per cent stimulated the multiplication of *Mycobacterium tuberculosis*. On the other hand, the data of Harvey and Taylor showed (1934) the multiplication of microbes *(Vibrio phosphorescens)* to decrease in the case of D_2O concentrations from 36 to 86 per cent. Contrasting with this, Hansen and Blegen (1935) demonstrated that the multiplication of microbes *(Staphylococcus albus, Eberthella typhosa)* was not inhibited even by D_2O concentrations over 92 per cent.

Following these pioneer attempts, there appeared especially in the last two decades, a number of communications concerning the biological effect of heavy water. Curtis and Cole (1942), Hodgkin and Huxley (1939), Tasaki (1950, 1955), Spyropoulos (1956, 1957, 1957a), Spyropoulos and Ezzy (1959) published data concerning the effect of heavy water on the activity of isolated nerves.

Katz and Crespy (1966) reported the effect of D_2O on different kinds of microbes, algae and plants.

Goodall (1958), Svensmark (1961) and Jenerich (1963, 1963a, 1964, 1964a) described the effect of heavy water on the mechanical work and action potential of striated muscle.

Stein and Forrester (1963), Siegel et al. (1964), Striguckij and Nikolskij (1967) published data on the inhibitory effect of high D_2O concentrations on the growth of plants.

Among the Hungarian authors Lábos (1968), Holland and Antoni (1968, 1970) as well as Ernst (1970, 1970a) reported experiments performed on biological objects with heavy water.

We have only very few data as to the effect of D_2O on sperms. Rostand (1935) described that frog sperm *(Rana temporaria)* remains able to move and fertilize in 99 per cent D_2O for 12 to 22 hours but thereafter it loses its motility and fertilizing ability. Amarose and Czajka (1962) reported the fact that the testis of mice that had drunk heavy water did not produce sperms if the body fluids reached a D_2O content of 30 per cent. According to Hughes and Calvin (1958), and Hughes et al. (1959, 1960) male mice when having drunk 30 per cent D_2O for six weeks exhibited aspermia and sterility.

These few and equivocal data prompted us to investigate the effect of D_2O on the lifetime and motility of frog sperms.

Methods*

The change in time of ripe, moving frog-sperms *(Rana esculenta, Rana ridibunda)* was investigated under isolated circumstances *(in vitro)* in Holtfreter fluid medium.

For the experiments a Holtfreter solution of a concentration ten times higher than usual was prepared. Its composition was as follows:

3.50 g NaCl
0.05 g KCl
0.10 g $CaCl_2$
0.20 g $NaHCO_3$ dissolved in 100 ml of bidistilled water.

For the examinations five 4 ml samples of different D_2O concentration were prepared. Their composition is shown in Table 1.

Two testes of the decapitated frog were isolated cut into pieces and about equal quantities smashed up by a glass rod in the solutions indicated in Table 1. Finally, the sperm suspensions were poured off from the tissue fragments.

* With the collaboration of Cs. Lajtai.

In this way 5 sperm suspension samples of equal volume and different D_2O concentration (0, 25, 50, 75 and 100 per cent) were obtained. The pH of the samples was between 7.2 and 7.4.

During the experiment we took great care that the samples be always in equal environmental circumstances. Sperms are extremely sensitive to heat and light. Therefore it was very important to ensure equal heat and light conditions. The samples were kept in a refrigerator in darkness at a temperature of +6 °C.

Table 1

The composition of solution samples

Holtfreter [1] solution (ml)	D_2O[2] (ml)	H_2O[3] (ml)	D_2O (per cent)
0.1	—	3.9	0
0.1	1	2.9	~25
0.1	2	1.9	~50
0.1	3	0.9	~75
0.1	3.9	—	~100

[1] concentration ten times higher than usual
[2] 99.8 per cent
[3] bidistilled H_2O

At the time of beginning of the experiment and then once every day the number of moving sperms was determined in a Bürker chamber. In a solution-sample of 1 μl the starting value (N_0) of the number of moving sperms was between 500 and 1000. Considering the starting value (N_0) of the moving sperms as the unit the values obtained at each point of time (N) were related to these starting values. Thus, the number of moving sperms was expressed in a relative value (K) at each point of the time of the determination. A total of 36 experiments were made and the average of the values measured at the same point of time for fluid media containing the same concentration of D_2O was plotted in a coordinate system (Fig. 1). The data were evaluated statistically with significant difference-calculation and "t"-test.

Results

36 experiments were performed with the sperm samples in the fluid media containing different concentrations of D_2O. In each experiment a D_2O-free fluid medium was used as control, and four further medium samples with 25, 50, 75 and 100 per cent D_2O content resp. The number of moving sperms was counted as described in "Methods", at the beginning of the experiment (N_0) and every day thereafter. The experimental results are shown in Fig. 1.

The abscissa of Fig. 1 shows the time in hours and the ordinate the relative values (K) of the number of moving sperms (N) related to the starting value (N_0). The values in the figure are the average values from the 36 experiments.

The control curve indicated by the solid line shows, with a rough approximation an exponential running.

The decrease of the relative number of the moving sperms is slower in a fluid medium containing D_2O than in the control experiments. If the concentration of D_2O is increased – as it can be seen in the figure – the number of moving sperms belonging to each point of time also increases. The standard

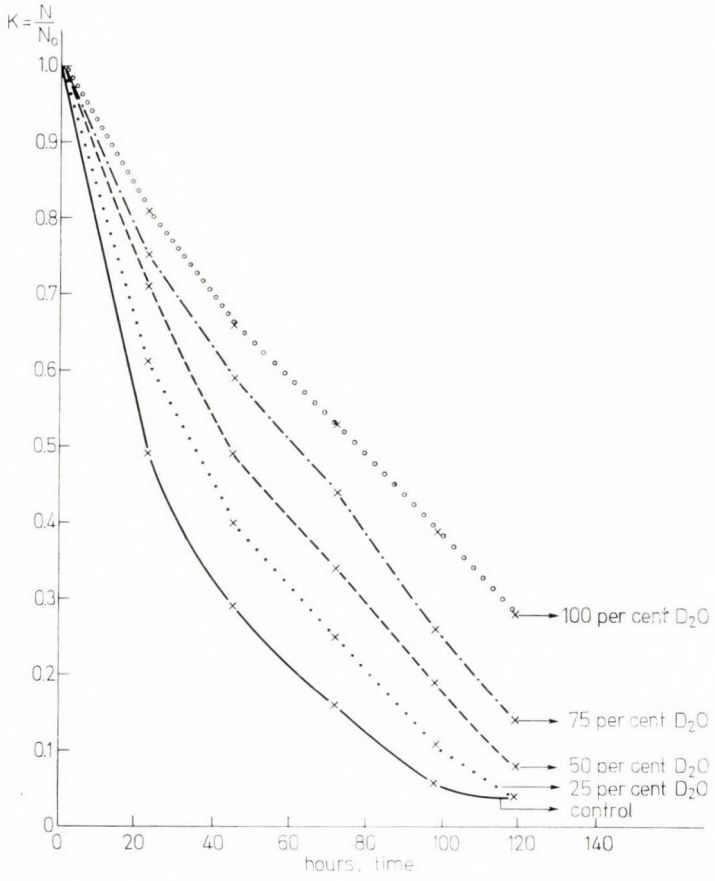

Fig. 1. Decrease in time of the number of moving sperms in fluid media of different D_2O concentrations

deviations belonging to each average value are relatively great because of the nature of the experimental object and because of the relatively significant error of the counting in Bürker chamber. The average values of the experimental data and the values of the standard deviation belonging to them are shown in Table 2.

The data of measurements were evaluated statistically with the aid of a two-sample "*t*"-test. The *K* values measured at each point of time with different

Table 2

The values of K at different points of time of the measurement with samples of different D_2O concentration

D_2O concentration (per cent)	Time (hours)				
	23	45	72	98	119
0 (control)	0.49±0.13	0.29±0.15	0.16±0.12	0.06±0.07	0.04±0.05
25	0.61±0.11	0.40±0.19	0.25±0.17	0.11±0.09	0.04±0.07
50	0.71±0.09	0.49±0.16	0.34±0.19	0.19±0.14	0.08±0.10
75	0.75±0.09	0.59±0.11	0.44±0.16	0.26±0.15	0.14±0.13
100	0.81±0.11	0.66±0.11	0.53±0.15	0.39±0.14	0.28±0.16

samples containing D_2O were compared with the K value measured in D_2O-free (control) sample. The results of this calculation are summarized in Table 3.

Table 3 shows the "t" values calculated from the experimental data for each point of time of the measurements performed with different concentrations

Table 3

The comparative statistical data of K values
($t - t_{(n-2)}$ values, P – the probability level of significance)

D_2O concentration (per cent)		Time (hours)				
		23	45	72	98	119
25	t	3.3	2.0	1.9	1.8	—
	P	0.01	—	—	—	—
50	t	6.4	3.8	3.6	3.4	1.4
	P	0.001	0.01	0.01	0.01	—
75	t	7.3	6.4	6.2	5.0	3.0
	P	0.001	0.001	0.001	0.001	0.01
100	t	8.8	8.4	8.7	8.7	5.8
	P	0.001	0.001	0.001	0.001	0.001

(t), as well as the levels of significance (probability) belonging to these "t" values (P).

It can be seen from the table that with samples of 25 per cent D_2O concentration the values differ significantly from the control values only in the first point of time of the measurement. With samples containing 50 per cent of D_2O a significant difference can be observed at a probability level of $P = 0.01$. With samples containing 75 and 100 per cent of D_2O the values similarly differ significantly from the control values at a probability level of 0.001 (except the 119 hour value of the sample containing 75 per cent of D_2O).

Thus, heavy water – at concentrations higher than 25 per cent – significantly increases the lifetime and the duration of motility, resp., of frog sperms. The significance becomes more and more expressed with increasing D_2O concentration.

On the basis of the experimental curves of Fig. 1 the time during which the number of moving sperms decreased to the half of the starting value (N_0), i.e. when the value of $K = \frac{N}{N_0}$ is 0.5, was determined by interpolation. This value is denoted with 0.5 K. The 0.5 K values determined in each D_2O-containing sample are shown in Table 4.

Table 4

The values of 0.5 K in samples of different D_2O concentration

D_2O concentration (per cent)	0.5 K (hours)
0 control	22
25	32
50	44
75	58
100	73

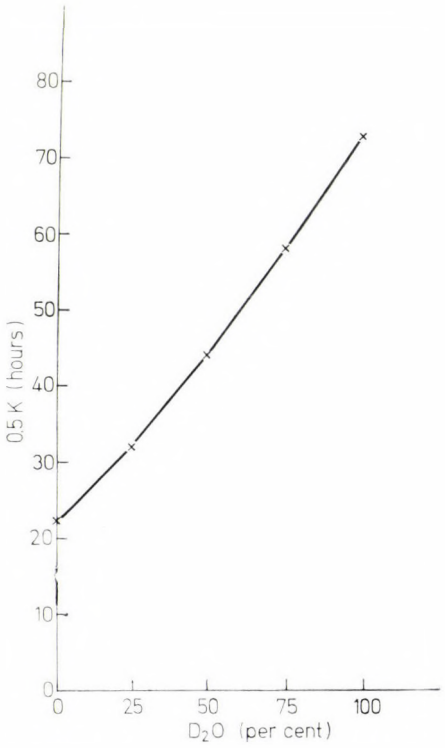

Fig. 2. Relation between D_2O concentration and the duration of the decrease to the half (0.5 K) of the number of moving sperms

It can be seen that with the increase of the concentration of D_2O the value 0.5 K also increases. The relation of the value 0.5 K to the concentration of D_2O is shown in Fig. 2.

Taking into consideration the error of counting in Bürker chamber and also the inaccuracy arising from interpolation, the relation between D_2O concentration and 0.5 K can be written, in the first approximation, as a linear relation. For the regression coefficient the experimental data gave the value of 0.51 ($b = 0.51$). So, the straight line appearing in Fig. 2 can be roughly described by the following relation:

$$y = 20.5 + 0.51\ x,$$

where y denotes the value of 0.5 K and x denotes the concentration of D_2O.

Discussion

A relatively great number of data has been published concerning the biological effect of D_2O, and in the last decade excellent summarizing works have appeared on this topic (Furness, 1960; Thomson, 1963). For all that, the question cannot be considered as clarified. The literature contains extremely few data concerning the effect of D_2O on sperms. According to Rostand's (1935) description the motility and fertilizing ability of frog sperms cease after 22 hours in a solution containing 99 per cent D_2O. Our data contradict to this. According to our measurements 80 per cent of the sperms remains moving after 22 hours in a fluid medium containing $\sim$100 per cent D_2O. The same value with the D_2O-free control sample is about 47 per cent. According to our experimental data 28 per cent of the sperms is still mobile even after 120 hours in this fluid medium. Thus, heavy water influences the motility of sperms in a favourable way, it increases the duration of motility. And motility is in close connection with fertilizing ability.

As aspermia and complete sterility was observed in mice drinking D_2O (D_2O of 30 per cent for 6 weeks) (Amarose, Czajka, 1962; Hughes, Calvin, 1958; Hughes et al., 1959, 1960), heavy water probably exerts an inhibitory effect on sperm production, but it influences the duration of the motility of ripe sperms in a favourable way. In the interpretation of this phenomenon a role must be undoubtedly ascribed to bioenergetic standpoints (Ernst, 1970, 1970a).

Our experimental data support the view (Fenn et al., 1942; Rabinowitz et al., 1958; Ernst, 1970a) that, in a biological system, the effect of various isotopes of the same element is different, and they support the validity of the isotope effect in biological systems.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 5 (4), pp. 485–487 (1970)

Submicroscopic Transverse Organization of the Cross-striated Fibril

Preliminary Report

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(Received July 30, 1970)

A contribution to the clarification of the question of the about 400 Å cross striation (Draper et al., 1949; Ernst, 1963; Huxley, 1953, Sjöstrand, 1962) obtained on striated fibrils has been made by our earlier work. In this shadowing of electron microscopic section was used to demonstrate (Trombitás, 1969) that the about 400 Å periodicity in the electron micrographs represents real structural elements, viz. a system of 400 Å transverse elements at right angles to the longitudinal direction. According to Ernst and his coworkers (Ernst et al., 1969) the submicroscopic structure of native striated fibrils consists of a network of longitudinal and transverse elements.

We have raised the following question: do the surfaces of transverse fractures of the fibrillary fragments produced by homogenization show the presumable transverse structure?

Our experiments were made on frog sartorius *(Rana esculenta)*. The slightly stretched muscle was fixed in glutaraldehyde, minced with a safety razor blade, and then homogenized, for one hour, in pH 7.4 cacodilate buffer with a Potter type homogenizer operated by hand. The homogenate was embedded in araldite in the usual manner. This homogenization resulted in isolated fragments of 1–2 sarcomeres.

Electron microscopic study of the fractures of the fibrils in thin sections showed that the fractures were mainly transverse, regular, with their straight border lines at right angles to the longitudinal axes of the fibrils. In Fig. 1 the transverse lines of 400 Å periodicity can be clearly seen both in the I and the A bands. In the right upper corner of the figure the fracture of the I band occurred along a transverse line. At the other end of the same fibril the surface of fracture shows a recess of about 400 Å. A similar phenomenon can be seen also in Fig. 2. At the bottom of this figure the sarcomere broke at about one third of its length; here, in the middle of the fibril, a projection of about 400 Å can be observed. The middle of this fibril broke at the I–A border and, at the left edge of the lower broken surface, 2 steps of the scale corresponding to the transverse line broke off the I band.

According to our experiments a fibril may break at any point of the sarcomere, thus either in the I or the A band, in a transverse direction, producing the regular surface of fracture mentioned above.

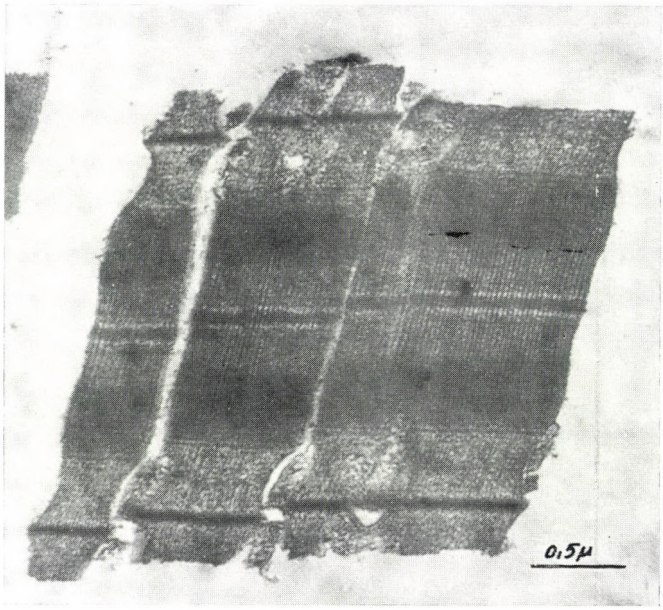

Fig. 1. Transverse line system of about 400 Å, both in the I and the A bands; fractures along the transverse lines

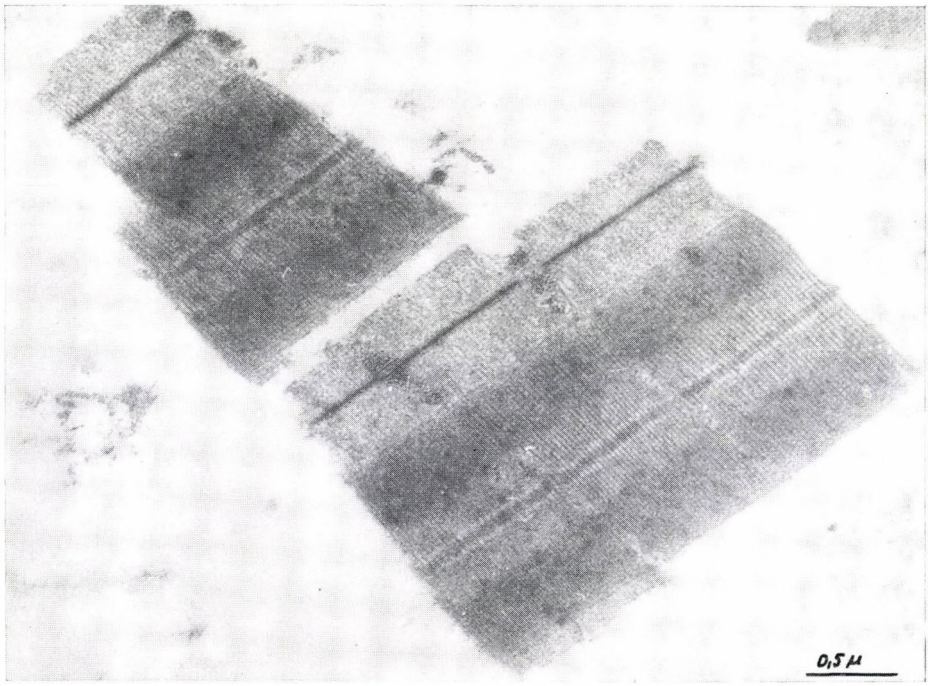

Fig. 2. Transverse surfaces of fracture, perpendicular to the longitudinal axes of the fibrils

The above-described results lend support of the concept that, in addition to its longitudinal organization, the fibril in the native state also has a submicroscopic transverse organization cutting at right angles the longitudinal axis.

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Printed in Hungary

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

A kézirat nyomdába érkezett: 1970. IX. 25. — Terjedelem: 10 (A/5) ív, 45 ábra (1 színes)

70.70459 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György

Hoppe-Seyler's Zeitschrift für Physiologische Chemie

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Vol. 351. No. 9 **Contents** September 1970

Walter de Gruyter & Co · Berlin 30

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