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Inhibition of D-Glyceraldehyde-3-phosphate Dehydrogenase by ATP and Quinaldate

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(Received November 8, 1978)

The inhibition of D-glyceraldehyde-3-phosphate dehydrogenase by ATP is of purely mixed type with respect to NAD ($K_i = 4.9$ mM), purely uncompetitive with respect to D-glyceraldehyde-3-phosphate ($K_i = 9.4$ mM) and partially uncompetitive with respect to inorganic phosphate ($K_i = 6.0$ mM).

Quinaldate is a purely mixed type inhibitor with respect to both NAD ($K_i = 10.0$ mM) and D-glyceraldehyde-3-phosphate ($K_i = 15.3$ mM), whereas purely non-competitive with respect to inorganic phosphate ($K_i = 11.0$ mM). In the presence of quinaldate a lag period is observed in the time course of enzyme reaction. The duration of this lag period depends on both quinaldate and substrate concentrations.

Introduction

Several authors analyzed the inhibition of GAPD** by ATP and their results are rather contradictory. The type of inhibition seemed to depend on the medium used and/or on the reversibility of the inhibition. The inhibition of enzymes isolated from rabbit muscle or yeast was found to be competitive with respect to NAD in the presence of mercaptoethanol (Yang, Deal, 1969), non-competitive if mercaptoethanol was absent (Sapag-Hagar, 1969), but mixed type in TES buffer (Oguchi et al., 1973). The pig muscle enzyme, in Tris buffer, is non-competitively inhibited with respect to NAD in the absence of mercaptoethanol, due to the irreversibility of inhibition by ATP under these circumstances. However, the inhibition is of mixed type with respect to NAD in the presence of mercaptoethanol, when the ATP induced inhibition is reversible (Ovádi et al., 1971). Mixed type inhibition was also found between ADP or AMP and NAD, in the case of rat muscle enzyme (Nagradova et al., 1969), between

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** *Abbreviations:* GAP, D-glyceraldehyde-3-phosphate; GAPD, D-glyceraldehyde-3-phosphate dehydrogenase (EC.1.2.1.12); TES, N-tris (hydroxy-methyl)-methyl-2-amino-ethan sulfonic acid.

ATP and P_i in the case of rabbit muscle enzyme in TES buffer and non-competitive inhibition with respect to GAP in the same conditions (Oguchi et al., 1973).

It has been shown that quinaldate (quinoline-3-carboxylate), one of the physiological end-products of trytophan catabolism, inhibits GAPD (Kőnig et al., 1975), but the type of inhibition was not analyzed. The enzyme is also inhibited by 4-quinolone-3-carboxylates (Yoshimoto, Hansch, 1976).

The aim of this work was to determine the type of inhibition by ATP and quinaldate towards the substrates of GAPD in glycine buffer, under conditions of reversible inhibition (cf. Tomova et al., 1977), in order to prepare the way for double inhibition experiments with these two components, (Lien et al., 1979).

Materials and methods

Four times recrystallized pig muscle GAPD was used (Elődi, Szörényi, 1956). Enzymic activity was measured spectrophotometrically in standard assay mixture (Keleti, Batke, 1965) in 0.1 M glycine buffer, pH 8.5, at 25°C. The molar specific activity of the enzyme preparations was 18 000–24 000 mole NADH/mole [enzyme × min. The molar extinction coefficient of NADH was taken to be $6.22 \times \times 10^3\ M^{-1}\ cm^{-1}$ (Horecker, Kornberg, 1948); enzyme concentration was calculated by using the coefficient $A^{1\ mg/ml}_{1\ cm,\ 280\ nm} = 1.0$ (Fox, Dandliker, 1956) and 145 000 for molecular weight (Elődi, 1958).

GAP was prepared according to Szewczuk et al. (1961).

NAD and ATP were Reanal, quinaldic acid (used as its Na-salt) was Aldrich-Europe preparation of 85%*, 90%** and 99% purity, respectively.

The measurements were carried out in an Opton PMQ II spectrophotometer with a Servogor automatic recorder. Calculations were performed with a Hewlett-Packard 9810 A desk calculator, using linear regression by the least squares method.

The data are the mean of 2–6 independent experiments.

Results and discussion

In order to determine the type of inhibition the initial (steady state) velocities of GAPD were measured in the presence of different concentrations of the two inhibitors, varying the concentration of one of the substrates and maintaining the concentration of the other two substrates at a constant, saturating level.

The types of inhibition were determined from Lineweaver–Burk plots (Lineweaver, Burk, 1934) and the values of K_i were derived from Dixon plots

* Determined enzymatically. The unknown contaminants do not inhibit GAPD activity (cf. its molar specific activity).

** Determined spectrophotometrically. The inhibition type of contaminating ADP or AMP is the same as that of ATP.

(Dixon, 1953), except for uncompetitive inhibitions. In the case of uncompetitive inhibitions the following relationships were used (Keleti, 1976):

$$K_i = \frac{a[I]}{b-a}$$

if the inhibition is partially uncompetitive, and

$$K_i = \frac{e[I]}{f-e}$$

if the inhibition is purely uncompetitive, where K_i is the inhibition constant, [I] the concentration of the inhibitor, a and b are the intercepts on the abscissa and e and f are the intercepts on the ordinate of the Lineweaver–Burk plot in the absence and presence of inhibitor, respectively.

The inhibition by ATP is purely mixed type with respect to NAD (Fig. 1), purely uncompetitive with respect to GAP (Fig. 2) and partially uncompetitive with respect to P_i (Fig. 3). The curves in the insert of Fig. 3 can also be fitted to straight lines. However, in this case the last points of *all* curves deviate in the same direction (i. e. it cannot be considered as random error and the deviation would be much greater than in the inserts of Figs 1, 2, 4–6). Double inhibition experiments (Fig. 1. B. in Lien et al., 1979) are also consistent with the partial type of ATP inhibition.

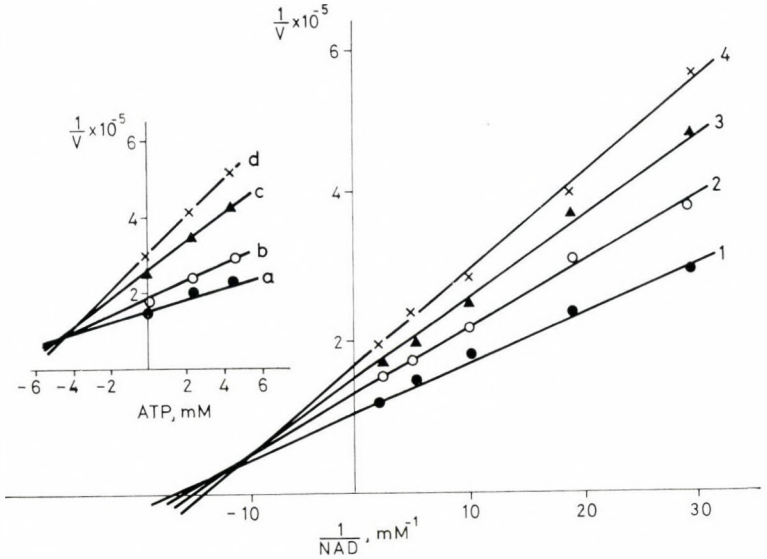

Fig. 1. Inhibition of GAPD by ATP using NAD as variable substrate. The reaction mixture contained 10 mM P_i, 2 mM GAP in 0.1 M glycine buffer, pH 8.5. The ordinate is in units of minute × mol^{-1} substrate. ATP concentrations = 1: 0; 2: 2.3 mM, 3: 4.5 mM, 4: 9.0 mM. Insert: Dixon plot. NAD concentrations = a: 0.2 mM, b: 0.1 mM, c: 0.05 mM, d: 0.03 mM

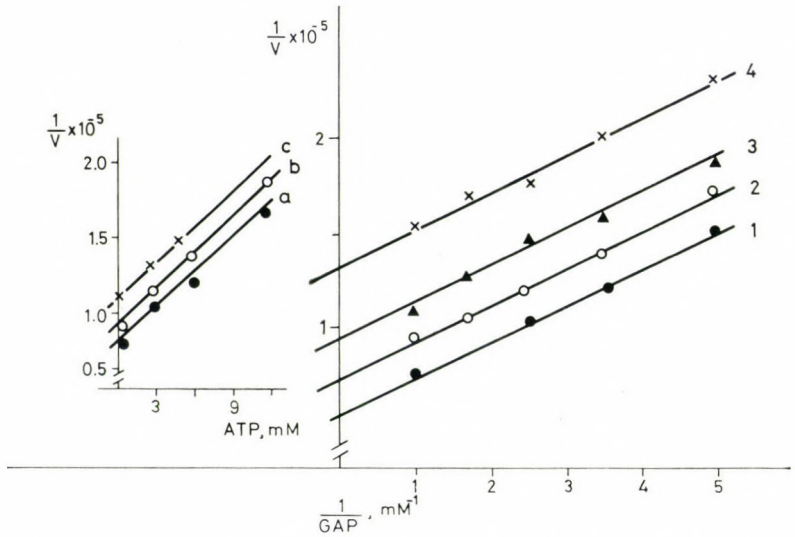

Fig. 2. ATP inhibition of GAPD using GAP as variable substrate. The reaction conditions and ordinate were the same as in Fig. 1 except that 2 mM NAD was employed and GAP concentration was varied. ATP concentrations = 1: 0; 2: 3.0 mM; 3: 6.0 mM; 4: 12 mM. Insert: Dixon plot. GAP concentrations = a: 1 mM; b: 0.6 mM; c: 0.4 mM

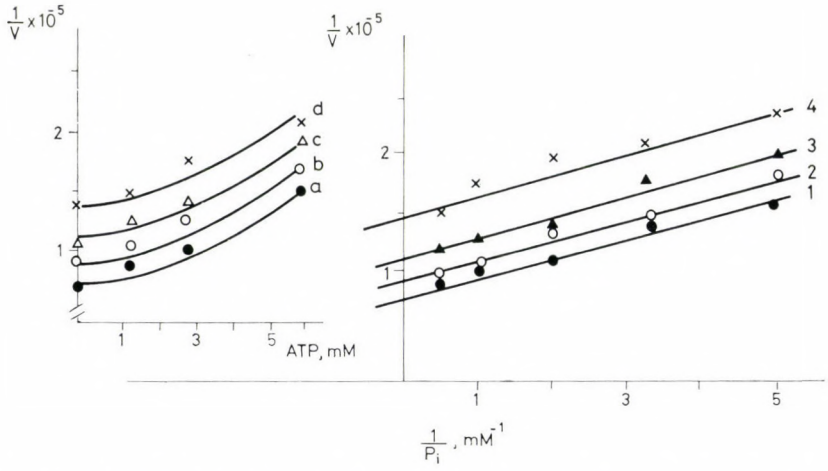

Fig. 3. ATP inhibition of GAPD using P_i as variable substrate. The reaction conditions and ordinate were the same as in Fig. 1 except for NAD concentration, which was raised to 2 mM and P_i concentration which was varied. ATP concentrations = 1: 0; 2: 1.4 mM, 3: 2.9 mM, 4: 5.8 mM. Insert: Dixon plot. P_i concentrations = a: 2 mM, b: 1 mM, c: 0.5 mM, d: 0.3 mM

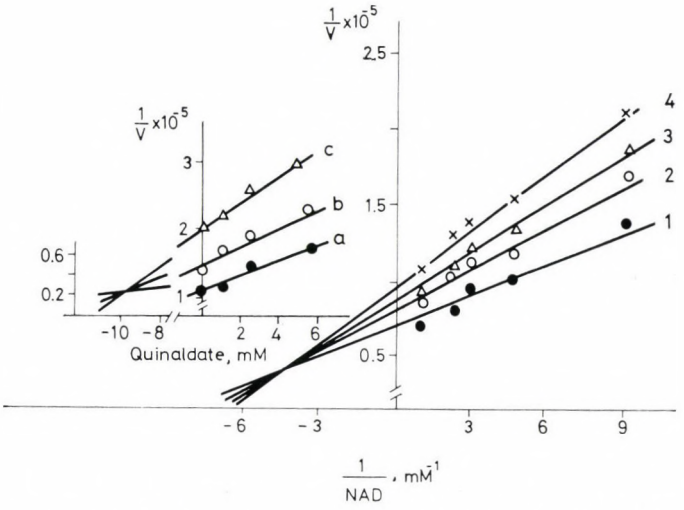

Fig. 4. Inhibition of GAPD by quinaldate using NAD as variable substrate. The reaction conditions and ordinate were the same as in Fig. 1. Quinaldate concentrations: 1: 0; 2: 1 mM, 3: 2.5 mM, 4: 5.0 mM. Insert: Dixon plot. NAD concentrations = a: 0.2 mM; b: 0.1 mM; c: 0.05 mM

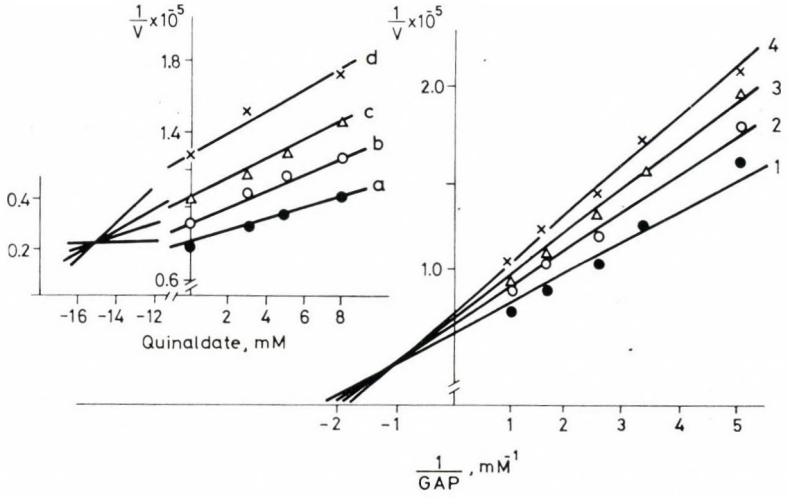

Fig. 5. Quinaldate inhibition of GAPD using GAP as variable substrate. The reaction conditions were the same as in Fig. 2, ordinate as in Fig. 1. Quinaldate concentrations: 1: 0; 2: 3 mM;, 3: 5.0 mM; 4: 8.0 mM. Insert: Dixon plot. GAP concentrations: a: 1 mM; b: 0.6 mM; c: 0.4 mM; d: 0.3 mM

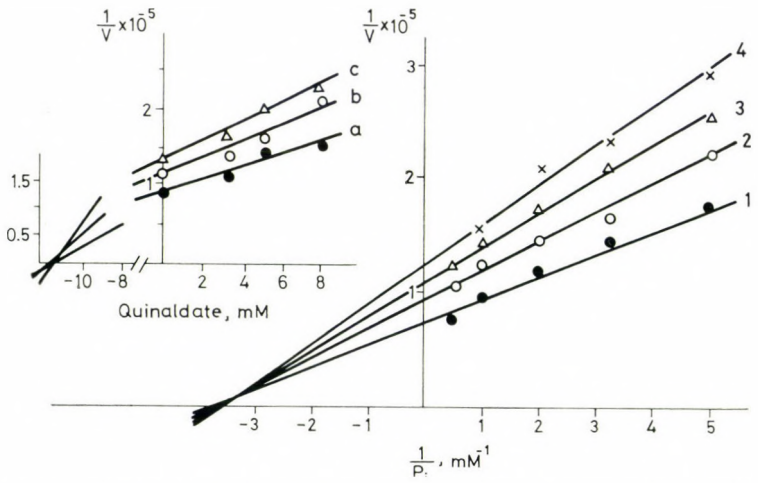

Fig. 6. Inhibition of GAPD by quinaldate using P_i as variable substrate. The reaction conditions were the same as in Fig. 3, ordinate as in Fig. 1. Quinaldate concentrations: 1: 0; 2: 3.3 mM; 3: 5.0 mM; 4: 8.0 mM. Insert: Dixon plot. P_i concentrations: a: 1 mM; b: 0.5 mM; c: 0.3 mM

Similar experiments were performed with quinaldate. Quinaldate inhibits GAPD in a purely mixed manner with respect to NAD (Fig. 4) and to GAP (Fig. 5). The inhibition with respect to P_i is purely non-competitive (Fig. 6).

The types of inhibition and the inhibition constants are summarized in Table 1.

That quinaldate is a pure type inhibitor with respect to all substrates indicates that none of the enzyme-substrate-inhibitor complexes is able to generate products. On the other hand, ATP is a partial type inhibitor with respect to P_i, which means that the ATP-enzyme-P_i complex is not completely inactive. The finding that the inhibition types of both inhibitors with respect to the different

Table 1

Types of inhibition of pig muscle GAPD by ATP and quinaldate

Substrates	ATP		Quinaldate	
	Type of inhibition*	K_i (mM)	Type of inhibition	K_i(mM)
NAD	Purely mixed	4.9	Purely mixed	10.0
GAP	Purely uncompetitive	9.4	Purely mixed	15.3
P_i	Partially uncompetitive	6.0	Purely non-competitive	11.0

* The nomenclature of inhibition types was described previously (Keleti, Telegdi, 1966).

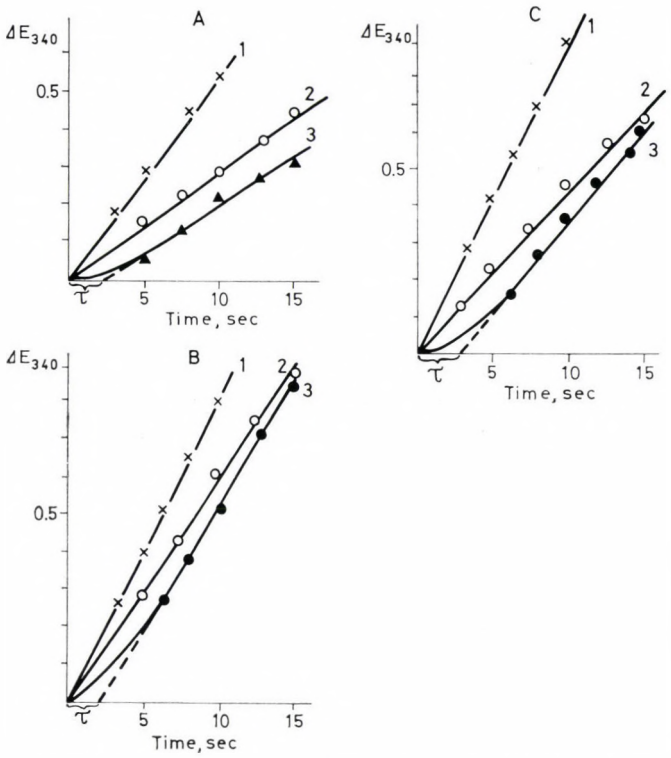

Fig. 7. "Lag" time of inhibition of GAPD by quinaldate. The activities of GAPD were measured at 0.05 mM NAD concentration (A), 0.2 mM GAP concentration (B), 0.2 mM P_i concentration (C). Other substrates were at saturating concentrations in 0.1 M glycine buffer, pH 8.5, at 25 °C. The enzyme reaction was started by addition of 6.9×10^{-6} mM GAPD. 1 — no inhibition; 2 — 6.0 mM ATP; 3 — 5.0 mM quinaldate

substrates are mixed, non-competitive or uncompetitive indicates that in all cases (except the ATP-enzyme-P_i complex) inactive substrate-enzyme-inhibitor complexes are formed. However, the uncompetitive type of inhibition by ATP with respect to GAP and P_i shows that ATP can be bound only by the enzyme-substrate complex (enzyme-GAP or enzyme-P_i), but not by the free enzyme. These results are in accord with the assumption that neither ATP (Ovádi et al., 1972), nor quinaldate (Kőnig et al., 1975) is bound at any of the substrate-binding sites of the enzyme.

The above findings and the data showing that the type of inhibition of quinaldate with respect to the coenzyme is similar for several different dehydrogenases support the hypothesis that this compound is a general inhibitor of dehydrogenases which acts at the catalytic site (Kőnig, 1975, Kőnig et al., 1975).

With quinaldate the steady state of the enzymatic reaction began after a lag time (Fig. 7). The initial velocities were determined in the presence of fixed con-

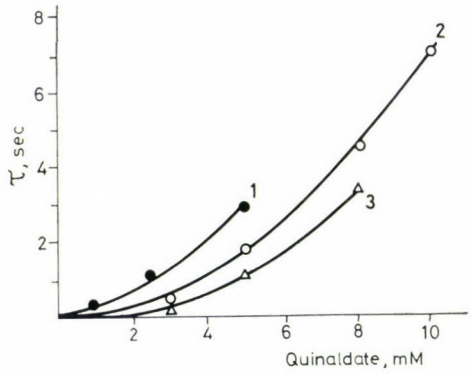

Fig. 8. Effect of inhibitor concentration on "lag" time. The initial velocities were measured at fixed substrate concentration: 1 — 0.03 mM of NAD; 2 — 0.2 mM of P_i; 3 — 0.2 mM of GAP. The other substrates were used in saturating concentrations. Quinaldate concentrations as indicated on the abscissa. In 0.1 M glycine buffer, pH 8.5. "τ" was determined graphically as in Fig. 7.

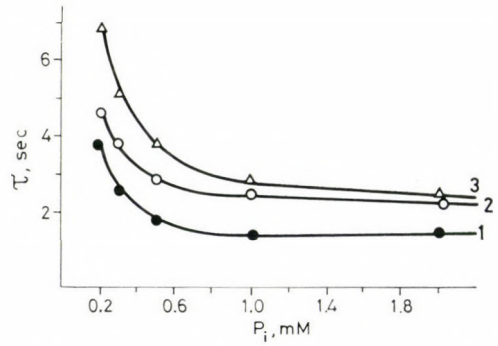

Fig. 9. Effect of substrate concentration on "lag" time. Quinaldate concentrations: 1: 5.0 mM; 2: 8.0 mM; 3: 10.0 mM. Other conditions as in Fig. 8.

centrations of substrate and various quinaldate concentrations. The duration of the lag phase seems to be a parabolic function of inhibitor concentration (Fig. 8). If the concentration of P_i was varied and quinaldate concentration was kept constant, the dependence of the lag phase on substrate concentration seemed to be hyperbolic (Fig. 9).

This feature of quinaldate inhibition was taken into account inasmuch as initial velocity data were taken after the lag phase from the linear, steady state part of the time course. The existence of the lag phase is consistent with the assumption that the association rate constant of the enzyme + inhibitor step is several orders of magnitude higher than the association rate constant of the enzyme + substrate step. Therefore, the enzyme-inhibitor complex is formed first, and this is

of course inactive. Later, the enzyme-inhibitor, enzyme-substrate and inhibitor-enzyme-substrate complexes become equilibrated and are present in amounts depending on their dissociation constants and on the concentration ratio of inhibitor and substrate.

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Double Inhibition of D-Glyceraldehyde-3-phosphate Dehydrogenase and Lactate Dehydrogenase

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The simultaneous action of ATP (partially uncompetitive inhibitor with respect to P_i) and quinaldate (purely non-competitive inhibitor with respect to P_i) on D-glyceraldehyde-3-phosphate dehydrogenase was analyzed kinetically. The interaction constant [as defined by Keleti and Fajszi (1971) Math. Biosci. *12* 197] of the two inhibitors for the D-glyceraldehyde-3-phosphate dehydrogenase-P_i complex is greater than 1, which means that the two inhibitors act antagonistically. The kinetic analysis of the double inhibition shows that there is no ATP-enzyme-quinaldate ternary complex, but a quaternary complex with P_i is formed. The interaction of the two inhibitors on the enzyme-P_i complex depends on substrate (P_i) concentration. The antagonistic effect of the two inhibitors becomes additive at low P_i concentrations (about 1 mM).

The simultaneous action of oxalate (purely uncompetitive inhibitor with respect to NAD) and quinaldate (partially mixed type inhibitor with respect to NAD) on lactate dehydrogenase was also analyzed. Oxalate and quinaldate act antagonistically on lactate dehydrogenase. However, at low NAD concentrations (about 0.06 mM) or at high quinaldate and low oxalate concentrations (around 7 and 1.7 mM, respectively) the antagonism turns into the simple summation of the effects of the two inhibitors.

Introduction

Many drugs exert their effect by influencing appropriate enzymes. Modern medicaments sometimes comprise two or more enzyme inhibitors acting simultaneously. Moreover, in the living cell several metabolites which may also behave as enzyme modifiers, act simultaneously on one and the same enzyme. Therefore, to study the effect of two or more modifiers on an enzyme is warranted.

The theoretical analysis of double inhibitions showed that modifiers, due to their interaction, may exert different kinetic effects on enzyme action depending on their types of inhibition. The study of these effects may elucidate the changes in steric structure caused by one or another inhibitor or by the substrate, the mecha-

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nism of action of inhibitors and some peculiar results of the modifier interactions (Keleti, Fajszi, 1971; Fajszi, 1976; Fajszi, Keleti, 1976).

D-glyceraldehyde-3-phosphate dehydrogenase is one of the key enzymes of the glycolytic pathway and, like other enzymes, it is affected *in vivo* by more than one modifier at the same time. The aim of this work is to examine the simultaneous effect of two inhibitors encountered in living cells, quinaldate and ATP*, on the activity of GAPD.

The inhibitory effect of ATP and quinaldate on GAPD, when added separately, has been analyzed in detail (Lien, Keleti, 1979). Theoretical consideration show that a triple faced enzyme inhibitor relation may exist if at least one of the inhibitors is of the partial type (Fajszi, Keleti, 1976). Since the inhibition of ATP is partially uncompetitive with respect to P_i, the latter was chosen as variable substrate.

With LDH quinaldate is a partially mixed type inhibitor in respect of NAD. Consequently, when we analyzed the simultaneous action of oxalate and quinaldate on LDH, NAD was used as variable substrate.

In this paper we study the interaction of the two inhibitors on GAPD and LDH and the change of their interaction as a function of substrate concentration.

Materials and methods

Four times recrystallized pig muscle GAPD was used (Előd i, Szörényi, 1956). Enzymic activity was measured spectrophotometrically in a standard assay mixture (Keleti, Batke, 1965), in 0.1 M glycine buffer, pH 8.5 or pH 7.5, at 25 °C. The molar specific activity of the enzyme preparations at pH 8.5 was 18 000 – 24 000 mole NADH/mole enzyme × min. The absorption of GAPD was taken to be $A^{1mg/ml}_{1cm,\ 280nm}$ = 1.0 (Fox, Dandliker, 1956) and its molecular weight = 145 000 (Előd i, 1958).

Pig muscle lactate dehydrogenase was used after 4 recrystallizations (Jécsai, 1961). Enzymic activity was measured spectrophotometrically in standard assay mixture (Jécsai, 1961), in 0.1 M glycine buffer, pH 10.0, at 20 °C. The molar specific activity of the enzyme preparations was 8500 – 12 000 mole NADH/mole enzyme × min. The absorption of LDH was taken to be $A^{1\ mg/ml}_{1\ cm,\ 280\ nm}$ = 1.29 (Jécsai, 1961), and its molecular weight 140 000 (Jaenicke, Knof, 1968).

The molar extinction coefficient of NADH was taken to be $6.22 \times 10^3\ M^{-1}\ cm^{-1}$ (Horecker, Kornberg, 1948).

GAP was prepared according to Szewczuk et al. (1961), NAD (85% pure), ATP (90% pure) and Na-oxalate (95% pure) were Reanal preparations. Lactic acid (98% pure) was purchased from Roth or Riedel-de-Haen. Quinaldic acid was an Aldrich-Europe or Koch-Light preparation of 99% purity.

* *Abbreviations:* GAP, D-glyceraldehyde-3-phosphate; GAPD, D-glyceraldehyde-3-phosphate dehydrogenase (EC.1.2.1.12); LDH, lactate dehydrogenase (EC.1.1.1.27).

The measurements were carried out in an Opton PMQ II spectrophotometer equipped with a Servogor automatic recorder. Calculations were performed with a Hewlett–Packard 9810 A desk calculator and plotter using Gregory–Newton interpolation and linear regression by the least squares method.

The analysis of double inhibition was performed as described earlier (Keleti, Fajszi, 1971; Fajszi, 1976; Fajszi, Keleti, 1976).

Other conditions were as defined previously (Lien, Keleti, 1979).

Results and discussion

Initial velocity measurements of the GAPD reaction were performed in the presence of both inhibitors, ATP and quinaldate, in different concentrations. The experimental data are plotted according to Yonetani and Theorell (1964), (Fig. 1).

The straight lines in Fig. 1. A indicate that quinaldate is a pure type inhibitor of GAPD with respect to the substrate, P_i, if the latter is used in suboptimal concentration. On the other hand, the curves in Fig. 1. B indicate that ATP is a partial type inhibitor, in agreement with experiments in which the two inhibitors were used separately (Lien, Keleti, 1979).

The intercepts on the ordinate of Fig. 1. A are further plotted as a function of ATP concentration (Fig. 2). The resulting monotonously increasing hyperbola indicates that the interaction constant of the two inhibitors for the enzyme-substrate complex, β, is between 1 and ∞ (Keleti, Fajszi, 1971). This means that the binding of one inhibitor hinders the binding of the other, i.e. the two inhibitors act antagonistically.

Two plots are used to examine whether both ternary (ATP–GAPD-quinaldate) and quaternary complexes (ATP–GAPD-quinaldate) exist (Fajszi, 1976).

$$\Big|_{P_i}$$

Fig. 3 shows the double reciprocal plot of initial velocity in the presence of both inhibitors as a function of the concentration of the variable substrate. The parallel straight lines indicate that the dissociation constant of ATP from the ternary complex equals infinity.

Fig. 4 shows the plot $(1/[I_1])(1/v_{1,2}-1/v_2)$ *vs.* $1/[P_i]$. The straight lines parallel with each other and the abscissa again indicate that the dissociation constant of ATP from the ternary complex equals infinity.

Since these results suggest that a ternary complex containing both inhibitors does not exist in the given system, the two inhibitors can interact only at the quaternary complex containing the substrate.

These results are in good agreement with those obtained with the two inhibitors separately (Lien, Keleti, 1979), namely that ATP is uncompetitive inhibitor of GAPD with respect of P_i and therefore is unable to bind to the free enzyme. Consequently, a ternary complex with the two inhibitors not containing substrate, cannot be formed.

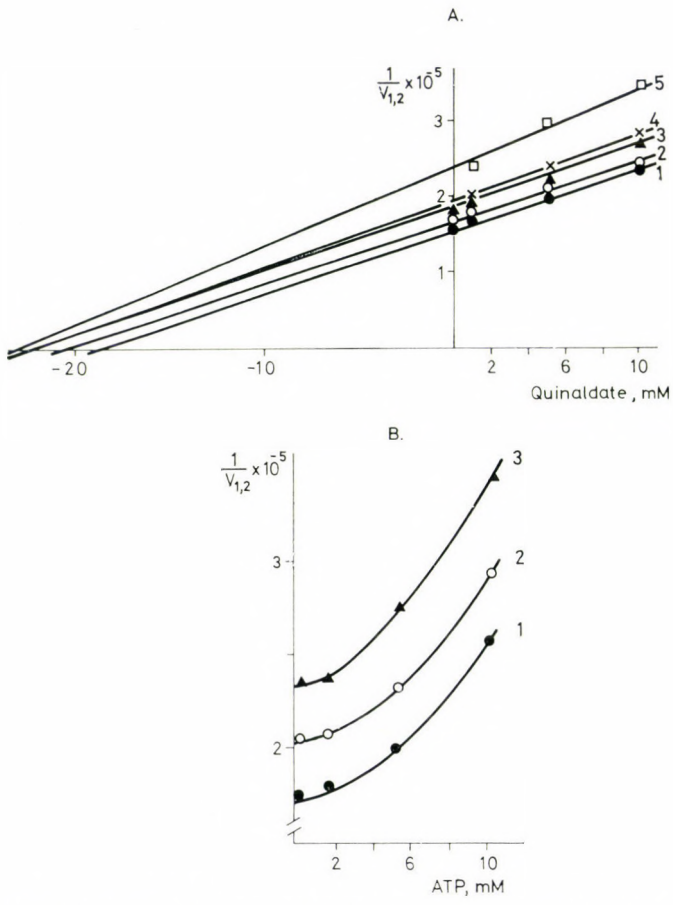

Fig. 1. Yonetani—Theorell plot of double inhibition of GAPD by quinaldate and ATP. The P_i concentration was 2 mM, the other substrates were employed at saturating concentration. [GAPD] = 6.9×10^{-6} mM. 0.1 M glycine buffer, pH 8.5, at 25 °C. $v_{1,2}$ = initial velocity in the presence of both inhibitors. A — $1/v_{1,2}$ *vs.* quinaldate concentration. ATP concentrations = 1: 0; 2: 1.5 mM; 3: 2.6 mM; 4: 5.3 mM; 5: 10.0 mM. B — $1/v_{1,2}$ *vs.* ATP concentration. Quinaldate concentrations = 1: 1.0 mM; 2: 5.0 mM; 3: 10.0 mM

We analyzed the interaction of ATP and quinaldate on GAPD as a function of P_i concentration (Fig. 5). At P_i concentrations higher than 1 mM, the two inhibitors act antagonistically, i.e. $v_0 v_{1,2}/v_1 v_2 > 1$. However, the antagonism of the two inhibitors turns into the simple summation of their effect ($v_0 v_{1,2}/v_1 v_2 = 1$) at the "characteristic substrate concentration" (cf. Fajszi, Keleti, 1976) which in the case of GAPD, is about 1 mM P_i (i.e. near physiological concentration). At this substrate concentration quinaldate and ATP act independently of each other, hence their effects are additive.

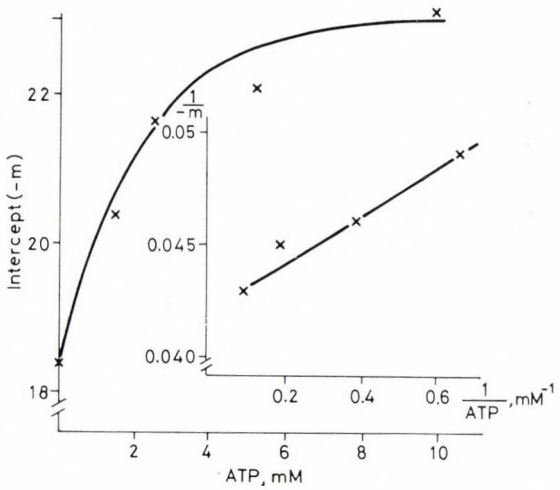

Fig. 2. Determination of interaction constant of the two inhibitors according to Keleti and Fajszi (1971). Intercepts in Fig. 1.A with the abscissa (−m) were plotted against the concentration of ATP at quinaldate concentrations: 0, 1, 2, 5 and 10 mM. Substrate (P_i) concentration was 2 mM. Other conditions as in Fig. 1. The straight line of the double reciprocal plot [ATP] *vs.* −m (insert) indicates that the curve in Fig. 2 is indeed a hyperbola

The double inhibition of LDH with NAD as variable substrate and oxalate and quinaldate as inhibitors was also studied. Novoa et al. (1959) found oxalate to be an uncompetitive inhibitor with respect to NAD. Kőnig et al. (1975) have shown that quinaldate is a mixed type inhibitor of LDH with respect to NAD.

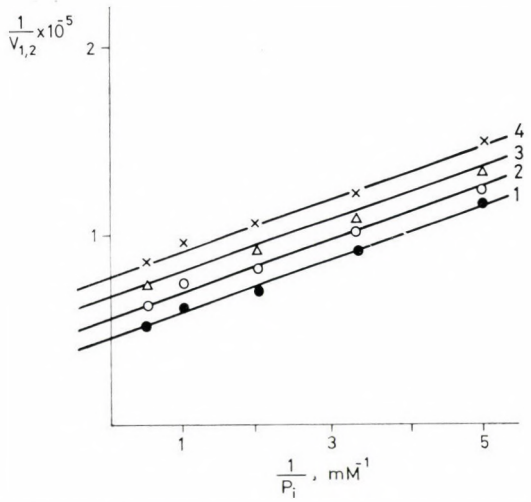

Fig. 3. Double reciprocal plot of $v_{1,2}$ against P_i concentrations. Initial velocities were measured with 1.5 mM ATP and the following quinaldate concentrations = 1: 0; 2: 1.0; 3: 3.0 4: 5.0 mM. Other conditions as in Fig. 1

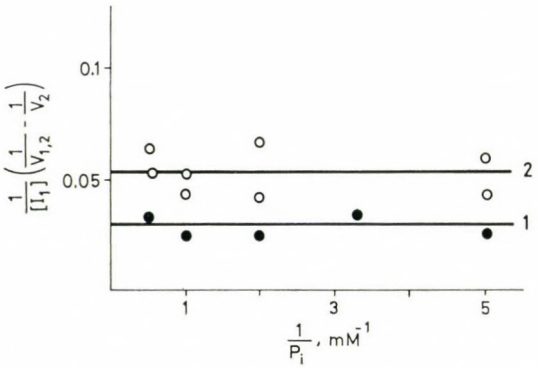

Fig. 4. Diagnostic test of GAPD-ATP-quinaldate complex. $[I_1]$ = concentration of quinaldate; v_2 = initial velocity of GAPD in the presence of ATP and $v_{1,2}$ = initial velocity in the presence of both quinaldate and ATP; 1: 3.3 mM quinaldate + 8.0 mM ATP; 2: 5.0 mM quinaldate + 5.0 mM ATP. Other conditions as in Fig. 1

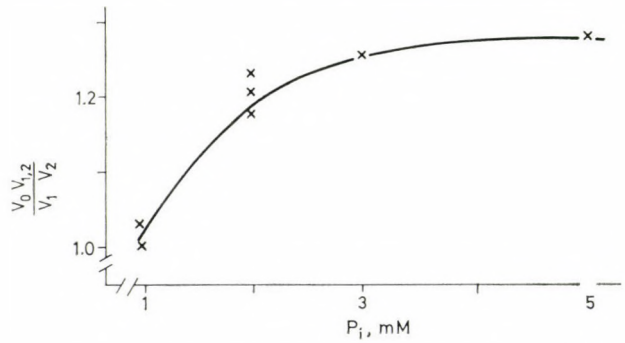

Fig. 5. Effect of substrate concentration on the interaction of two inhibitors on GAPD. Initial velocities of GAPD were measured in the presence of 2.5 mM quinaldate (v_1), 5.0 mM ATP (v_2), and both ($v_{1,2}$) as well as in the absence of inhibitors (v_0). The concentration of GAPD was 6.9×10^{-6} mM. The measurements were carried out in 0.1 M glycine buffer, pH 7.5 at 25 °C

Our experiments agreed with these results. Furthermore, we proved that oxalate is a purely uncompetitive whereas quinaldate is a partially mixed type inhibitor.

Fig. 6 shows that the two inhibitors interact on LDH in the same way as on GAPD: lowering the concentration of the substrate the antagonism between the two inhibitors turns into additivity. At high quinaldate concentration (7 mM) and low oxalate concentration (1.7 mM) practically the simple summation of the effects of the two inhibitors can be demonstrated independently of substrate concentration.

These results are consistent with the theoretical predictions that the antagonism or synergy of two inhibitors may turn into additivity or *vice versa* depen-

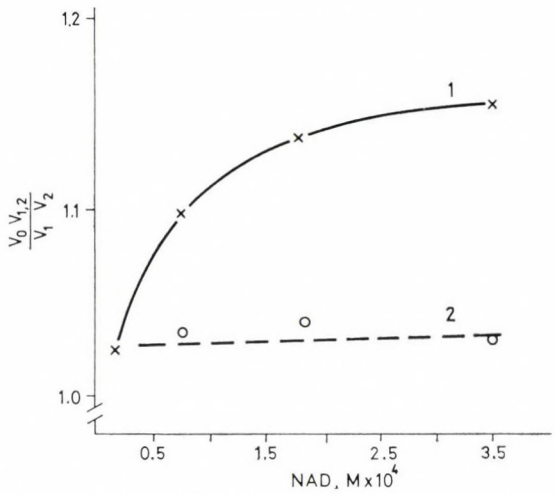

Fig. 6. Effect of substrate concentration on the interaction of two inhibitors on LDH. Initial velocities of LDH were measured in the presence of: 1: 6.8×10^{-3} M oxalate and/or 3.5×10^{-3} M quinaldate, 2: 1.7×10^{-3} M oxalate and/or 7.0×10^{-3} M quinaldate. The concentration of LDH was 8.6×10^{-6} mM. The measurements were carried out in 0.1 M glycine buffer, pH 10.0 at 20 °C

ding on substrate concentration (Fajszi, Keleti, 1976). Moreover, our data confirm that this phenomenon may be observed if at least one of the inhibitors is a partial type inhibitor; in fact two different dehydrogenases showed this effect.

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pH and Temperature Dependence of the Double Inhibition of D-Glyceraldehyde-3-phosphate Dehydrogenase by ATP and Quinaldate

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ATP and quinaldate, two inhibitors of D-glyceraldehyde-3-phosphate dehydrogenase, act antagonistically if $[P_i] > 1$ mM, at pH 7.5 and 25 °C. However, the type of interaction of the two inhibitors changes with pH and temperature. Antagonism is strongest at pH values near neutrality and decreases at higher pH. Above pH 9.5 the two inhibitors act nearly additively. At pH 8.5 and $[P_i] = 5$ mM there is antagonism above 20 °C between the two inhibitors whereas there is synergy below 20 °C.

A lag period in the time course of the enzyme reaction was detected when both inhibitors were present. The lag period is a function of pH. Below pH 8.5 the pH-dependence of the lag period resembles a titration curve with a $pK_{app} > 8$.

Introduction

ATP*** is a partially uncompetitive and quinaldate is a purely non-competitive inhibitor of GAPD with respect to P_i (Lien, Keleti, 1979). The analysis of the double inhibition of GAPD by ATP and quinaldate has shown that the two inhibitors act antagonistically on the enzyme-substrate complex if $[P_i] > 1$ mM (Lien et al., 1979). It has been deduced theoretically that the type of double inhibition may be altered by changing the substrate concentration (Fajszi, Keleti, 1976). Indeed, it has been shown experimentally that the antagonism of ATP and quinaldate on GAPD activity changes into the simple summation of their effect if P_i concentration is lowered (Lien et al., 1979).

The theory of double inhibitions cannot predict whether the interaction of two inhibitors changes or not with pH or temperature. The aim of this work was to tackle this question experimentally.

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*** *Abbreviations:* GAP, D-glyceraldehyde-3-phosphate; GAPD, D-glyceraldehyde-3-phosphate dehydrogenase (EC.1.2.1.12)

Materials and methods

Four times recrystallized pig muscle GAPD was used (Elődi, Szörényi, 1965).

Enzymic activity was measured spectrophotometrically in a standard assay mixture (Keleti, Batke, 1965), in 0.1 M glycine buffer (pH 7.0–9.5), between 10 and 40 °C as stated at the individual experiments. The molar specific activity of the enzyme preparations at pH 8.5 and 25°C was 18 000–24 000 mole NADH/mole enzyme × min. The molar extinction coefficient of NADH was taken to be 6.22 × $\times 10^3$ M^{-1} cm^{-1} (Horecker, Kornberg, 1948). The absorption of GAPD was $A^{1\ mg/ml}_{1\ cm,\ 280\ nm} = 1.0$ (Fox, Dandliker, 1956) and its molecular weight 145 000 (Elődi, 1958).

GAP was prepared according to Szewczuk et al., (1961).

NAD and ATP were purchased from Reanal, quinaldate from Aldrich-Europe; they were of 85%, 90% and 99% purity, respectively.

The measurements were carried out in an Opton PMQ II spectrophotometer equipped with a Servogor automatic recorder. Calculations were performed with a Hewlett–Packard 9810 A desk calculator and plotter, by using linear regression by the least squares method.

Other conditions were as defined previously (Lien, Keleti, 1979).

Results and discussion

Enzyme activity is influenced by the pH since enzyme proteins and substrates possess ionizable groups. The pH dependence of GAPD action, reflects on the one hand, the phosphate ionization of GAP, NAD and inorganic phosphate, and the pK of some histidyl and cysteinyl residues of the enzyme on the other (Keleti, Batke, 1967). We analyzed the pH dependence of the double inhibition of GAPD by ATP and quinaldate in the presence of 2 mM P_i, where at pH 7.5 and 25 °C marked antagonism between the two inhibitors could be detected (Lien et al., 1979).

Fig. 1 shows that in the pH-range studied the antagonism is the strongest at pH 7.0 and monotonously decreases with increasing pH. At pH 9.5 the effect of the two inhibitors is practically additive.

The temperature dependence of GAPD action was determined previously in the absence of inhibitors (Keleti et al., 1972; Tro, Keleti, 1974), as well as in the presence of an equimolar (i.e. 4 moles of ATP per mole of enzyme) amount of ATP (Keleti et al., 1972). We determined the thermodynamic parameters of GAPD action in the presence and absence of ATP and/or quinaldate. The Arrhenius plots (not shown) were in all cases linear in the given temperature range (10–40 °C). The thermodynamic parameters of inhibited and non-inhibited GAPD action are summarized in Table. 1. The difference in activation enthalpies is small compared with the relatively large error of their determination. Therefore, the difference in activation energy of non-inhibited and inhibited reactions was determined by the differential method elaborated previously (Keleti et al., 1972). The experimental

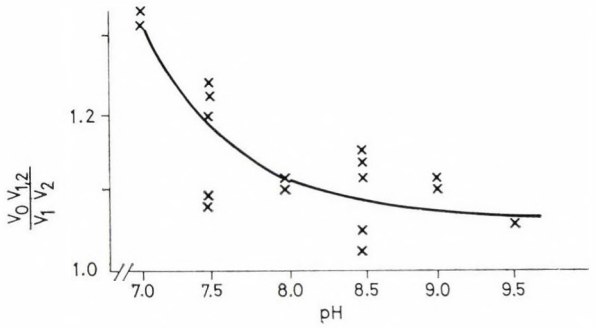

Fig. 1. Effect of pH on the interaction of the two inhibitors. GAPD = 7.1×10^{-9} M, NAD= = 2 mM, GAP = 1 mM, ATP = 3 mM, quinaldate = 5 mM, P_i = 2 mM, 0.1 M glycine buffer, 25 °C. v_0: initial velocity in the absence of inhibitors; v_1: initial velocity in the presence of quinaldate; v_2: initial velocity in the presence of ATP $v_{1,2}$: initial velocity in the presence of both quinaldate and ATP

Table 1

Thermodynamic parameters of GAPD action in the presence and absence of inhibitors

Inhibitor	ΔH^* kcal/mole	ΔS^* cal/mole × °K	ΔG^* kcal/mole at 25 °C	ΔE_{act}^* kcal/mole
—	10.9±0.5**	−11.2±0.2**	14.4±0.4**	
3.3 mM quinaldate	10.0±0.3	−16.2±0.4	15.0±0.5	0.6
6.0 mM ATP	11.1±0.3	−15.4±0.4	15.2±0.4	1.2
3.3 mM quinaldate + + 6.0 mM ATP	12.1±0.7	−13.0±0.2	15.2±0.4	2.1

* Difference of activation energies of the non-inhibited and inhibited reactions as determined by the differential method (Keleti et al., 1972). Experimental conditions see in legend to Fig. 2.

** Values from the literature: ΔH^* = 10.4 kcal/mole (Keleti et al., 1972), ΔS^* = = −11.2 cal/mole × °K (Keleti et al., 1972), ΔG^* = 14 kcal/mole at 25 °C (Tro, Keleti, 1974).

data are presented in Fig. 2 and Table 1. The difference in activation energy of inhibited and double-inhibited enzymatic reaction is not additive. Therefore it was of interest to examine the change of interaction of the two inhibitors as a function of temperature.

Fig. 3 shows that by increasing the temperature above 20 °C the synergy of the inhibitors turns into antagonism. This indicates that the triple-faced enzyme-inhibitor relation (Fajszi, Keleti, 1976) can be observed not only by varying the substrate concentration, as it was theoretically predicted, but also by changing the temperature.

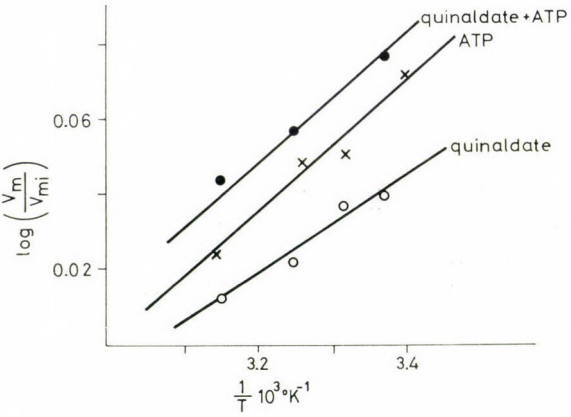

Fig. 2. Determination of the difference in activation energy of inhibited and non-inhibited GAPD reaction. V_m and V_{mi} are the maximum velocities in the absence and presence of inhibitor, respectively. GAPD = 6.9×10^{-9} M, GAP = 2 mM, NAD = 2 mM, P_i = varied substrate 0.2—5 mM, ATP = 6 mM, quinaldate = 3.3 mM, 0.1 M glycine buffer, pH 8.5

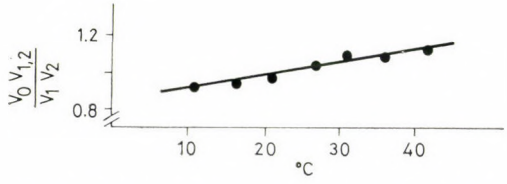

Fig. 3. Temperature dependence of interaction of ATP and quinaldate on GAPD. GAPD= = 6.9×10^{-9} M, GAP = 2 mM, NAD = 2 mM, P_i = 5 mM, ATP = 6 mM, quinaldate = = 3.3 mM, 0.1 M glycine buffer, pH 8.5

It is to be noted that the pH-dependence of the interaction of the inhibitors is very similar to its P_i concentration dependence (Lien et al., 1979). It is possible that the pH change alters the available P_i concentration by shifting the ionization of phosphate, which mimics the concentration change at a given pH. In contrast, the change in temperature shifts the interaction of inhibitors from antagonism not only to additivity but even to synergy. This may be due to the effect of temperature on the steric structure of GAPD. Consequently, the geometry of active site may also be effected, which in turn may cause a qualitative change in the interaction of ligands bound at this site.

These results warn us to exercise great caution in evaluating *in vitro* results of enzyme inhibition in respect of *in vivo* consequences. In the living cell where several modifiers are simultaneously present and the local substrate concentration, pH and temperature may differ from the bulk average, the interaction of modifiers e.g. various drugs, may be at variance with that determined *in vitro*. The simultaneous action of several herbicides or insecticides may differ in the temperate

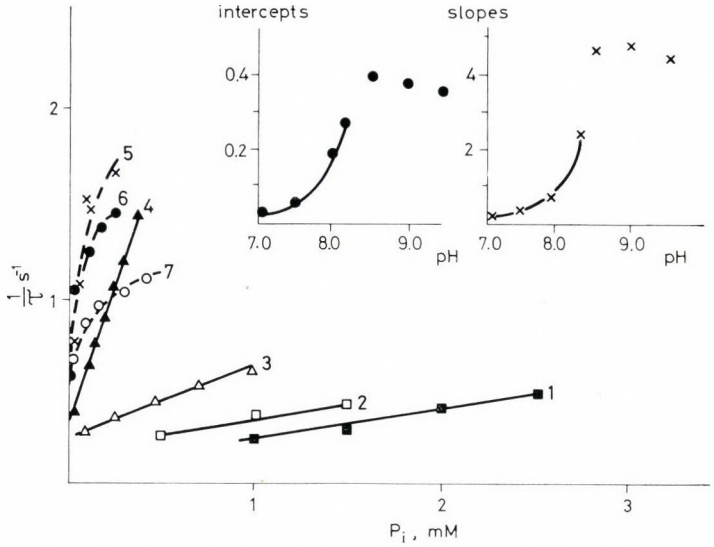

Fig. 4. Dependence of the reciprocal lag phase on inorganic phosphate concentration in double inhibition at different pH values. Quinaldate = 2.5mM, ATP = 3.0 mM, other conditions as in Fig. 1, except [P_i] which is varied. 1 : pH 7.0; 2 : pH 7.5; 3 : pH 8.0; 4 : pH 8.25; 5: pH 8.5; 6: pH 8.8; 7: pH 9.3. Inserts: intercept on the ordinate and slope of straight lines as a function of pH

zones and in the tropics, before or after fertilization, in alkaline or acidic type arable land.

It has previously been demonstrated that the enzymatic reaction catalyzed by GAPD commences only after a lag phase if the enzyme is inhibited by quinaldate (Lien, Keleti, 1979). This lag phase is longer if ATP is present, although ATP alone fails to elicit a similar effect. The lag phase is also a function of both [P_i] (Lien, Keleti, 1979) and pH. This latter is illustrated in Fig. 4, which shows that the substrate concentration dependence of the reciprocal lag phase is a linear function of [P_i] at low pH. The pH dependence of the ordinate intercept and of the slopes are illustrated in the insert of Fig. 4.

The intercept on the ordinate of the $1/\tau$ *vs.* [P_i] plot represents the maximal lag phase extrapolated to zero substrate concentration, i.e. this value is already independent of substrate concentration. Therefore the pH-dependence of lag phase cannot be interpreted in terms of a change in the ionization of P_i. The slopes of the straight lines indicate the sensitivity of the lag phase towards the change in substrate concentration at a given pH value.

If we assume that the lower parts of the curves (where the $1/\tau$ *vs.* [P_i] plots are linear) can be fitted to a titration curve, we obtain $pK_{app} > 8$. The similarity of the two curves suggests that both the duration of the lag phase and its sensitivity to changes in substrate concentration depend on the ionization of the same group(s).

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A Fluorescence Energy Transfer System for Studying the Functional Properties of Chromatin

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3Y1-B-1 rat embryo fibroblasts were used for double fluorescent labeling. The labeling was carried out with fluorescamine and ethidium bromide as an energy donor-acceptor pair.

The fluorimetric measurements proved the existence of an energy transfer process. The parameters of this transfer depended on both cell and dye concentrations. It is this character that makes this labeling technique promising as a means of distinction between cells with different structures.

Introduction

The functional properties of the chromatin, i.e. of the histones that are complexed with DNA in a repeating pattern, termed as nucleosomes, as well as those of some proteins having regulatory or enzymic functions in connection with gene expression are poorly understood in eukaryotic systems (Kornberg, 1977). The main reason for our vague knowledge is the lack of a suitably sensitive and *in vivo* applicable method of monitoring DNA-protein interactions. As has recently been shown, double fluorescence labeling techniques can succesfully be applied to study *in vitro* the interactions between bacterial DNA-dependent RNA polymerase and different template DNAs (Damjanovich et al., 1977). One of the fluorescence dyes, fluorescamine, is known to form an irreversibly bound fluorescence adduct with the enzyme. In such studies ethidium bromide is used to label the DNA (Stein et al., 1974; Jovin, Striker, 1977). Fluorescamine and ethidium bromide have very suitable spectral properties for the forming of an energy donor-acceptor pair (Damjanovich et al., 1977). The Förster-type singlet-singlet energy transfer studies carried out with DNA and RNA polymerase from prokaryotic systems enabled us to obtain information about the DNA-protein interaction at an

Abbreviations: MEM, Minimal essential medium; PBS, Phosphate-buffered saline; EB, Ethidium bromide.

Å level. This communication describes the application of this method to the study of nucleic acid-protein interactions in eukaryotic systems. Our primary aim was to detect energy transfer processes between the two dyes inside the eukaryotic cell.

Materials and methods

3Y1–B–1 rat embryo fibroblasts, isolated by Kimura and Itagaki (Kimura, Itagaki, 1975), were taken from a continuous line, cultured in Dulbecco's modified MEM containing 10% fetal serum. After passage, the cells were cultured for over 3 days at 33 °C. Then 0.25% trypsin (Difco) in 0.1 M PBS, pH 7.2, was used to make a cell suspension which was then centrifuged and washed three times with serum-free Dulbecco's modified MEM. Finally, the centrifuged cells were suspended in 0.1 M PBS, pH 7.2.

The labeling of cells with fluorescamine and ethidium bromide was done in the following way: 0.1 ml of 10^{-2} M fluorescamine (Hoffman La Roche) dissolved in dimethylsulfoxide (Merck, spectroscopic grade) was rapidly mixed with 1.9 ml of fibroblast cell suspension. 0.1 ml of this fluorescamine-labeled cell suspension was further diluted with 0.55 ml PBS and stained with 0.05 ml ethidium bromide. The final ethidium bromide concentration varied between 0.1×10^{-5} and $1.8 \times \times 10^{-5}$ M. In the case of controls containing one or none of the dyes an equivalent amount of the suitable solvent(s) was added to the samples.

The double-labeled cells were observed in a Zeiss microscope fluorimeter. The plasma showed the greenish-bluish fluorescence of fluorescamine. In contrast to the method of Hawkes et al. (1976; Hawkes, Bartholomew, 1977) in our case no preferential binding of the fluorescamine had occurred inside the plasma membrane. It was clearly demonstrated by the red fluorescence of the nuclei that ethidium bromide labeled mainly the chromatin.

Control experiments showed that none of the fluorescence intensities changed during the course of the experiment.

0.3 ml of the suspensions was placed in a Hitachi MPF–4 fluorescence spectrometer and fluorescence intensities were measured at 33 °C. Light scattering and inner filter effects were negligible at the excitation wavelength of 385 nm. The fluorescence intensities of the suspensions were measured at the emission maxima of both fluorescamine (470 nm) and ethidium bromide (600 nm.) The samples were excited at 385 nm, i.e. around the excitation maximum of fluorescamine.

The transfer efficiency of fluorescence energy transfer (E) can easily be calculated from the following equation (Cantor and Tao, 1972):

$$\frac{F_A^D}{F_A^O} = 1 + \frac{\varepsilon_D}{\varepsilon_A} \cdot E$$

where F_A^O and F_A^D are the fluorescence intensities of the fluorescence acceptor in

the absence and in the presence of fluorescence donor, respectively. ε_D and ε_A are the molar absorption coefficients of the donor and acceptor dyes at the excitation wavelength, respectively.

Results and discussion

Cell suspensions were made from Fischer rat embryo fibroblasts and labeled with fluorescamine and ethidium bromide as described in Methods.

Fluorescence energy transfer was determined by comparing the emission intensities of ethidium bromide fluorescence in the absence (F_A^O) and in the presence (F_A^D) of fluorescamine. Samples were excited at the excitation maximum of fluorescamine, where ethidium bromide is only very poorly excited. Since fluorescamine also has some emission at 600 nm, pertinent controls with suspensions made from fluorescamine-labeled cells served to get a corrected value of F_A^D. Table 1 shows the fluorescence intensities of suspensions in which the cell number and the concentrations of fluorescamine and ethidium bromide were the variable parameters.

The unlabeled samples showed very low intensity values at both 470 and 600 nm. The fluorescamine emission at 470 nm was rather sensitive to the cell concentration. The 600 nm fluorescence was somewhat less sensitive to the ethidium bromide concentration in the absence of fluorescamine when cell concentration was similarly altered. Applying double-fluorescence labeling, the 470 nm emission, i.e. the fluorescamine fluorescence, decreased in the presence of ethidium bromide.

Table 1

The fluorescence intensities of rat embryo fibroblasts labeled with fluorescamine and ethidium bromide

The figures in the Table stand for the fluorescence intensities measured at 600 nm (numerator) and at 470 nm (denominator) using an excitation wavelength at 385 nm

Final cell concentration		5×10^5/ml		10^5/ml		5×10^4/ml	
Fluorescamine concentration during labeling		None	5×10^{-4}M	None	5×10^{-4}M	None	5×10^{-4}M
EB concentration during labeling	None	0.5 / 4.5	32.0 / 505.0	0.3 / 2.5	8.3 / 127.7	0.2 / 2.1	6.7 / 96.7
	1.8×10^{-5}M	11.5 / 3.0	62.0 / 365.0	10.5 / 4.0	20.0 / 90.0	9.5 / 3.2	18.3 / 63.7
	0.9×10^{-5}M	7.0 / 3.5	57.0 / 475.0	5.5 / 2.5	16.0 / 106.7	5.5 / 1.5	15.0 / 85.0

Higher ethidium bromide concentrations caused a greater decrease of this fluorescence. On the other hand, the emission at 500 nm significantly increased if both dyes were present. This phenomenon strongly suggested the existence of a Förster-type singlet-singlet energy transfer, with fluorescamine as energy donor and ethidium bromide as the acceptor. The sensitivity of the transfer to the changing of the variable parameters, i.e. the concentrations of cells and dye, is clearly shown.

Table 2, demonstrates the sensitivity of $F_A^D/F_A^O - 1$ to the cell number and ethidium bromide concentration. The difference in the values of $F_A^D/F_A^O - 1$, which is proportional to the transfer efficiency, E, at different cell and ethidium bromide concentrations can be attributed to unequal labeling since the ratio of dye concentration to cell number was changed.

Table 2

The effect of cell number and ethidium bromide concentration on $F_A^D/F_A^O - 1$

Ethidium bromide and fluorescamine concentrations were as in Table 1. F_A^O is the fluorescence intensity of ethidium bromide at 600 nm after labeling the cell only with ethidium bromide. (To get F_A^O, the fluorescence of unlabeled cells was substracted from the fluorescence intensities measured at 600 nm in the presence of EB.) F_A^D is the fluorescence intensity of ethidium bromide at 600 nm in the presence of both dyes. (To calculate F_A^D, the fluorescence intensity of cells labeled only with fluorescamine was substracted from the fluorescence intensities measured at 600 nm in the presence of both dyes.)

Final cell concentration		5×10^5/ml	10^5/ml	5×10^4/ml
EB concentration during labeling	1.8×10^{-5}M	1.73	0.14	0.25
	0.9×10^{-5}M	2.85	0.48	0.57

The Tables demonstrate the existence of energy transfer and the dependence of transfer efficiency upon the donor as well as acceptor concentrations. Fluorescamine is known to react rapidly with primary amines, forming a highly fluorescent adduct with excitation and emission maxima at 385 nm and 470 nm, respectively. The intracellular environment provides a great variety of primary amine targets for fluorescamine. The ethidium bromide molecules are reversibly intercalated in or bound to the double helical DNA and RNA. Their reaction with DNA wrapped up in nucleohistones differs to some extent from that with free DNA (Olmsted, Kearns, 1977). However, there is an enhancement of fluorescence upon intercalation attributed to a reduction of the rate of excited-state proton transfer to solvent molecules (Bontemps, Fredericq, 1974). Thus, only those ethidium bromide molecules which are intercalated with double helical nucleic acids will emit the transferred energy with a high quantum efficiency. The limiting distance of the Förster-type singlet-singlet energy transfer is about 60 Å (Stryer, Haugland, 1967). These facts restrict energy donors to those fluorescamine molecules that are bound in close proximity to the nucleic acids. However, *in vivo* experiments show that the

affinity of ethidium bromide to DNA is about 10 times higher than that to RNAs (Angerer, Moudrianakis, 1972; Tritton, Mohr, 1973; Bontemps, Fredericq, 1974; Genest, Fichadou, 1977; Plumbridge, Brown, 1977). The above considerations support our assumption that the observed energy transfer mainly occurred in the chromatin.

Along with the concentration of dyes the energy transfer also depends on the concentration of cells in the suspension. Apparently there is an optimum for these three parameters: the concentration of the cells, that of the donors and that of the acceptors. The variation of cell concentration was limited by the spontaneous sedimentation and the light scattering of cells. Between $10^4 - 10^5$ cells/ml none of the above occured. A higher fluorescamine concentration resulted in a higher probability to label the protein efficiently inside the nucleus. The possible disturbance caused by the effect of inner filter could be avoided by labeling the cell suspensions with a higher concentration of fluorescamine and diluting the samples before labeling with ethidium bromide. The most important factor proved to be the concentration of ethidium bromide. Separate titrations are necessary to establish the saturation of intercalation sites of DNA with ethidium bromide. In this way dissipation of the energy provided by the donor fluorescamine can be prevented.

The above double fluorescence labeling procedure and the exploitation of fluorescence energy transfer as a "spectroscopic ruler" (Stryer, Haugland, 1967) can be used to measure the proximity of DNA and proteins inside the cell nucleus. Experiments are in progress to differentiate between transformed and controll cell lines. A microscope fluorimetric version of the above method has also been worked out to study the cell nuclei of rats at different stages of ageing.

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Depolymerization of Actin in Concentrated Solutions of Divalent Metal Chlorides

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Actin transferred to concentrated (0.3—1.2 M) $MgCl_2$ solutions depolymerizes completely. When protected by a high excess of ATP, actin in this $MgCl_2$-depolymerized state is stable for several days in the cold. In the absence of excess ATP it slowly denatures.

Chiroptical data and proteolysis experiments show that $MgCl_2$-depolymerized actin is in a native, folded state, although its helix content is considerably decreased.

By dissolving F-actin pellets or actin precipitated in paracrystalline state in concentrated $MgCl_2$ solutions in the presence of ATP, very concentrated (100—200 mg/ml) monomeric actin solutions can be prepared.

$CaCl_2$ and $MnCl_2$ have similar effects although these were not studied in detail.

Introduction

Actin exists, depending on solute conditions, in several different forms. In salt free solutions, well above the isoleectric pH (pH 4.75), actin is monomeric ("G-actin"). Lowering the pH or addition of salts transforms actin to the polymeric, fibrous state (F-actin). At the time of the discovery of actin (Straub, 1942) it was already noted that $MgCl_2$ and $CaCl_2$, at a concentration of about 0.01—0.05 M, precipitate F-actin. This effect was studied later in detail by Bárány et al. (1954). It was Hanson (1973) who discovered that in $MgCl_2$-precipitated actin F-actin filaments form different types of ordered aggregates.

All these effects can be interpreted by assuming that binding of cations decreases the net negativ charge of actin removing thereby electrostatic repulsions preventing monomer-monomer and/or filament-filament interactions (Mommaerts, 1952; Bárány et al., 1954). This general assumption recieved detailed support with respect to divalent cations by the ion binding studies of Martonosi et al. (1964) and recently by the very detailed studies of Strzelecka-Golaszewska et al. (1978 a, b). These studies showed a strict paralellism between the degree of ion binding and polymerization. The studies of Strzelecka-Golaszewska (1978 a) further showed a fine correlation between the type of paracrystalline structure and concentration of divalent cations present.

In the paper of Bárány et al. (1954) it has been noted that still higher concentrations of $MgCl_2$ or $CaCl_2$ (0.1 – 0.6 M) dissolved the precipitated actin, in all probability in a depolymerized form. We began to study this depolymerization of actin by high concentrations of $MgCl_2$ in an attempt to crystallize G-actin. We did not reach this ultimate goal, but several interesting observations were made on the behavior of actin in concentrated $MgCl_2$ solutions. These will be reported here.

Materials and methods

Actin was extracted from acetone-dried muscle powder according to Bárány et al. (1954) with one ultracentrifugation cycle added according to Mommaerts (1952). Since this procedure gives a completely homogeneous actin preparation (as assessed by SDS gel electrophoresis) without any additional precautions, it seems to us worthwhile to give a detailed description of the procedure as we use it now.

Minced muscle extracted with Guba-Straub solution (0.15 M KCl – 0.3 M potassium phosphate, pH 6.5) can be stored indefinitely in a deep-freeze. The frozen mass is thawed and washed with 3 volumes of distilled water for 10 min with intensive strirring. The mass is pressed through a cloth, resuspended in 5 volumes of 0.4% $NaHCO_3$ and stirred for 15 min under cooling. This step is repeated once followed by 2 – 3 washigs with cooled distilled water. This is followed by dedyhration with acetone involving 6 – 8 washings with 3 volumes of cooled acetone and-stirring for 10 min. Acetone should be pressed out gently. The residue is then air dried.

For extraction of actin the powder is suspended in 20 volumes of 0.2 mM ATP solution, pH 7.0, that has been cooled very carefully to 0 °C, mixed throughly and left standing for 15 min with careful cooling. After filtering by suction the crude G-actin is polymerized by the addition of 0.05 M KCl. After 30 min at room temperature F-actin is peletted by 90 min centrifugation at 100 000 *g*. The pellets and the centrifuge tube are gently rinsed with destilled water, the pellets dispersed in a Potter-Elvehiem-type homogenizer in 0.2 mM ATP, pH 7.0, and dialyzed overnight against the same solution. The depolymerized actin is clarified by centrifugation for 90 min at 30 000 *g*.

Preparation of *highly concentrated $MgCl_2$-depolymerized actin solutions:* Purified actin is polymerized by addition of 8 mM $MgCl_2$ or 50 mM KCl and pelleted by ultracentrifugation at 100 000 *g* for 90 min. The pellet is collected on a small wetted piece of ordinary cellophane with the help of a small spatula and prepared for dialysis against a solution of 0.6 – 1.2 M $MgCl_2$ containing 5 – 15 mM ATP and 5 – 20 mM tris – HCl buffer. The dialysis solution is adjusted to pH 8.0 – 8.2. After 24 – 48 hours of dialysis the content of the cellophane bag becomes completely liquid. Undissolved components (supposedly denatured proteins and dust) are eliminated by centrifugation at 13 000 *g* for 30 min. In some experiments a few mM mercaptoethanol was included in the dialysis solution but

this proved to be unnecessary. By this procedure depolymerized action solutions approaching the concentration of the ultracentrifuged pellets (100–150 mg/ml) are obtained. Concentrations up to 230 mg/ml can be reached if actin is precipitated with 50 mM $MgCl_2$ prior to ultracentrifugation.

Myosin was prepared according to Portzehl et al. (1950) and clarified by centrifugation at 15 000 *g* for 30 min.

Subfragment–1 (S–1) of myosin was prepared according to Weeds and Taylor (1975) and lyophilized.

Determination of *protein concentration* was made in several different ways, according to the requirements of the particular experiments.

For the most exacting chiroptical experiments the method of Houk and Ue (1974) was used with correction for light scattering according to Winder and Gent (1971). The extinction coefficients given by Houk and Ue (1974):

$\varepsilon_{289} = 1.11$ ml × mg^{-1} cm^{-1} and $\varepsilon_{290} = 0.63$ ml × mg^{-1} cm^{-1} were used. Spectral measurements in these experiments were made with a Hitachi PES–3T recording spectrophotometer.

In the solubility experiments (Fig. 2) extinction obtained at 290 nm against an appropriate blank solution was used without light scattering correction. Although, as verified in separate experiments, actin depolymerized by $MgCl_2$ showed a difference spectrum with reference to ordinary G-actin in the region of 280–290 nm, this difference was less than 2% at 290 nm. Therefore we used the above extinction coefficient without correction.

In some experiments actin concentration was determined by the microbiuret method of Itzaki and Gill (1964).

Activation of myosin ATP-ase by actin was measured in a total volume of 2 ml containing 2 mM ATP, 2 mM $MgCl_2$, 20 mM Tris-acetate buffer, pH 7.0, 50 μg lyophilized S-1 and various concentrations of actin. Incubation time was 15 min at 20 °C. The reaction was stopped by addition of an appropriate amount of ammonium molybdate solution and phosphorus was estimated without deproteinization as described by Tashima (1975).

Chiroptical measurements: ORD in the visible range was measured in the Perkin–Elmer 141 M spectropolarimeter in a 1 dm jacketed cell. ORD and CD spectra in the ultraviolet range were recorded in a JASCO ORD (UV) CD–5 spectropolarimeter in 10 mm and 2 mm jacketed cells. The instruments were calibrated with sucrose and d-10-camphor sulfonic acid solutions (Cassim, Yang, 1969). The mean residue weight of actin was taken as 115. The refractive index correction term $\frac{3}{n^2+2} = 0.79$ was used for the determinaxtion of the Moffiit-Yang plots in the visible range.

SDS-gel electrophoresis was carried out according to Weber and Osborn (1969).

$MgCl_2$ was analytical grade; the concentrations of the solutions were checked pycnometrically using tabulated data (Hodgman 1952).

Viscosity of depolymerized actin solution was measured in an Ubbelohde viscometer at 0 °C with an outflow time of about 150 sec.

ATP sensitivity tests were carried out essentially as described by Szent-Györgyi (1951): the drop of viscosity on addition of ATP to the actomyosin formed with the actin tested was measured in an Ostwald type viscometer (outflow time 66 sec) at 0 °C in the following system: 0.26 mg/ml myosin, 0.38 M KCl, 2.5 mM phosphate buffer, pH 7.2, and various concentrations of actin.

Results

High concentrations of $MgCl_2$ (0.3 – 1.3 M) completely depolymerize actin.

Fig. 1 shows the reduced viscosity of different highly concentrated, $MgCl_2$-depolymerized actin preparations (obtained as described in "methods") as a function of protein concentration. Note that with the exception of the lowest concentration (4.8 mg/ml) the curve extends to concentrations that cannot be reached by G-actin prepared in a traditional way. In spite of this the curve extrapolates to an intrinsic viscosity of 0.0316 dl/g which is practically the same as the lowest value reported in the literature for the intrinsic viscosity of G-actin (Cohen, 1966). Note also that the most concentrated actin solution measured in the experiment was 73 mg/ml (!). As the reduced viscosity of F-actin at 1 mg/ml concentration is about 5.00, the presence of even 1 % of F-actin would set this point grossly out of line.

Fig. 2 demonstrates the transition of actin from the precipitated, paracrystalline to the depolymerized state on raising the concentration of $MgCl_2$.* Actin

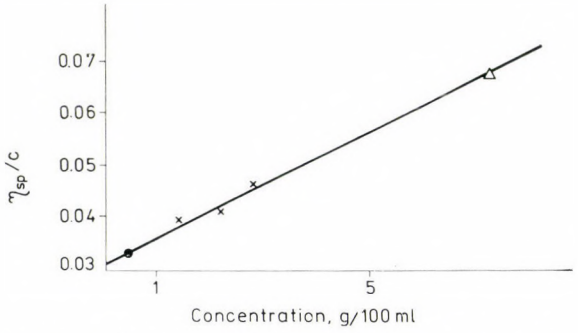

Fig. 1. Intrinsic viscosity of depolymerised actin. Different points were obtained with different preparations, measured under slightly different conditions ●, 0.36 M $MgCl_2$, 20 mM phosphate buffer, pH 6.5; x, 0.55 $MgCl_2$; △, 0.77 M $MgCl_2$; x and △, 20 mM Tris-HCl buffer, pH 8.2; ATP, 0.8 – 5.5 mM. 5 mM mercaptoethanol throughout. The line fitted by least square methods gives: $\frac{\eta}{c} = 0.0316 + 4.93 \times 10^{-3} C$

* A similar behavior was experienced with $CaCl_2$ and $MnCl_2$ but detailed experiments were carried out with $MgCl_2$ only.

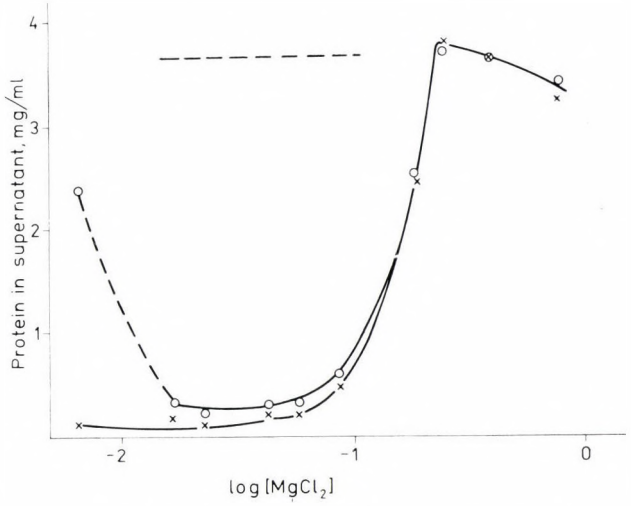

Fig. 2. Solubility of actin as a function of $MgCl_2$ concentration. G-actin was polymerized by addition of $MgCl_2$ to a final concentration of 6.5 mM. To this actin a concentrated ATP solution, pH 8.2, was added to give in the final sample a concentration of 0.16 mM. To equal aliquots of this actin varying amounts of $MgCl_2$ were added and the samples were complemented with distilled water to equal final volumes. After standing overnight in the refrigerator the samples were centrifuged at 13 000 *g* for 30 min. Aliquots were taken from the supernatants and the samples were immediately centrifuged at 54 000 *g* for 180 min. Protein concentrations of the supernatants of this "low speed" and "high speed" centrifugations were measured at 290 nm after appropriate dilution with ATP-$MgCl_2$ mixtures of a composition identical to that of the respective samples. O, centrifugation at 13 000 *g*; X, centrifugation at 54 000 *g*. The horizontal dotted line gives the total actin concentration of the samples calculated from the concentration of the G-actin solution used in the experiment

samples containing different concentrations of $MgCl_2$ were centrifuged with low and subsequently with high centrifugal force. The protein concentration in the supernatant of the high centrifugal force centrifugation gives the amount of the monomeric actin present in the system. The supernatant of the low speed centrifugation contains besides monomeric actin also the non-aggregated F-actin, if present.

At high $MgCl_2$ concentrations the protein concentrations in the high and low centrifugal force supernatants are practically identical, no detectable amount of F-actin is found in the solubilized fraction. At these high $MgCl_2$ concentrations actin seems to go from the aggregated, paracrystalline state directly into the monomeric form.

The extremely low viscosity of actin in concentrated $MgCl_2$ solutions *per se* could be due to denaturation of actin. It is well known e.g. that actin denatured by EDTA is not precipitated and has a very low viscosity. Chiroptical experiments (Fig. 3 and Table 1), however, show that this $MgCl_2$-depolymerized actin is not denatured. The far UV CD spectrum of $MgCl_2$-depolymerized actin (Fig. 3) is

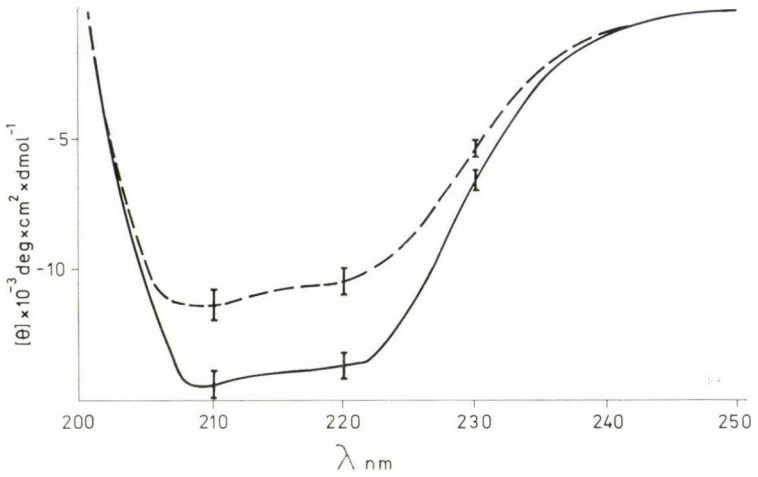

Fig. 3. Far ultraviolet CD spectrum of G-actin in the presence and absence of 0.8 M $MgCl_2$. Dotted line, 0.8 M $MgCl_2$ present; continous line, no $MgCl_2$. The curves represent mean values of six independent measurements with different preparations. Vertical bars, mean square root deviations. ATP, 100–200 μM; Tris-HCl buffer, pH 8.2, 5 mM; protein, 6.7–10.5 μM. For other details of the measurement see "Methods"

Table 1

Summary of the chiroptical parameters of G-actin in the presence and absence of 0.8 M $MgCl_2$

Actin concentration, 11.9–59.5 μM; ATP 100–200 μM; Tris-HCl, pH 8.2, 5 mM; temperature, 10 °C

All values are the means of six independent measurements with mean square deviations. For further details see "Methods"

Optical parameter	$MgCl_2$ absent	0.8 M $MgCl_2$
	degr×cm²×dmol⁻¹	
Moffitt–Yang parameters: b_0 a_0	−154±10 −264±15	−122±11 −305± 6
$[m']_{546\ nm}$	51.1±2.5	− 57.5±1.5
$[m']_{229\ nm}$	− 6 300±300	− 5 300±400
$[\theta]_{222\ nm}$	−13 200±500	− 9 900±700
$[\theta]_{290\ nm}$	−14 500±700	−11 400±800

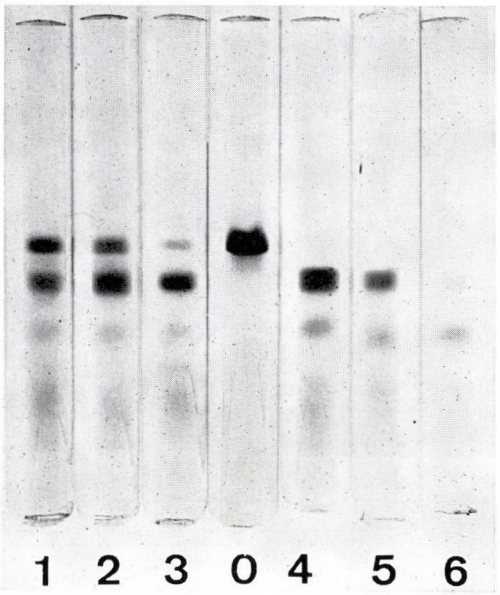

Fig. 4. Limited tryptic proteolysis of actin in the presence of 0.6 M $MgCl_2$. Digestion was carried out at 24 °C, with actin solutions of 3.8 mg/ml containing 0.5 mM ATP, 5 mM mercaptoethanol and 1 mM Tris-HCl buffer, pH 8.2. Actin to trypsin ratio 10 : 1 for control G-actin, 10 : 2 for actin in 0.6 M $MgCl_2$. Digestion was arrested at 10, 20 and 60 min by pipetting 0.1 ml digest in to 0.9 ml of a mixture of 1% SDS and 1% mercaptoethanol, 30 mM Tris-malate, pH 7.0, at 100 °C and incubation at 100 °C for 5 minutes. Aliquots of 50 μl were subjected to electrophoresis. 1—3, G-actin (control); 4—6, actin in the presence of 0.6 M $MgCl_2$ (in the order of increasing digestion times). Tube marked 0, undigested G-actin

typical of a folded globular protein although it is significantly different from that of G-actin.* On the basis of Fig. 3 and the chiroptical data summarized in Table 1 actin in the $MgCl_2$-depolymerized state has a significantly lowered helical content. If we attribute the differences observed simply to a transition of part of the peptide backbone from helical to unordered conformation, this change affects roughly 10% of the backbone peptide units. As G-actin has a helix content of round 45% only (Wu, Yang, 1976) this change is important.

Proteolysis experiments also point to an altered conformation which, however, is far from complete unfolding. As shown in Fig. 4 when actin is digested with trypsin in the presence of 0.6 M $MgCl_2$ and ATP, apparently the same resistant core is obtained as described by Jacobson and Rosenbusch (1976) for the proteolysis of "ordinary" salt-free G-actin. Note that the trypsin concentration in the case of $MgCl_2$-depolymerized actin was doubled as compared to the control. This was done because in the case of synthetic substrates we observed a roughly 50% appa-

* The CD spectrum of G-actin obtained in these experiments was practically identical with that published by Wu and Yang (1976).

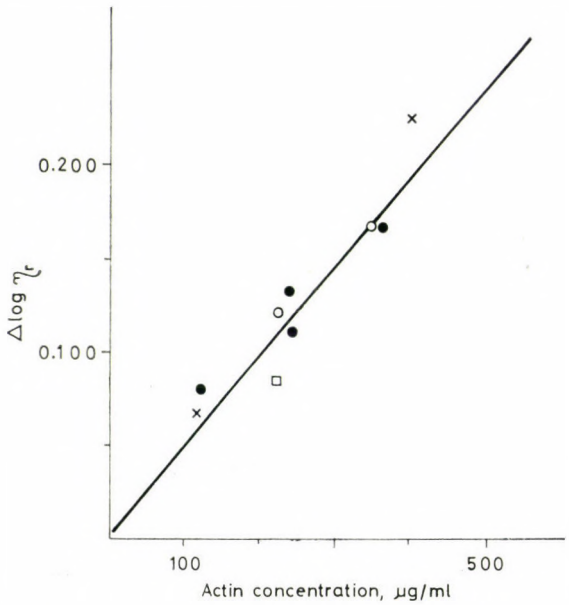

Fig. 5. Reversibility of depolymerization by $MgCl_2$ as tested by ATP sensitivity. Three pellets obtained by ultracentrifugation of equal aliquots of F-actin were homogenized in salines containing 0.55 M $MgCl_2$, 1.6 mM ATP, 4 mM mercaptoethanol and different buffers. After centrifugation at 13 000 *g* for 30 min the depolymerized actin solutions of about 25 mg/ml concentration were stored in the refrigerator. On the days of storage indicated below, 0.3 ml aliquots were diluted with distilled water to 25 ml and dialyzed against 1500 ml of 0.1 M KCl, 2.5 M phosphate buffer, pH 7, for 24 hours or more. The actual concentrations of actin solutions thus obtained were measured by the microbiuret method (Itzaki, Gill, 1964). Ordinate, log η_r (minus the log η_r value of myosin present); abscissa, final concentration of actin in μg/ml. ○, seven-day storage in the presence of 5 mM phosphate buffer, pH 6.9; □, three-day storage in the presence of 5 mM Tris-HCl buffer, pH 8.15; x and ●, two different untreated control F-actin preparations. The straight line was fitted to all points with the least square method and a slope of 0.49 was obtained

rent depression of trypsin activity in the presence of 0.6 M $MgCl_2$. As judged from the time dependence of the changes in the electrophoretic pattern, proteolytic degradation is more intensive in the case of Mg-depolymerized actin. In contrast to G-actin, in which the core formed is practically completely resistant to further proteolysis, in the case of $MgCl_2$-depolymerized actin the core formed is a transitory product, which slowly degrades to low molecular weight fragments.

Actin containing only about stoichiometric amounts of free ATP, as used during preparation, develops an intensive turbidity when transferred to high concentrations of $MgCl_2$. When $MgCl_2$ is eliminated by dialysis against a low ionic strenght buffer, all protein precipitates. In the presence of five to tenfold excess of ATP, however, actin at low temperature is quite stable in $MgCl_2$ solutions and when returned by dialysis to salines favorable to polymerization it forms

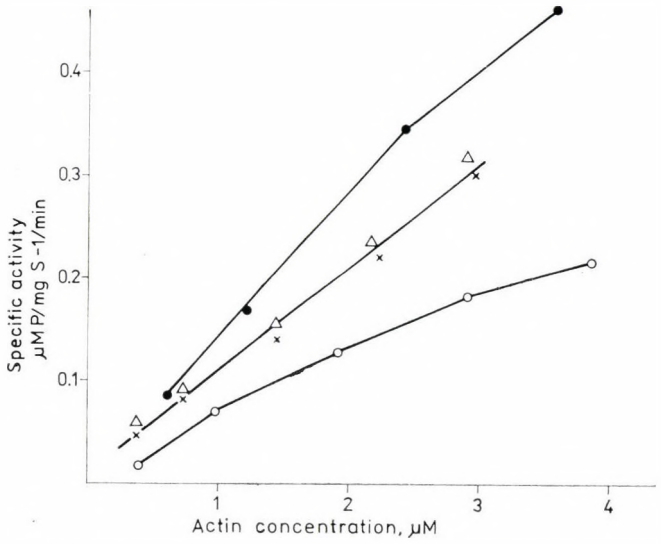

Fig. 6. Reversibility of depolymerization of actin by $MgCl_2$ as tested by the ATP-ase activating effect of actin. Ultracentrifugated pellets of F-actin were homogenized in 0.6 M $MgCl_2$, 4.23 mM ATP, pH 8.2, to give a final concentration of about 30 mg/ml, and dialyzed against the above solution for 24 hours. The depolymerized actin solutions were clarified by centrifugation for 30 min at 13 000 *g* and kept in the refrigerator. At the times of storage indicated, the solutions were diluted to about 2 mg/ml protein concentration and dialyzed for 24 hours against a large volume of 0.05 M KCl. Before estimation of protein concentration according to Houk and Ue (1974) the solutions were clarified as above, although there was no conspicous precipitate or turbidity. To a constant concentration of S-1, various amounts of F-actin treated as specified below were added. Abscissa, actin concentration μM; ordinate, specific activity, μM P/mg S-1 min. The activity of S-1 alone was negligible under the conditions used. Actin kept in Mg-depolymerized state for x, 24 hours; △ for 48 hours. Controls: ●, freshly purified actin (extracted from aceton powder the day before ATP-ase measurement); ○, actin kept for four days in the refrigerator in the form of ultracentrifuged pellet (an aliquot of the preparation symbolized by △). On the day of the experiment, the pellet was homogenized in 0.05 M KCl and clarified by centrifugation as described above. Bacterial growth in the stored pellet was prevented by adding 0.5% sodium azide to the F-actin prior to ultracentrifugation

normal F-actin. This latter point is demonstrated by experiments in which concentrated actin solutions were kept depolymerized by $MgCl_2$ for different periods of time, then returned to polymerizing solutions by dilution and subsequent dialysis.

In Fig. 5 the sate of actin thus treated is shown as judged from its actomyosin-forming ability measured by viscometry. (Szent-Györgyi, 1951). This experiment shows that actin is quite stable up to seven days in $MgCl_2$-depolymerized form. The points of reverted actins and of control actin can be fitted to a single straight line. The slope of this line, 0.49 is somewhat lower as compared to the value of 0.54 obtained by Szent-Györgyi, but at the time of his experiments actins were

contaminated by various unknown amounts of tropomyosin, since no special care was taken to avoid contamination.

When the state of the repolymerized actins is tested by the effect of actin on myosin ATP-ase activity (Fig. 5), reversibility is not so good. The reverted actins strongly activate the ATP-ase activity of S-1, but definitely less so (by 20–30%) than freshly prepared actin does. On the other hand, the same actin preparation, kept in the form of ultracentrifuged pellet (in a carefully closed centrifuge tube, a procedure generally regarded as a safe way to keep actin) for four days, is a much inferior activator of ATP-ase than if kept in a depolymerized state for two days and then reveted by two days of dialysis.

Discussion

As shown in this paper actin precipitated by appropriate concentrations of divalent metal chlorides dissolves in native monomeric state when the $MgCl_2$ concentration is raised to several tenths of moles per liter. It is interesting to compare this behavior of actin to that of globular and fibrous proteins, respectively. Globular proteins in genral are not precipitated in $Me^{++}Cl_2$ solutions. If polymerization of actin is prevented by modification of the Tyr – 53 residue (Bender et al., 1976) or by complex formation with DN-ase-I (Mannherz et al., 1975; Hitchock et al., 1976), it will not precipitate at any concentration of $MgCl_2$ up to 1.0 M (unpublished observations).

On the other hand, "truly" fibrous proteins behave similarly to F-actin. More or less detailed data on this phenomenon are available in the literature for tropomyosin, for the various helical fragments of myosin and for paramyosin. These are all precipiteted by similar concentrations of $MgCl_2$. Thus precipitation of proteins under the conditions described here seems to depend somehow on their highly asymmetric form. The aggregates of the fibrous proteins referred to above formed at low concentrations of $MgCl_2$ dissolve at higher concentrations of $MgCl_2$ (unpublished observations).

In the case of actin we have detailed data on cation binding (Martonosi et al., 1964; Strzelecka–Golaszewska et al., 1978 b.). These data suggest that aggregation of F-action under the conditions described here is the result of neutralization of the overall net charge of the protein. The very low net charge makes the formation of rather weak intermolecular bonds possible. Precipitation of fibrous as opposed to globular proteins may be the result of a cooperation of a periodically repeated series of intermolecular bonds. Interfilament salt bonds through the divalent cation may play an eminent role (Strzelecka–Golaszewska et al. 1978 a).

Dissolution and depolymerization of aggregated F-actin upon rasing the concentration of $MgCl_2$ cannot be explained by the attractive and simple idea of a further, even weaker class of binding sites conferring a net positive charge to the protein (Bárány et al., 1954), because the concentration range of depolymerization (some tenth of a mole per liter) would give affiinities for these binding

sites with free energies of binding in the range of thermal energies (association constants of some $2-5\ M^{-1}$). Depolymerization-dissolution can be regarded instead as a sort of general salting-in effect. Accepting the recent, interesting new interpretation of protein solubility (Melander, Horváth, 1977 a, b) the dissolution of aggregated actin under the conditions described here may mean that hydrophobic interactions play very little role (if any) among the forces holding together the aggregated monomers. The very subordinate role of hydrophobic bonding in monomer-monomer interactions of F-actin can be supported further by contrasting the behavior of F-actin polymer with that of a simple oligomeric protein. In the detailed study of Hsu and Neet (1973) on the dissociation of aldolase tetramers in concentrated solutions of $MgCl_2$ only a partial dissociation, an equilibrium between all possible protomers was found, although $MgCl_2$ concentrations up to 2.0 M and very low protein concentrations were studied. It can be assumed, by analogy to oligomeric proteins of known three-dimensional structure, that the subunits of aldolase are held together mainly by hydrophobic interactions on the contact surfaces, hence the high resistance against the dissociating effect of concentrated $MgCl_2$.

We are much indebted to Miss Ágnes Koródi and Miss Mária Magyar for skilful technical assistance.

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Access to copies of papers under publication from the Nencki Institute, Poland, Warszawa, quoted in this paper was of invaluable help in preparing this manuscript. For this and for her many useful comments on our work we are very much indebted to Dr Strzelecka-Golaszewska.

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Thymus Actin: Preparation and Characterization

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A combination of the C-γalumina adsorption technique of Lindberg, U. Skoog, L. 1970. Eur. J. Biochem. *13* 326—335 with the traditional actin purification procedure (polymerization-depolymerization) yielded a simple method for the preparation of actin from fresh or acetone-dried thymus tissue.

Actin obtained by this procedure from thymus was homogeneous and comigrated with skeletal actin in SDS gel electrophoresis. In isoelectric focusing it was shown to contain β and γ actin.

Thymus actin polymerized poorly or not at all. It was native, however, as judged from its DN-ase I inhibiting activity which equalled that of skeletal actin. It also activated skeletal myosin ATP-ase but to a lesser extent than skeletal actin.

On addition of HMM to thymus G actin, decorated filaments formed abundantly.

Introduction

The abundance of cellular actin in thymus tissue was discovered by the unexpected finding of Lazarides and Lindberg (1974) showing that the inhibitor of pancreatic DN-ase I discovered by Lindberg in thymus (Lindberg, Skoog, 1970) was in fact actin.

The preparative procedure of Lindberg starts with adsorption to and elution from C-γ-alumina gel. This is followed by a series of conventional purification procedures.

A problem constantly encountered in Lindberg's procedure has been the formation of an inactive aggregated protein. Since the monomeric form of actin is the active inhibitor (Mannhez et al., 1975; Hitchkock et al., 1976), the aggregated inactive protein can be assumed to be polymerized actin. These considerations led us to try to prepare thymus actin by combining the very effective adsorption step of the Lindberg and Skoog (1970) technique with polymerization-depolymerization cycles (Mommaerts, 1952) routinely used with muscle actin. This way we developed a simple and effective preparation procedure as described below together with the characterization of purified thymus actin.

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Materials and methods

Thymus was obtained from freshly slaughtered calves and was immediately packed in ice until use. It was used either immediately for preparation or after storage in a deep freezer at -18 °C for up to several months. Crude extract was prepared from untreated tissue or from tissue dehydrated with acetone (five washings with ice cold acetone after grinding).

DN-ase I was obtained from "Zavod Meditsinskykh Preparatov, Leningrad". It was further purified in our laboratory.

Skeletal actin was prepared from rabbit muscle powder using one polymerization-depolymerization cycle. Rabbit skeletal myosin was prepared according to Portzehl et al. (1950), HMM according to Bálint et al. (1975).

DNA (highly polymerized, from chicken erythrocytes), and ATP were from Reanal, Budapest. C-γ-alumina gel was prepared according to Willstätter and Kraut (1923) and used without ageing. Guanidine HCl and urea were from Serva, Heidelberg.

SDS gel electrophoresis was carried out essentially according to Weber and Osborne (1969). Isolectric focusing was done in the presence of urea as described by O'Farrel (1975) with staining according to Malik and Berrie (1973).

Viscosity was measured in an Ubbelohde type viscometer with an outflow time of 60–90 sec depending on the temperature.

ATP-ase activity was measured in a total volume of 2 ml. The reaction mixture contained 0.8 mg/ml myosin, 0.14 mg/ml actin (if not stated otherwise), 2 mM $MgCl_2$, 10 mM tris HCl buffer, pH 7.4, and 2 mM ATP, ionic strength 0.07. Incubation was for 5–20 minutes at 22 °C. Phosphorus determination was done according to Fiske and Subbarow (1925). DN-ase-I inhibiting activity was measured and expressed as specific inhibiting activity according to Lindberg (1967). Protein was determined either according to Gornall et al. (1949) or by a microbiuret test (Itzaki, Gill, 1964) or by ultraviolet absorption.

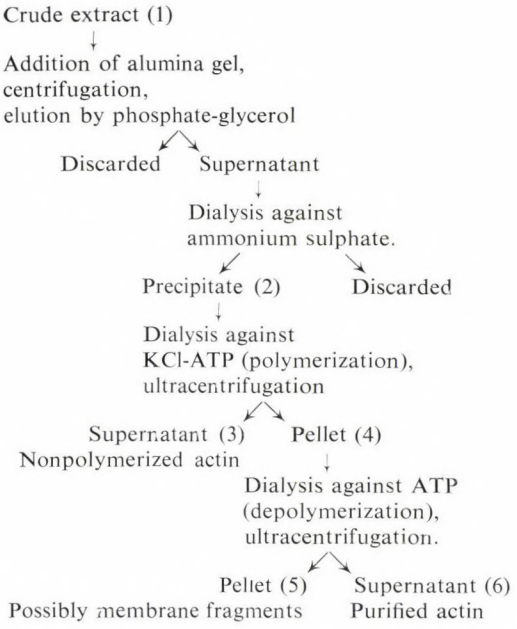
Fixation and „decoration" of actin preparations by HMM, negative staining and observation of specimens were done according to Huxley (1963).

Results and discussion

I. *Preparation*

Scheme 1 shows the flow-sheet of the purification procedure. In Fig. 1 the SDS polyacrylamide gel electrophoretograms of the different fractions obtained are presented.

For the preparation of crude extracts fresh or frozen and thawed thymus tissue was ground and extracted for 30 sec with 1% glycerol, 0.02 M mercaptoethanol (1 : 5, w/v) in a Waring Blendor. The homogenate was centrifuged for 30 min at 10 000 *g*. When the crude extract was prepared from acetone dried



Scheme 1
The preparation procedure of thymus actin

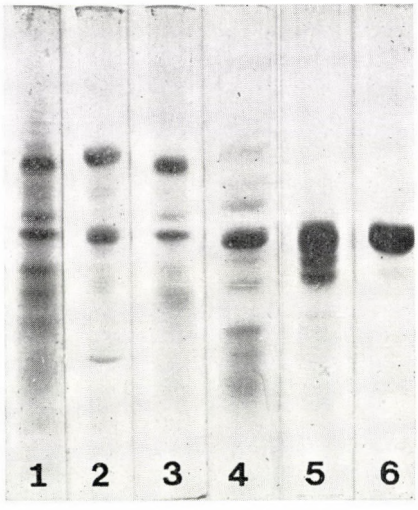


Fig. 1. SDS polyacrylamide gel electrophorestograms of the fractions shown in Scheme 1

Table 1

The activating effect of thymus actin on the ATP-ase of rabbit skeletal muscle myosin (Mg-ATP-ase)

	Specific activity μ mole P/mg myosin. min	Activation %
Skeletal myosin	0.016	—
Skeletal myosin + thymus actin	0.035	216
Skeletal myosin + skeletal actin	0.19	1156

powder, a tenfold volume of neutralized 0.5 mM ATP was used for extraction with subsequent centrifugation. The crude extract (Fig. 1, fraction 1) was further purified according to Lindberg and Skoog (1970): a one to one mixture of crude extract and alumina suspensions was stirred for 5 minutes under cooling. The suspension was centrifuged at 10 000 *g* for 10 minutes. After being washed with 0.05 M phosphate buffer pH 6, the sediment was eluted with 0.15 M phosphate buffer, pH 6, containing 30% glycerol. The eluate was dialyzed against saturated ammonium sulfate solution for 36 hours at 4 °C. The precipitate was collected by centrifugation (30 000 *g*, 15 min) (Fig. 1, fraction 2).

At this point our method followed the conventional actin purification procedure: the ammonium sulfate precipitate was taken up in 0.5 mM ATP, pH 7, and dialyzed against 0.5 mM ATP in 0.1 M KCl (pH 7) for 36 hours. These unusual long dialysis times were found to be necessary to have an optimal yield. Polymerized actin was pelleted by centrifugation at 100 000 *g* for 2 hours. The pellet (Fig. 1, fraction 4) was homogenized in 0.5 mM ATP, pH 7, and dialyzed against the same solution for 24 hours. The dialyzed solution was centrifuged as above to remove any undepolymerized material. A small pellet, looking like F-actin was formed at this step (Fig. 1, fraction 5). The supernatant (Fig. 1, fraction 6) consisted of homogeneous actin that was used for the characterization of thymus actin.

An important component with the electrophoretic mobility of actin remained in the supernatant after the first ultracentrifugation (see: Fig. 1, fraction 3). Under these conditions skeletal actin would sediment out. The considerable amount of native actin contained in this fractions could be detected also by its high specefic inhibiting activity (20 000 U/mg). There was considerable amount, of actin-like protein in the pellet after the second ultracentrifugation (Fig. 1 fraction 5) too. Under these conditions muscle G-actin would have stayed in the supernatant. This actin-like component was consistently accompanied by two lower molecular weight proteins. This pellet did not dissolve when homogenized in either polymerizing or depolymerizing salt solution but gave a turbid suspension.

When the preparation was started from acetone powder, this fraction was practically absent. We assume that this fraction represents some kind of membrane fragment.

II. *Characterization of thymus actin*

Our final preparation comigrated with skeletal actin in SDS gel electrophoresis (Fig. 2 A). When studied by isoelectric focusing (Fig. 2 B) in the presence of 8 M urea, it showed the characteristic double band (β and γ actin) well distinguishable from the skeletal actin. (Whalen et al. 1976). The ratio of β to γ actin was found to be 2 : 1 by planimetry of densitometer tracings. A similar heterogeneity and distinction from skeletal actin in isoelectric focusing was recently been found by different authors for many nonmuscle actins (Schachat et al., 1977; Landon et al., 1977, Garrels, Gibson, 1976).

The DN-ase I inhibiting activity of this preparation equals that of skeletal actin (G form) and is about twice as high (98 000 U/mg) as the specific activity of a Lindberg-type preparation (48 000 U/mg). As this latter is electrophoretically also homogenous (not shown) the difference is most probably due to the completely monomeric and/or native state of our preparation.

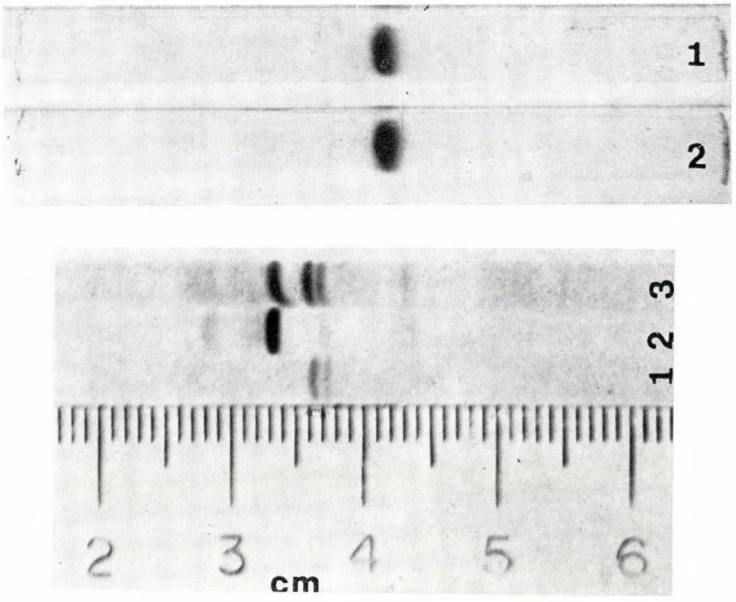
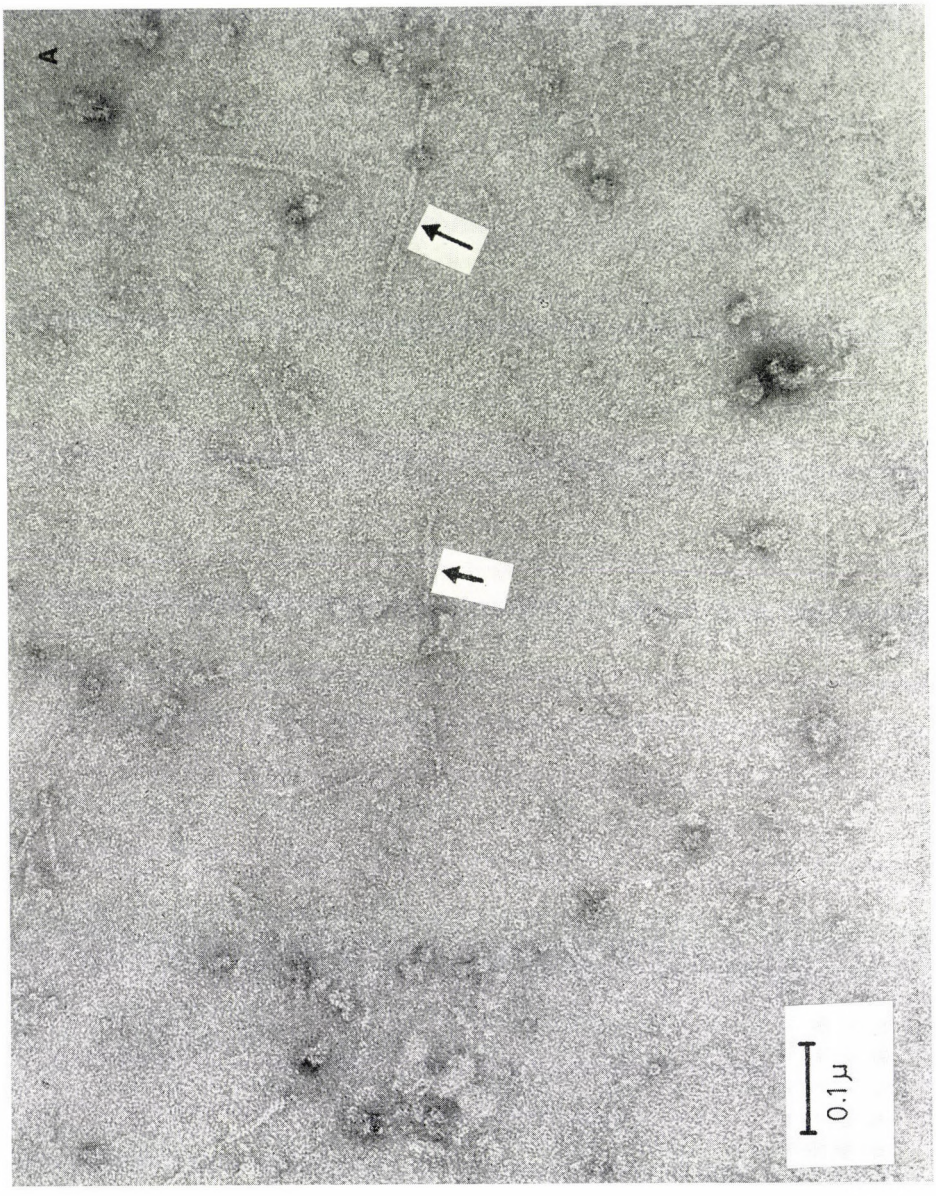


Fig. 2. Electrophoretic comparison of thymus actin and skeletal actin. A). Coelectrophoresis of the two actins in SDS gel. 1: thymus actin, 2 : 1 to 1 mixture of skeletal and thymus actin; B. Isoelectric focusing of skeletal and thymus actin. 1: thymus actin, 2: skeletal actin, 3: 1 to 1 mixture of skeletal and thymus actin. For technical details of the electrophoresis procedures see Materials and methods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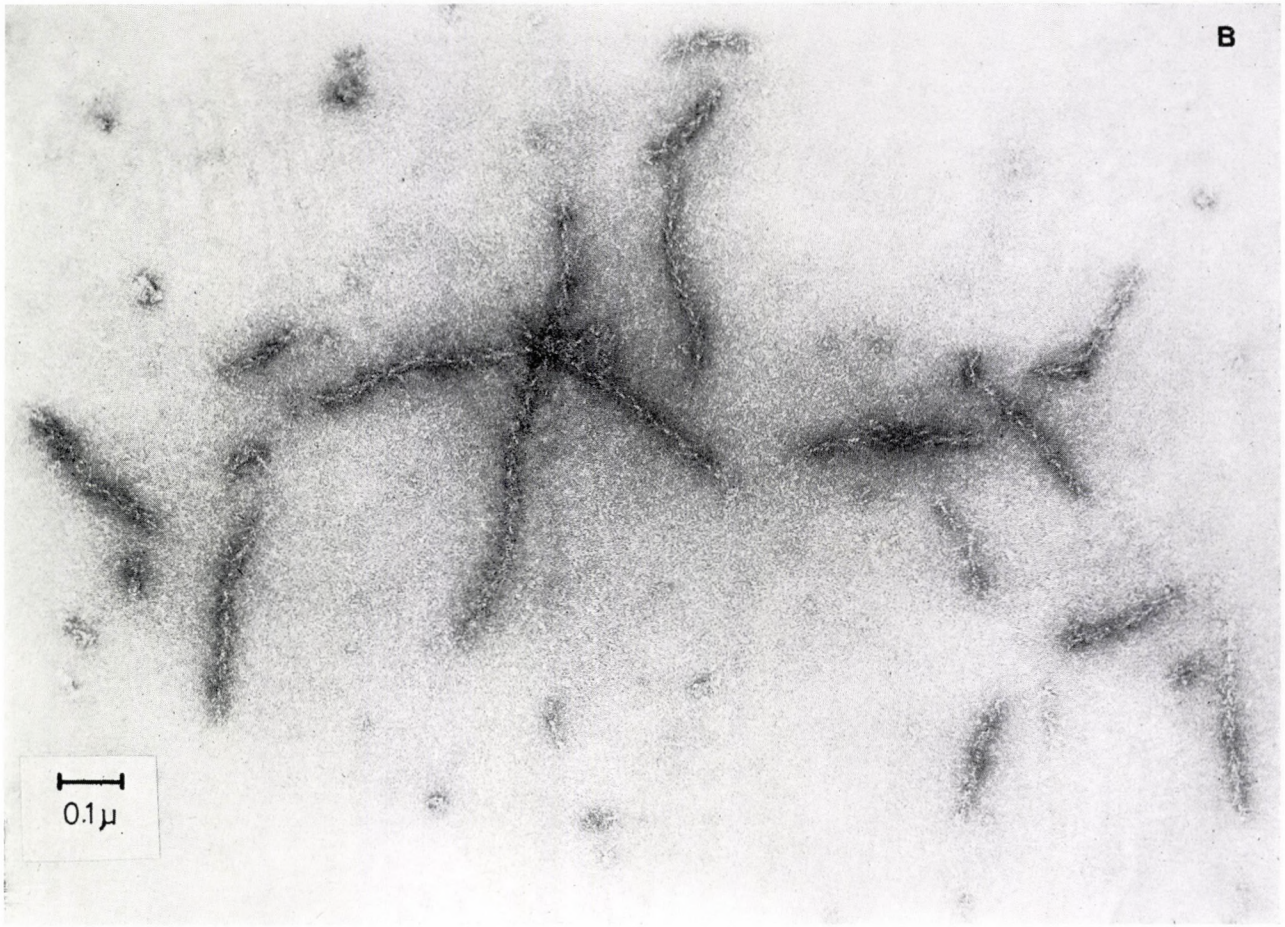

Fig. 3. Electron microscopic observations on thymus actin. A: Thymus actin (pellet after first ultracentrifugation; fraction 4 in Fig. 1). B: The same as A decorated by HMM

Our actin contained 0.76 – 0.85 mole of bound ATP per mole of actin, measured as described by Asakura (1961). Low concentrations of EDTA caused complete denaturation: when thymus actin and skeletal actin were kept in the refrigerator in the presence of 1 mM or 3 mM EDTA at pH 7 for 48 hours, the DN-ase inhibiting activity was completely lost. Control actin treated in a similar way without EDTA showed no decrease of its inhibitor activity.

While many of the properties of thymus actin are closely related to those of skeletal actin, activation of myosin ATP-ase and polymerizability are markedly different.

Although thymus actin had a considerable activating effect on (skeletal) myosin ATP-ase (Table 1) this was rather low, when compared to the 12 fold activation obtained under our experimental conditions for skeletal actin. Similar low activations of ATPase were described for several nonmuscle actins (Pollard, Weihing, 1974). A detailed kinetic analysis of activation of myosin ATPase by thymus actin, as summarized by Korn (1978) for several different cellular actins, was not made.

An even more serious deviation from the behavior of skeletal actin was found in the case of polymerizability. We could not polymerize purified thymus actin (Fig. 1, fraction 6) under a variety of conditions of saline concentration and temperature. The purified actin preparation showed no increase in viscosity even after 48 hours, when 0.1 M KCl was added to a 1 mg/ml actin solution in 0.2 mM ATP. When actin was kept in polymerizing salines for 48 hours and then centrifuged for 2 hours at 100 000 *g* only a negligible amount of F actin (transparent pellet) was formed. The same negative results were obtained when different concentrations up to 6 mM of $MgCl_2$ or $CaCl_2$, alone or with 0.1 M KCl, were tested as polymerizing media.

This unpolymerizability can not be attributed to the relatively low concentrations of actin tested (up to 2 mg/ml) since according to the data of Korn (1978) this concentration is well above the "critical concentrations" for different kinds of cellular actins. In the studies of Bray and Thomas (1976) actin extracted in a monomeric state from cultured fibroblasts and brain by extremely gentle methods, was found to polymerize when concentrated to 1 mg/m or more.

The unpolymerizability of our preparations is not due to denaturation either since our thymus actin gave full DNase I inhibiting activity (equal to that of skeletal G actin) showing that it is native and completely nomomeric. Denatured as well as polymeric actins have no inhibitor activity (Mannherz et al., 1975, Hitchkock et al., 1975).

We have at present no explanation for the unpolymerizability of pure thymus actin. We examined in the electron microscope the pellet obtained after the first ultracentrifugation (Fig. 1, fraction 4). In this state the bulk of thymus actin was sedimented out in contrast to the final preparation.

This pellet obtained was homogenized in 0.1 M KCl. It showed (Fig. 3 A) instead of the long filaments characteristic of skeletal F-actin some short filaments (arrows), together with a considerable amount of amorphous aggregated material.

The picture obtained is very similar to the one observed by Nachmias and Asch (1976) for Physarum actin in an aggregated state. When skeletal rabbit HMM was added to the resuspended pellet the characteristic „decorated" actin filament picture was obtained (Fig. 3 B). This decorated filaments seemed to be in the average longer than the filaments seen in Fig. 3 A but still shorter than skeletal actin filaments. It is possibile that the decorated filaments were formed at least partly by transformation of the amorphous material seen without added HMM.

Rather unexpectedly, thymus actin was readily polymerized when HMM was added: a mixture of thymus actin and HMM (at a ratio of 1 : 2 or 1 : 3), after standing overnight in 0.1 M KCl in the refrigerator, gave a heavy pellet on ultracentrifugation. No precipitate was formed when thymus actin or HMM was treated separately under identical conditions. In the supernatant no actin was detected by electrophoresis and the resuspended pellet gave in the electron microscope characteristic decorated actin filaments in abundance.

Recently Schwartz et al. (1977) produced evidence that actin in nerve ending extracts polymerized poorly but addition of skeletal HMM elicited the formation of typical decorated filaments.

Data on the polymerizability of cellular actins are conflicting. Several authors have described actins which polymerized poorly or not at all under conditions that are known to polymerize skeletal actin (Abramowitz et al., 1975; Muszbek at al., 1976, Bray, Thomas, 1976). Korn, on the other hand, in a recent review (Korn, 1978) is quite cathegorical about the complete polymerizability of all cellular actins. It is interesting to point out, however, that the bulk of cellular actins *in vivo* is in all probability monomeric in spite of rather high intracellular concentrations (Bray, Thomas, 1976). The reason for this is by no means clear. Hence we feel that our thymus actin preparation showing complete unpolymerizability in vitro in spite of its native state warrants further study.

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An Adenosine Triphosphate Dependent Deoxyribonuclease with Adenosine Triphosphatase Activity from *Bacillus cereus*

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An adenosine triphosphate-stimulated deoxyrib onuclease was purified to about 4200 fold from *Bacillus cereus*. The enzyme activity of the crude extract increased by a factor of about 5 after dialysis. One of the low molecular weight inhibitors of the crude extract was found to be inorganic phosphate. During enzyme purification two nucleases were identified. One of them was specific to denatured DNA and the other one degraded both denatured DNA and native DNA. The activity towards native DNA could be increased several times by ATP. Through all steps of purification the ATP-independent DNase always accompanied the ATP-dependent one and the ratio of their activity was found to be constant. The ATP-dependent DNase also possessed ATPase activity stimulated both by native and denatured DNA. The fact that ATPase was stimulated by DNA and went together with ATP-dependent DNase during purification suggests that these functions belong to the same enzyme complex. Maximal activity of ATPase had broader pH, Mg^{2+} and ATP concentration ranges than that of DNase. Cooperation of the two functions may be limited only to a narrow range of ATP concentration. K_m for ATPase was 1.6×10^{-4} M ATP.

Introduction

ATP-dependent DNase seems to be the enzyme involved in genetic recombination (Oishi, 1969; Greth, Chevallier, 1973; Clark, 1971; Vovis, Buttin, 1970). Hovewer, the mechanism of action of this so called "recombination enzyme" of bacteria has not yet been elucidated in detail. Among the broad spectrum of activities towards different DNA substrates the enzyme possesses, in addition to the ATP-dependent DNase activity, a DNA-dependent ATPase activity as well (Vovis, Buttin, 1970; Anai et al., 1970; Nobrega et al., 1972; Smith, Friedman, 1972; Goldmark, Linn, 1972; Rosamond, Lunt, 1977). In enzymes purified to homogeneity (Doly, Anagnostopoulos, 1976) or to near homogeneity (Goldmark, Linn, 1972; Eichler, Lehman, 1977) the DNase and ATPase activities seemed to be tightly associated and therefore not to be separable. The ATP-dependent DNase reaction is accompanied by conformational changes of the DNA (Vovis, Buttin, 1970). The question arises whether or not the ATPase activity is stringently coupled with the degradation of DNA. There are indications that uncoupling of the DNase

and ATPase activities can be achieved by the use of crosslinked DNA (Karu, Linn, 1972; Orlosky, Smith, 1976), by RNA–DNA hybrids (Karu et al., 1973) or by DNA binding protein (MacKay, Linn, 1976).

Some characteristics of the ATP-dependent DNase from Bacillus cereus have already been described (Bánfalvi et al., 1975). In the present paper partial purification of B. cereus ATP-dependent DNase, DNA-dependent ATPase and further differences between the properties of the two activities will be reported.

Materials and methods

Bacteria

Bacillus cereus 130 and *Bacillus cereus* NRRL B–569 were grown in 1% Casamine medium containing 10 g Bactocasamine acid, 2.72 g KH_2PO_4 0.35 g $MgSO_4$, 1.25 g sodium citrate, 4 g glucose, 1 ml of trace element solution,* and 1 ml of ferrous ammonium sulfate solution,** pH 7.2, per liter. *Bacillus cereus* 130 thy was grown in 0.25% Casamine medium which contained 100 μg trimetoprim and 1 μg thymin per ml.

Chemicals. Reanal (Budapest) supplied ATP as disodium salt, DEAE cellulose, ammonium sulfate, egg white lysozyme, and phenol (freshly distilled before use). Pronase was purchased from Calbiochem, bovine serum albumin and Norit A from Serva. Streptomycin sulfate and trimetoprim were the products of EGYT (Budapest), thymine was from Merck Co. ^{3}H-thymidine was purchased from UVVR (Prague).

$\gamma-^{32}$P–ATP (200 Ci per ml) was prepared according to Post and Sen (1976).

Buffer solutions. Buffer A consisted of 20 mM Tris–HCl, pH 7.5, containing 2 mM 2-mercaptoethanol, 0.1 mM EDTA and 10% v/v glycerol and Buffer B of 20 mM Tris-HCl, pH 7.5 containing 2 mM 2-mercaptoethanol, 0.1 mM EDTA and 30% v/v glycerol. Buffered SSC contained 150 mM NaCl, 15 mM sodium citrate, pH 7.0.

Preparation of DNA substrates

Native ^3H–DNA. ^{3}H labelled DNA was isolated from *B. cereus* 130 thy– according to Ohlbaum et al. 1977). Unlabelled B. cereus DNA was isolated from wild type *B. cereus* 130.

* Trace element solution contained 0.1 mg $ZnSO_4 \cdot 7\ H_2O$, 0.1 mg $CoCl \cdot 6\ H_2O$, 1 mg $MnCl_2 \cdot 4\ H_2O$, 0.001 mg $CuSO_4 \cdot 6\ H_2O$ and 0.1 mg ammonium molibdate per 100 ml.

** Ferrous ammonium sulfate solution contained 0.156 g $(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6\ H_2O$ and 0.168 g citric acid per 100 ml.

Heat denatured ^3H-DNA

Native ^3H-DNA was denatured by heating it before use at 100 °C for 10 minutes at a concentration of 100 μg per ml in SSC buffer. This was followed by rapid chilling in ice.

Determination of DNA

The amount of DNA was determined by the method described by Burton (1956).

Assay of ATP-dependent DNase

Exonuclease activity was tested by the release of acid soluble oligonucleotides. The standard assay mixture contained in a volume of 0.15 ml : 3–4 nmol (in nucleotide-equivalents) *B. cereus* ^3H-DNA ($2-3 \times 10^4$ cpm per nmol), 30 nmol ATP, 6 μmol $MgCl_2$, 7.5 μmol Tris-HCl buffer, pH 8.0, and 0.1–0.5 unit of enzyme. A control assay without ATP was always performed. The reaction mixture was incubated at 35 °C, stopped by cooling the sample in ice with addition of 0.15ml cold 2 M perchloric acid and 500 μg unlabelled *B. cereus* DNA in 0.1 ml. After an incubation in ice for 10 minutes the samples were centrifuged at 5000 × *g* for 10 minutes. From the supernatant aliquots of 0.2 ml were pipetted into counting vials which contained 3 ml methanol and 5 ml scintillation mixture (4.2 g 2,5-diphenyl-oxazole and 0.05 g 1,4-bis/4-methyl-5-phenoxazole-2-yl/benzene in 1 liter of toluene). Radioctivity was measured in a Beckman liquid scintillation counter. One unit is that amount of enzyme which degrades 1 nmol of DNA in nucleotide-equivalents to acid soluble products in 20 minutes under the conditions mentioned above. The radioactivities of the samples without ATP were substracted from those containing ATP.

Assay of DNA-dependent ATPase

ATP ase activity was assayed by measuring the amount of inorganic $^{32}P(^{32}P_i)$ released from $\gamma-^{32}P-ATP$. The reaction mixture (0.15 ml) contained 3–4 nmol unlabelled B. cereus DNA, 0.5 nmol $\gamma-^{32}P-ATP$ ($1-2 \times 10^5$ cpm per nmol), 150 nmol unlabelled ATP, 6 μmol $MgCl_2$, 7.5 μmol Tris-HCl buffer, pH 8.0, and 0.1–0.5 unit of enzyme. After incubation at 35 °C for 20 minutes the reaction was terminated by the addition of 0.2 ml activated charcoal suspension (4% in 0.1 M HCl) and 0.05 ml methanol. After standing for 15 min at 0 °C, the charcoal was removed by centrifugation at 4000 × *g* for 20 minutes and the $^{32}P_i$ remaining in the supernantat was measured in an aqueous solution by means of the Cerenkov effect. ATPase activity was always determined in the absence and presence of linear duplex DNA, and DNA-dependent ATPase activity was defined as the difference between the two activites. One unit is that amount of enzyme which degrades 1 μmol of ATP to ADP and P_i in 20 minutes under the conditions mentioned above.

Determination of protein

Protein was determined either spectrophotometrically at 280 μm or by the method of Lowry et al. (1951) using a calibration curve with human serum albumin as a control.

Purification of the ATP-dependent DNase from Bacillus cereus

Bacillus cereus strain NRRL B−569 was grown at 35 °C in 1% Casamine medium in a Gallenkamp orbital shaker (130 rpm). The layer of the medium was 2.5 cm deep. The log phase cells (5×10^8 cells per ml) were harvested by centrifugation and washed once with 0.5% NaCl−0.5% KCl solution and once with cold Buffer A. The cells were resuspended by adding 2 ml of Buffer A to each g of cell (wet weight). The cell suspension was stored at −20 °C. All other operations were carried out at 0−4 °C. Centrifugations were done in a Beckman J−21 centrifuge at 12 000 × *g* for 20 minutes unless otherwise stated.

Lysis

The frozen cell suspension was slowly thawed at 4 °C. 10 ml aliquots were disrupted with ten pulses, 30 sec each, from an Artek Sonic 300 Dismembrator with the intermediate tip at 60% of the maximal frequency. The debris were removed by centrifugation. The supernatant was dialyzed against 200 volumes of Buffer A (Crude extract = Fraction I).

Ultracentrifugation

Fraction I was centrifuged at 110 000 × *g* for 60 minutes in a Beckman model L ultracentrifuge. The pellet was discarded and the supernatant was saved (Fraction II).

Streptomycin sulfate precipitation

Freshly prepared 10% streptomycin sulfate solution in Buffer A was slowly added to Fraction II with constant stirring over a 10 min period. The volume of the added streptomycin sulfate was 1/20 of Fraction II. After mixing for another 20 min the suspension was centrifuged and the pellet which contained the nucleic acids was discarded. The supernatants were pooled (Fraction III).

First ammonium sulfate fractionation

To Fraction III solid pulverised ammonium sulfate was added slowly over a 10 min period to give 50% saturation (31.3 g to 100 ml). After constant stirring for a further 20 min the suspension was centrifuged. The supernatant was saved (Fraction IV).

Second ammonium sulfate fractionation

Solid ammonium sulfate was slowly added to Fraction IV and brought to 60% saturation (6.6 g to 100 ml) as described above and then centrifuged. The precipitate was resuspended in 5 ml Buffer A and dialyzed against 5 liters of Buffer A (Fraction V).

First DEAE cellulose column chromatography

A DEAE cellulose column (1.6 cm × 12 cm) was equilibrated with Buffer A. Two ml of Fraction V were diluted tenfold with Buffer A and loaded onto the column at a rate of 1.0 ml per min. The column was washed with 100 ml of Buffer A, then a linear gradient (200 ml total) from o to 0.5 M NaCl in Buffer A was applied and 2 ml fractions were collected. In order to overcome the observed instability of the enzyme in this step of purification, to each fraction 2 ml glycerol was added. The active fractions were pooled and dialyzed against 100 volumes of Buffer B containing 30 percent glycerol (Fraction VI).

Second DEAE cellulose colum chromatography

A DEAE cellulose column (0.8 cm × 6 cm) was equilibrated with Buffer B. Fraction VI was loaded onto the column at a rate of 0.8 ml per min. After washing the column with 30 ml of Buffer B a linear gradient elution toward 0.6 M NaCl in Buffer B was carried out (40 ml total) and 2 ml fractions were collected. The active fractions were pooled and dialyzed against 100 ml of Buffer B (Fraction VII).

Results

Properties of the enzyme

Inhibitors of ATP-dependent DNase in B. cereus crude extract.

After dialysis of the *B. cereus* NRRL B – 569 crude extract against 100 volumes of Buffer A, the activity of the ATP-dependent DNase increased about fivefold, suggesting the presence of a dialyzable inhibitor.

Nature of inhibitors

The analysis showed that in the crude extract there was a heat-resistant, probably an inorganic substance that inhibited the DNase. We tested two inorganic compounds, sodium phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 8.0) and NaCl for their inhibitory effects. Fig. 1 shows that phosphate buffer, at a concentration of 5×10^{-2} M, inhibited the activity of the enzyme by about 50%, whereas NaCl had

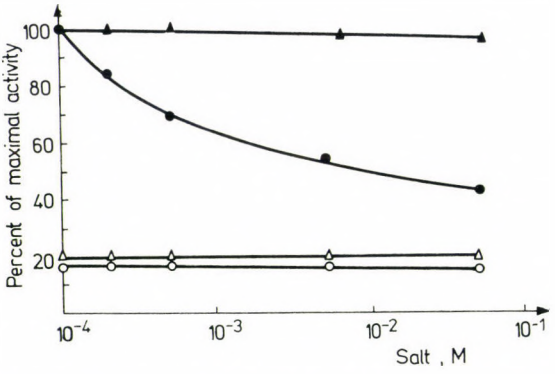

Fig. 1. Inhibitory effect of salts on ATP-dependent DNase. The dialyzed crude extract was preincubated with various concentrations of phosphate (Na_2HPO_4/NaH_2PO_4, pH 8.0) or NaCl. Salts were dissolved in Buffer A. DNase activity in the presence of NaCl without ATP (△), with ATP (▲) and in the presence of sodium phosphate without ATP (○) and with ATP (●)

Table 1

Purification of ATP-dependent DNase-DNA-dependent ATPase from Bacillus cereus NRRL B-569

	Protein, mg	DNase			ATPase		
		Units of activity	Units per mg	Degree of purification	Units of activity	Units per mg	Degree of purification
I. Crude extract	598	347	0.58	1.00	31.7	0.053	1.00
II. Supernatant after ultracentrifugation	576	627	1.08	1.8	—	—	—
III. Supernatant after precipitation with streptomycin	450	1555	3.46	5.9	—	—	—
IV. Supernatant after 50% saturation with ammonium sulfate	364	1393	3.83	6.6	—	—	—
V. Precipitate after 60% saturation with ammonium sulfate	105	1218	11.6	20	—	—	—
VI. 1st DEAE-cellulose column chromatography	5.4	789	146.2	252	10.4	1.86	35
VII. 2nd DEAE-cellulose column chromatography	0.206	506	2453.3	4220	1.15	5.62	106

no inhibitory effect in the concentration range tested (In Fig. 1 only the date obtained with NaCl at concentrations from 10^{-4} to 10^{-1} M are shown). The inorganic phosphate content of the crude extract was determined before and after dialysis and was found to be 2.8×10^{-4} M and 4.5×10^{-5} M, respectively. As can be seen in Fig. 1 re-addition of the dialyzed P_i, to the enzyme causes an inhibition of about 20%. These data suggest that the crude extract probably contained more than one inhibitory factor under our experimental conditions, in contrast to the finding of Ohi and Sueoka (1973), no inhibition was found with low molecular weight DNA (digested with endonuclease I).

We observed that at the early stage of purification the total activity of the ATP-dependent DNase increased (Table 1). The activity after streptomycin treatment was 5 times higher than that of the crude extract. This increase can be attributed to the cellular DNA present which competed with the small amount of radioisotopically labelled DNA added to the reaction mixture.

Detection of two nucleases

Fractions obtained after the first DEAE-cellulose chromatography (see Purification) were checked for nuclease activity both with native and denatured DNAs. DNase activity was detected both in the presence and in the absence of ATP. As Fig. 2 shows, DNase activity towards denatured DNA was eluted at 0.22 M NaCl. The ATP-dependent DNase activity towards native DNA as a substrate was eluted with 0.28 M and 0.32 M NaCl, in two separate peaks. As shown in Fig. 2, ATP-independent DNase could be detected in the latter peaks as well, but the rate of DNA degradation was found to be much

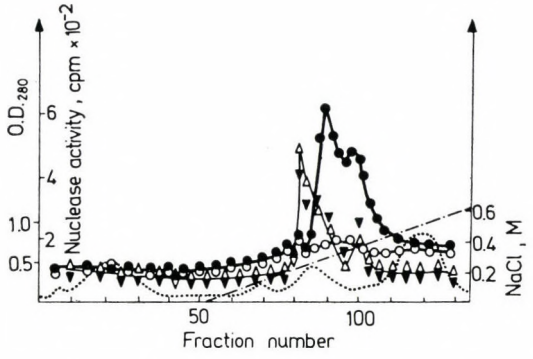

Fig. 2. First DEAE-cellulose chromatography of ATP-dependent DNase and substrate specificity of the enzyme for double and single stranded DNA. Fraction V was loaded onto a DEAE-cellulose column as described in section "Purification . . .". Nuclease activity was measured with native DNA in the absence of ATP (○), in the presence of ATP (●), with denatured DNA in the absence of ATP (△) and in the presence of ATP (▲) as described in "Methods". Protein was determined spectrophotometrically at 280 nm (. . .); NaCl gradient (— . —)

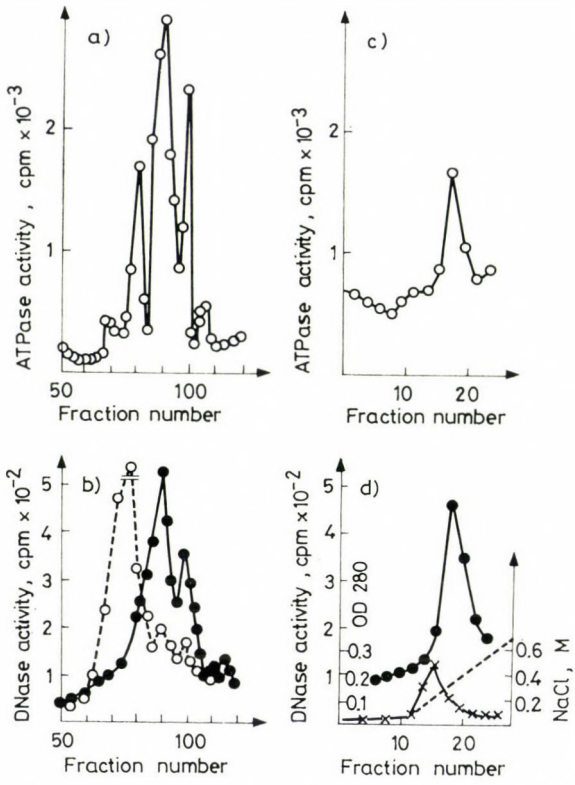

Fig. 3. The copurification of ATP-dependent DNase and DNA-dependent ATPase after the first and second DEAE-cellulose column chromatographic runs. A: DNA-dependent ATPase activity after the first DEAE-cellulose chromatography (○); B: ATP-dependent DNase activity towards native DNA (●) and towards denatured DNA (○) in the fractions from the first DEAE-cellulose chromatographic run; C: DNA-dependent ATPase activity (○) in the fractions from the second DEAE-cellulose chromatographic run. Pooled fractions (98—103) of the first chromatographic run were loaded onto the column as described in section "Purification . . ."; D: ATP-dependent DNase activity towards native DNA (●) in the fractions from the second DEAE-cellulose chromatographic run. Protein was determined spectrophotometrically at 280 nm (×); NaCl gradient (- -). The purification procedure and the measurement of DNase and ATPase activities are described in "Methods"

lower. These data suggest that we have separated two kinds of enzyme, an ATP-dependent DNase having some ATP independent nuclease activity, too, and a nuclease specific for denatured DNA. Fractions separated by DEAE cellulose chromatography and containing DNase were also tested for ATPase activity. As shown in Fig. 3 A ATPase activity was detected in each peak with DNase activites. Fig. B presents the same peaks with respect to their DNase activity towards both native and denatured DNA. From the three major peaks in Fig. 3 B the first represents the ATP-independent DNase specific to the denatured DNA,

the second and third peaks the ATP-dependent DNase. The two latter peaks (fractions 88–96 and 98–106) were then subjected separately to a second DEAE-cellulose chromatography. The pooled material from fractions 88–96 (middle peak) lost both its DNase and ATPase activities after the second chromatography, but the other pooled material (from fractions 98–106) maintained both its ATP-dependent DNase (Fig. 3 D) and DNA-dependent ATPase activities (Fig. 3 C). The DNA-dependent ATPase activity after the second chromatography (Fig. 3 C) coincided with the ATP-dependent DNase activity presented in Fig. 3 D.

The reason of presenting the properties of the nuclease and phosphohydrolase of Fraction VII together is that the DNase and ATPase activities went together through all the purification steps mentioned above.

Substrate specificity

The nucleolytic activity was tested with native and heat denatured *B. cereus* ^{3}H–DNAs. The time course of the reaction is presented in Fig. 4. The quantity of the nucleotides released by the ATP-dependent enzyme was about 5–7 times higher with double stranded DNA than with single stranded DNA as described earlier (Bánfalvi et al., 1976). The enzyme could hydrolyze ATP to P_i and ADP and this ATPase activity was stimulated by a factor of 2–3 in the presence of double or single stranded DNA.

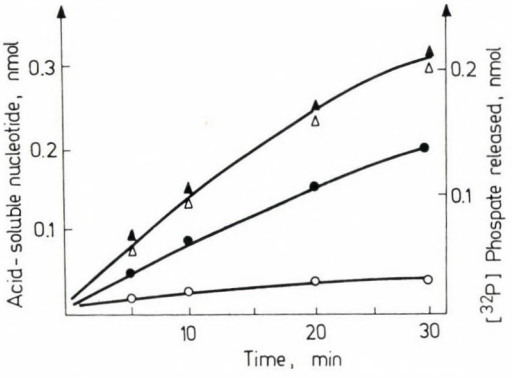

Fig. 4. Kinetics of ATP-dependent DNase and DNA-dependent ATPase activities towards native and denatured DNA. DNase and ATPase assays were carried out with native and denatured DNA as described in "Methods". The samples were taken at the intervals indicated. Degradation of denatured DNA (○), degradation of native DNA (●). Hydrolysis of ATP in the presence of denatured DNA (△) and in the presence of native DNA (▲)

Mg^{2+} dependency

The enzyme needs Mg^{2+} both for its nucleolytic and phosphohydrolytic activities. Fig. 5 represents the ATP-dependent and ATP-independent nuclease activities of the purified enzyme at different Mg^{2+} concentrations. The Mg^{2+} optima of the ATP-dependent and ATP-independent DNases were the same, 10 mM as determined with native DNA. It can be seen that the DNase activity with and without ATP rapidly decreased at Mg^{2+} concentrations higher than 10 mM.

There was a difference between the Mg^{2+} requirement of the ATP-dependent DNase and the DNA-dependent ATPase. At high concentrations of Mg^{2+} (40–120 mM) the degradation of DNA was inhibited, while the rate of ATP hydrolysis was not influenced (Fig. 6).

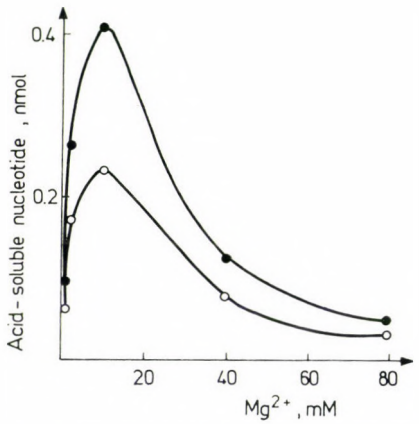

Fig. 5. Variation of ATP-dependent and ATP-independent DNase activities at different Mg^{2+} concentrations. DNase activity was measured with 0.4 unit of enzyme under the assay conditions described in "Method". Mg^{2+} concentration changed between 1–80 mM. ATP-independent DNase activity (○), ATP-dependent DNase activity (●)

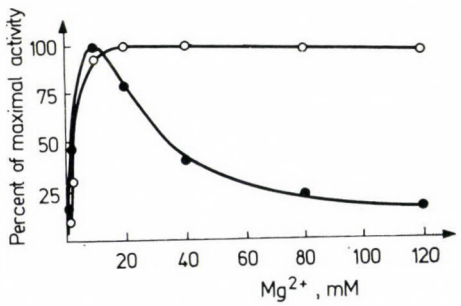

Fig. 6. Dependence of DNase and ATPase activities on Mg^{2+} concentration. ATP-dependent DNase and DNA-dependent ATPase assay conditions were those described in "Methods". Mg^{2+} concentration increased from 2 to 120 mM. The relative activities of DNA-dependent ATPase (○) and ATP-dependent DNase (●) were compared

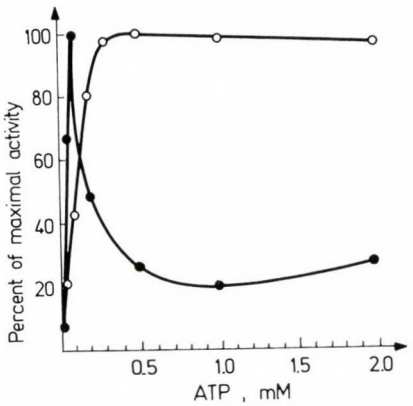

Fig. 7. Effect of ATP concentration on the ATP-dependent DNase and DNA-dependent ATPase reactions. The reaction mixture (0.15 ml) contained 3 nmol *B. cereus* ^{3}H-DNA, 1.5 μmol $MgCl_2$, 7.5 μmol Tris/HCl, pH 8.0, and 0.3 units of DNase. The reactions were terminated after incubation for 20 minutes at 35 °C with perchloric acid in the case of DNase and with Norit A in the case of ATPase as described in "Methods". ATP concentration changed between 10 μM—2 mM. ATPase (○), DNase (●)

ATP dependency

Fraction VII had its optimal DNase activity at about 0.1 mM ATP concentration (Fig. 7). At 1 mM ATP concentration the level of DNase activity fell to 20% of the maximal activity in the case of the purified enzyme. At higher concentrations of ATP the DNase activity of the purified enzyme decreased much more rapidly than in crude extracts. The narrow range of ATP concentration for maximal activity has been reported also for enzymes purified from other bacteria (Vovis, Buttin, 1970; Goldmark, Linn 1972; Chestukhin, et al., 1972; Doly, Anagnostopoulos, 1976). Fig. 7 also shows that the ATPase activity was not inhibited at higher concentrations of ATP unlike the DNase activity. The K_M of the DNA-dependent ATPase was found to be 1.6×10^{-4} M ATP. The release of inorganic phosphate was parallelled by an increase in the amount of acid soluble material during the course of the reaction. The ratio of phosphate released from ATP to that released from oligonucleotides with an average length taken as 3—6 nucleotide residues was 3.2 at 0.5 mM ATP concentration (optimum for ATPase) and about 1 at 0.12 mM ATP concentration (optimum for DNase).

pH dependency

Enzyme activities were measured between pH 7 and pH 10 in 50 mM Tris-HCl buffer (Fig. 8). The optimum for the DNase was about pH 9.0 in accordance with the data obtained in crude extracts (Bánfalvi et al., 1975). While ATP-

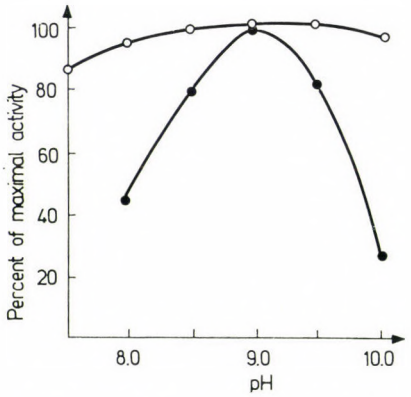

Fig. 8. Effect of pH on DNase and ATPase reactions. Assay conditions described in "Methods" were employed. DNase and ATPase activities were determined by using 0.05 M Tris/HCl at pH values from 7.5 to 10.0. DNA-dependent ATPase activity (○), ATP-dependent DNase activity (●)

dependent DNase activity had a relatively sharp pH maximum at pH 9.0, ATPase activity was not sensitive to pH and maintained its catalytic activity within wide pH range.

Discussion

The 4200 fold purification of ATP-dependent DNase from *B. cereus* may be attributed to a removal of inhibitory substances. ATP-dependent DNase inhibitors have been reported to occur in crude extracts of other microorganisms (Goldmark, Linn, 1970; Ohi, Sueoka, 1973). Goldmark and Linn (1970) found a heat-labile inhibitor in *E. coli*. Ohi and Sueoka (1973) detected an inhibitor in *B. subtilis* that consisted of fragmented DNA or DNA complexed with other compounds. We found no inhibition with low molecular weight DNA under our experimental conditions. Ultracentrifugation and streptomycin sulfate precipitation resulted in an increase in DNase activity, most probably due to the removal of DNA which would have competed with the small amount of radioisotopically labelled DNA in the reaction mixture. An increase in total DNase activity observed during the dialysis of the crude extract indicates that one or more small molecular weight substances had been removed. In accordance with this observation a slight inhibitory effect of inorganic phosphate was found.

DEAE-cellulose column chromatography separated two kinds of DNases which eluted close to each other. One of them was a nuclease specific for denatured DNA and with no requirement for ATP. The other DNase degraded preferentially linear double stranded DNA and its activity increased upon the addition of ATP. Similarly, two kinds of nucleases have been reported by Miller and Clark (1976).

The ATP-dependent DNase from *B. cereus* was eluted in two separate peaks with 0.28 and 0.32 M NaCl, respectively, in the first DEAE-cellulose chromatography. Two peaks of ATP-dependent DNase were also observed in *B. subtilis* (Ohi, Sueoka, 1973) and in *E. coli* (Friedman, Smith, 1972). In spite of the purification of the enzyme, the ATP-independent activity could not be separated from the ATP-dependent one. The optimal assay conditions for both activities were the same. Furthermore the ratio of ATP-dependent activities remained nearly constant during the purification procedure. Residual ATP-independent activity was also described for enzymes purified from other bacteria (Friedman, Smith, 1972; Doly, Anagnostopoulos, 1976). The ATP-dependent DNase possessed a DNA-dependent ATPase activity as well, as it has been described for other microorganisms (Vovis, Buttin, 1970; Anai et al., 1970; Smith, Friedman, 1972; Goldmark, Linn, 1972; Ohi, Sueoka, 1973; Doly, Anagnostopoulos, 1976). ATPase was eluted together with the DNase even after rechromatography of the ATP-dependent DNase. In spite of this fact the degrees of purification of DNase and ATPase were remarkably different: a 106 fold purification was achieved for ATPase and a 4200 fold purification for DNase. Maximal DNA-dependent ATPase and ATP-dependent DNase activities were measured at different ATP concentrations. Furthermore, hydrolysis of ATP under our conditions (pH 7.0, 1 mM ATP, 40 mM $MgCl_2$) took place without phosphodiester bound cleavage. Detection of two activities – ATP-dependent DNase and DNA-dependent ATPase – as well as the difference in the degree of their purification suggest the existence of a protein complex carrying independent and separable functions.

The hydrolysis of ATP was increased by the addition of either single-stranded or double-stranded DNA. The K_m for ATP was found to be 1.6×10^{-4} M which was similar to the value described for unwinding proteins of bacteria (Abdel-Monem et al., 1977; Young, 1978). The role of ATP in the degradation of DNA is still obscure. Complexes of ATP-dependent DNase bound to DNA do not seem to require either Mg^{2+} or ATP (Wilcox, Smith, 1976). Therefore the hypothesis that the binding is an ATP-dependent process is very unlikely. ATP may have other functions: either unwinding of double stranded DNA (Friedman, Smith, 1973; MacKay, Linn, 1974) and/or translocation of the enzyme along the DNA during its digestion (Winder, 1972).

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A Novel Method for the Purification of 15-Hydroxy-Prostaglandin-Dehydrogenase from Swine Lungs

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15-Hydroxy-prostaglandin-dehydrogenase was purified 3 to 5 fold and with almost 100% yield from swine lungs by a novel method of precipitation with Rivanol after which it was further purified (8—10 fold) by gel-filtration. The optimal stability parameters of the purified enzyme preparation were determined and the behaviour of PGDH in polyacrylamide-gel-electrophoresis was studied by local enzyme detection. The enzyme seems to be composed of subunits. K_M values of PGDH with respect to NAD, PGE_2 and $PGF_{2\alpha}$ were found to be 0.27, 0.017 and 0.038 mM, respectively.

Introduction

A number of methods for the purification of 15-hydroxy-prostaglandin-dehydrogenase (E.C. 1.1.1.141) are known from the literature (Änggård, Samuelsson, 1966, 1969; Marrazzi, Andersen, 1974; Hanson, 1976). These methods usually employ high speed centrifugation and precipitation techniques (salting out, organic solvents) for the middle-stage purification of PGDH-containing organ extracts. Instead of these rather tedious steps we succeeded in developing a novel method of selectively precipitating PGDH with acridin dyes, e.g. Rivanol (Tolnay et al., 1974) which was applied earlier for protein separations (Neurath, Brunner, 1969). This step with almost 100% yield leading to a 3 to 5 fold purification was followed by gel-filtration to obtain PGDH of high purity. The intermediate product was found to be stable for 4—5 months at $-15\,^{\circ}$ C.

Materials and methods

Rivanol (6,9-diamino-2-ethoxy-acridinium-lactate) was obtained from Serva GmbH; PGE_1, PGE_2 and $PGF_{2\alpha}$ from Chinoin Co., Budapest; NAD from Reanal Fine Chemicals, Budapest; Sephadex G-150 from Pharmacia AB; Aquacide III

Abbreviations: PGDH, 15-hydroxy-prostaglandin-dehydrogenase; NAD, nicotinamid-adenin-dinucleotide, oxidized; NADH, reduced NAD; PGE_1, PGE_2 and $PGF_{2\alpha}$, resp., prostaglandin E_1, E_2 and $F_{2\alpha}$, resp.; NBT, nitroblue-tetrazolium; PMS, phenazin-methosulphate; EDTA, ethylene-diamine-tetraacetic acid disodium salt; ME, 2-mercapto-ethanol.

from Calbiochem Corp.; NBT, amidoblack 6B, PMS and acrylamide-monomer from Fluka AG; molecular weight marker substances from Mann Co. All other substances were of analytical grade.

The activity of PGDH was measured by using a modified version of the method of Marrazzi and Matschinsky (1972) in a system containing 95 μM PGE_1, 1.1 mM NAD, 5.5 mM phosphate buffer, pH 7.4, 1.1 mM EDTA, 2.2 mM ME and PGDH. Incubation was at 44 °C for 30 min. Spectrophotometric determinations were made in 1.0 cm silica cells at 340 nm, measuring the absorption of NADH formed or in 0.5 cm glass cells at 500 nm after alkaline treatment of the 15-keto-PGE_1. 1 unit of PGDH is that amount of enzyme which oxidizes 0.12 n-mole of PGE_1 per min under the above conditions. The molar extinction coefficient of 15-keto-PGE_1 was taken as 16 900 $M^{-1} cm^{-1}$.

Sephadex columns were equilibrated with 0.05 M phosphate buffer, pH 7.4 containing 2 mM EDTA, 4 mM ME and 0.3 M NaCl. All other details are given in the legends to the figures. Polyacrylamide-gel-electrophoresis was carried out according to Williams and Reisfeld (1964). Local staining was made with amidoblack 6B while enzyme activity tests were carried out by introducing $PGF_{2\alpha}$ and NAD through diffusion into the disks and detecting the enzymatically formed NADH through a PMS-NBT electron carrying system. Size-isomers and charge-isomers of PGDH were detected according to Hedrick and Smith (1968). Protein was determined according to Lowry et al. (1951).

Results and discussion

Chopped swine lungs were extracted with 2 volumes of 0.01 M phosphate buffer, pH 7.4, containing 2 mM EDTA and 4 mM ME (solution "A") in a homogenizer for 8 min and centrifuged at 1200 × *g* for 45 min. All subsequent operations were carried out at 4 °C. The supernatant solution, after filtration through cheese cloth, was left to stand overnight. The pH was adjusted with 2 N HCl to 5.6–5.7. The precipitate formed in large amounts was centrifuged off for 15 min as above and discarded. After readjusting the pH to 6.2–6.3 with 2 N NaOH, 0.5 volume of 0.4% (w/w) aqueous Rivanol solution was added under continuous stirring. The precipitate containing PGDH was centrifuged as above, washed with water and suspended in solution "A" containing 0.25 M KBr. Protein extraction was carried out in an ice-cold beaker with 15 min stirring followed by centrifugation at 4300 × *g*. The supernatant solution was dialyzed overnight against 20 volumes of solution "A", centrifuged for 30 min at 4300 × *g* and lyophilized. The yield was 2.5–3.0 g intermediate product from 1 kg fresh swine lungs. This powder is highly soluble with a specific activity of 4–6 units/mg dry weight, and stable for 4–5 months at −15 °C.

The distribution of PGDH between precipitate and solution as a function of pH after precipitation with Rivanol is shown in Fig. 1. It can be seen that beyond pH 6.3 the PGDH-content of the precipitate did not increase further. For

future experiments a pH of 6.2 was chosen since pH values below 6.0 may cause activity losses. This step led to a 3–5 fold purification compared to the previous stage.

This product was further purified by gel-filtration on Sephadex G-150 (Fig. 2A). The bulk of PGDH was found in the descending branch of the last major peak. The contents of the enzymatically active tubes were pooled, dialyzed against 20 volumes of solution "A", concentrated with Aquacide III in a dialysis bag,

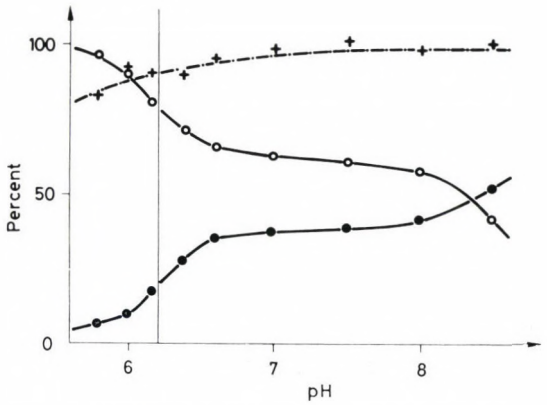

Fig. 1. Distribution of the protein content expressed in percentage of the initial amount, between the precipitate and the supernatant as a function of pH after precipitation with 0.13% (w/v) Rivanol. Precipitate, ——●—— supernatant, ——○——; PGDH activity extracted from the precipitate and expressed in percentage of the initial activity, ——+——The vertical line intersecting the abscissa at 6.2 indicates the pH value for optimal separation

brought to 0.3 M NaCl and rechromatographed (Fig. 2B). The PGDH-containing fractions were pooled, dialyzed and concentrated as above and stabilized by the addition of glycerol and NAD so as to obtain a final concentration of 50% (v/v) and 0.25 mM, respectively.

A summary of the purification steps and yields is given in Table 1.

The purified PGDH preparation keeps its activity without significant loss for 4–6 months if stored below 4 °C. Neither glycerol nor NAD alone could bring about a satisfactory stabilization (Fig. 3). A number of other additives were also tested as stabilizers (inorganic salts, amino acids, proteins, synthetic polymers, sugars, polyols etc.) without acceptable results.

The purified preparation was not yet homogeneous as indicated by polyacrylamide-gel-electrophoresis; activity staining showed two components.

In order to decide whether these bands corresponded to size-isomers or charge-isomers the purified PGDH was run with proper markers in a series of gels, each with a different acrylamide concentration (Fig. 4). The converging lines

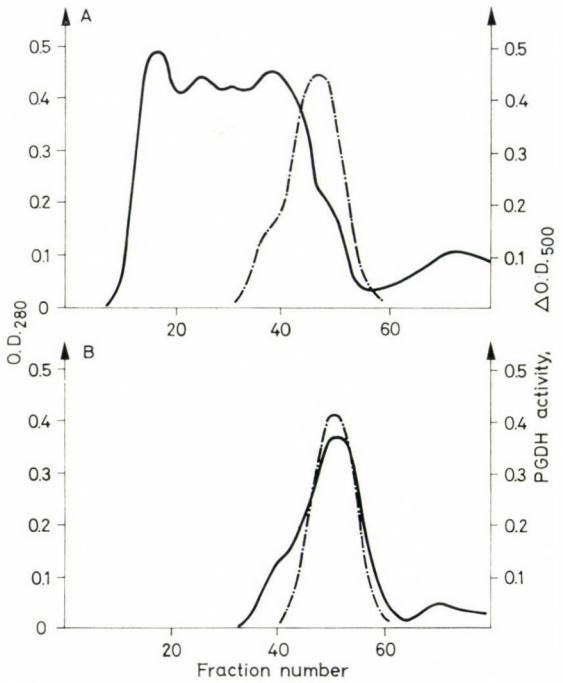

Fig. 2. Gel-filtration of crude PGDH on Sephadex G-150. Elution buffer: 0.01 M phosphate buffer, pH 7.4, containing 2 mM EDTA, 4 mM ME and 0.3 M NaCl. Starting material: 1 g lyophilized crude product dissolved in 25 ml buffer. A) Chromatography on a 2.4× 100 cm Sephadex G-150 column. Fraction volume, 5 ml; B) Rechromatography of the PGDH-containing fractions on a Sephadex G-150 column 1.5× 90 cm. Fraction volume, 1.5 ml. E_{280} (solid line) was measured from 1 : 5 and 1 : 2 dilutions of the fractions after chromatography A) and B), respectively. PGDH activity (Dotted line) was determined from 0.1 ml aliquots in a final volume of 0.9 ml

Table 1

Purification and yields of PGDH. Starting material: 1 kg (fresh weight) of swine lung tissue

Preparative step	Protein (g)	Activity (10^3 units)	Specific activity (units/mg protein)
Centrifuged homogenate	13	*	(1.7)**
pH 5.7 supernatant solution	8.5	21	2.5
After Rivanol fractionation	1.8	22	12
Lyophilized crude product	1.8	16	9 (6)***
Gel-filtration on Sephadex G-150	0.27	14	50
Rechromatography on Sephadex G-150	0.11	11	100

* Photometric evaluation could not be carried out precisely because of interfering factors.

** This value was obtained from the total activity measured before Rivanol treatment and the protein content of the centrifuged homogenate.

*** Expressed in units per mg dry weight.

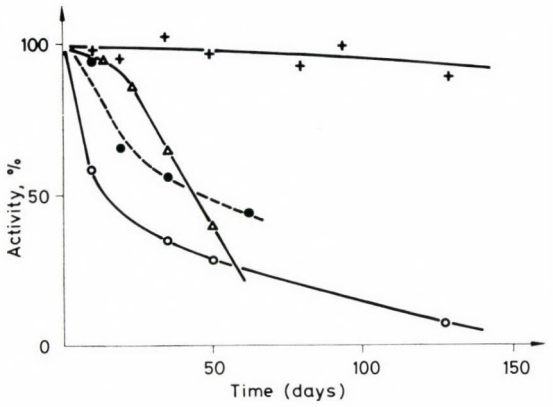

Fig. 3. Stability of the purified PGDH preparation during storage at 4 °C. Stabilizing agents: 50% (v/v) glycerol + 0.25 mM NAD, ——+——; 50% (v/v) glycerol, ——●——; 0.25 mM NAD, ——△——; without stabilizer, ——○——

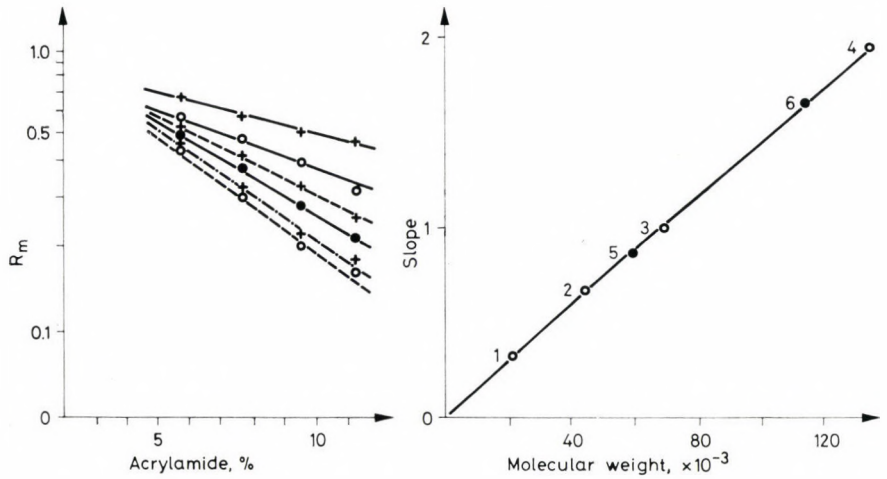

Fig. 4. Polyacrylamide-gel-electrophoresis of the purified PGDH in gels with different acrylamide concentrations. A) Relative mobilities of the components plotted against the acrylamide concentration of the gel. Symbols: PGDH-components detected by activity staining, ----+---- and -.-.-+-.-.-; soybean trypsin inhibitor (M. W. 20 000), ——+——; ovalbumin (M. W. 43 000), ——○——; Bovine serum albumin monomer (M. W. 68 000), ——●—— and its dimer, ----○----; B) Slopes of the lines characteristic of the proteins used for calibration plotted against their molecular weights. Soybean trypsin inhibitor, 1; ovalbumin, 2; bovine serum albumin; 3 and 4 for the monomer and dimer, respectively. Slopes of the two lines for PGDH define the points 5 and 6

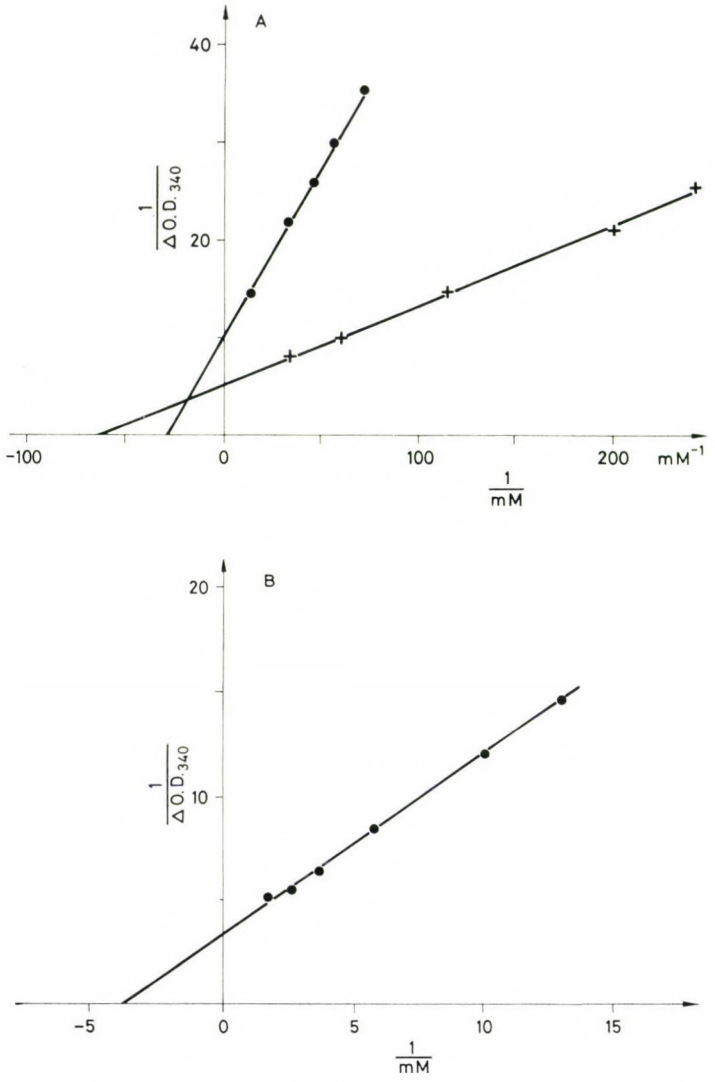

Fig. 5. Lineweaver-Burk plots of PGDH. Reaction time 30 min. A) 12 units/ml PGDH; 1.6 mM NAD; 4—50 μM PGE_2 ——+——; 20—100 μM $PGF_{2\alpha}$ ——●——; B) 12 units/ml PGDH; 0.1 mM PGE_2; 70—1000 μM NAD

of the marker compounds and active bands indicated that our purified PGDH consisted of size-isomers. Plotting the slope of the curves against molecular weights a value of 60,000 daltons was obtained for PGDH (main component) with an accompanying component of about 120,000 daltons. The molecular weight of the enzyme as estimated by gel filtration on Sephadex G-150 (Divald, 1977)

was 30,000 daltons which is in good agreement with the value reported by Jung et al. (1975).

Separation of the predominating PGDH component by preparative gel-electrophoresis of the purified PGDH yielded a pure PGDH component. Further work is in progress with this pure component.

Some enzymological parameters of pure PGDH were estimated by plotting Lineweaver-Burk diagrams with various kinds of prostaglandins and NAD, respectively. In these experiments PGDH was always saturated with the invariable substrate. Details are given in the legends to Fig. 5 (A, B).

The apparent K_M value of PGE_2 was found to be 0.017 mM, that of $PGF_{2\alpha}$ 0.038 mM, while that of NAD 0.27 mM.

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Heterogeneity in the Carbohydrate Moiety of the γ-Chain of Bovine Fibrinogen

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Two glycopeptides with identical amino acid sequence and carbohydrate composition, but of different sialic acid content were isolated from a combined tryptic-chymotryptic hydrolysate of bovine fibrinogen. Glycopeptide 1 contained two moles, glycopeptide 2 one mole of sialic acid. Both of them may have been derived from the γ-polypeptide chain according to their amino acid sequence: Gln-Val-Glu-Asn-Lys.

Fibrinogen, the clotting plasma glycoprotein is built up of three polypeptide chain-pairs [α "A", $_\beta$(B) and γ*] cross-linked by disulphide bridges. Two of them contain a carbohydrate moiety consisting of galactose, mannose, N-acetil-glucosamine and sialic residues, whereas the α-chain is free of carbohydrate. The 1-78 N-terminal amino acid sequence of the γ-polypeptide chain from human fibrinogen – bearing the carbohydrate moiety – has been determined (Iwanaga et al., 1968). The complete sequence of the β-chain (Lottspeich, Henschen, 1977) and of the γ-chain (Henschen, Lottspeich, 1977) of human fibrin has also been reported.

Several laboratories have been engaged in the structure determination of bovine fibrinogen. Mostly short glycopeptides were isolated and their amino acid sequences were determined (Haschemeyer et al., 1966; Mészáros, 1968). Recently, the partial amino acid sequences of the disulphide knot peptides from bovine fibrinogen have been determined (Timpl et al., 1977). These polypeptide sequences, however, are devoid of carbohydrate.

In this paper we report the isolation of a glycopeptide-pair with identical amino acid sequence, carbohydrate composition, but different sialic acid content, deriving from the γ-chain of bovine fibrinogen.

* This nomenclature for the chains has been recommended by the International Committee on Hemostasis and Thrombosis (Washington, 1967).

Experimental

Bovine fibrinogen was precipitated and purified from fresh citrated plasma (Blombäck, Blombäck 1957).

Combined tryptic-chymotryptic hydrolysis. Purified fibrinogen fraction 1–4 (20 g) was suspended in 0.1 M ammonium hydrogen carbonate solution (1 lit) and digested in the presence of trypsin (200 mg) and chymotrypsin (200 mg). After 12 hours, fresh enzymes (200 mg of each) were added and next day the digest was acidified with acetic acid to pH 4.0, and evaporated (with some butanol-1 to prevent foaming) to about 100 ml. The residue was centrifuged (3000 × *g*, 30 minutes) and the precipitate resuspended in 0.6 per cent acetic acid (60 ml). After centrifugation the clear supernatants were combined and used for gel chromatography. The precipitate was discarded as it did not contain any significant amount of carbohydrate.

Gel filtration. An aliquot (30 ml) of the clear supernatant from the enzymic digest was applied on the top of a 50 × 800 mm Sephadex G 25 fine column equilibrated with 0.6 per cent acetic acid. The carbohydrate-containing fractions (reagent: orcinol-sulphuric acid) were combined and freeze dried with a yield of about 150 mg substance.

Ion exchange chromatography. The glycopeptide fraction (30 mg) from gel filtration was applied on the top of a 9 × 750 mm Dowex 50 × 2 (200–400 mesh) column equilibrated with 2 M pyridine-acetic acid buffer, pH 3.1, dissolved and eluted with the same buffer. The elution was monitored with a differential refractometer. The orcinol sulphuric acid positive peaks were pooled, evaporated and the rest of buffer eliminated on a Sephadex G 15 fine column (15 × 700 mm) with water as eluant and freeze dried. The yield for glycopeptide-1+2 was 18 mg. Glycopeptides from peaks 3 and 4 derive from the β-cnain and will be discussed in a separate paper.

The first two not completely separated peaks contain glycopeptides from the γ-chain. They were resolved by rechromatography on a Dowex 50 × 2 column (11 × 2220 mm) in pyridine-acetic acid buffer, pH 2.6. Conditions were the same as in the first run. The yields are given in mg.

Paper electrophoresis. The homogeneity of the fractions from the ion exchange separation were controlled by paper electrophoresis. The runs were made in 2 N acetic acid at 10 V per cm for 4 hours. Detection was performed with periodate-benzidine reagent (Mowery, 1957) and with ninhydrin.

Digestion with carboxypeptidase B (Sajgó, Dévényi 1972). The peptide (0.2 mg) was dissolved in 50 μl of 0.1 per cent ammonium hydrogen carbonate solution, incubated at 38° with 0.5 U carboxypeptidase B (68 U/mg) for 30–300 minutes. Samples taken at different intervals were acidified with 10 μl of 0.1 N hydrochloric acid and after evaporation chromatographed on Silicagel G plates and Whatman CC 41 plates in solvents: A) n-butanol, acetic acid, water (4 : 1 : 1), B) n-butanol, methyl-ethyl-ketone, diethylamine, water (15 : 15 : 3 : 7.5), detection with alcoholic ninhydrin solution.

Amino acid analyses were performed as usual in an amino acid analyzer.

Amino acid sequence analysis. The manual Edman degradation (Sjöquist et al., 1961) was employed and the PTH-amino acids were identified by TLC.

Desialysation was done as described earlier (Mészáros et al., 1971).

Methylation analysis was performed by using 2 mg glycopeptide (Björndal et al., 1967; Hakomori, 1964).

Neutral sugars were determined in the form of alditol acetates (Savardeker, Sloneker 1965), by gas liquid chromatography, *aminosugars* were not determined.

Sialic acid was determined by the thiobarbituric acid method (Warren, 1959).

Results

Bovine fibrinogen was degraded by combined tryptic-chymotryptic digestion and the carbohydrate-carrying peptides were separated from the bulk of peptide fragments by gel filtration (Fig. 1). The crude product containing glycopeptides from both chains (β and γ) and some peptides, too, was further purified on a cation exchange column and separated into four fractions containing carbohydrate. At pH 3.1 (Fig. 2A) two lysine glycopeptides were present in peaks 1+2, while peaks 3 and 4 contained arginine glycopeptides deriving from the β-polypeptide chain. The lysine glycopeptides were separated in a somewhat more acidic buffer at pH 2.6 (Fig. 2B) on a Dowex 50×2 resin. The fractions were controlled for

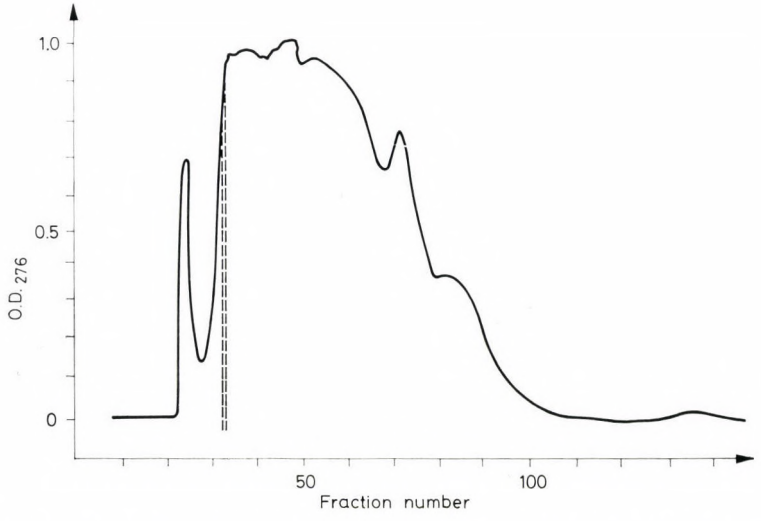

Fig. 1. Gel chromatography of an enzymatic digest of bovine fibrinogen (30 ml) on a Sephadex G-25 column (50× 800 mm) equilibrated with 0.6% acetic acid. The flow rate was 300 ml per h. Detection with Uvicord, fractions between the two vertical lines were collected. 25 ml fractions were collected

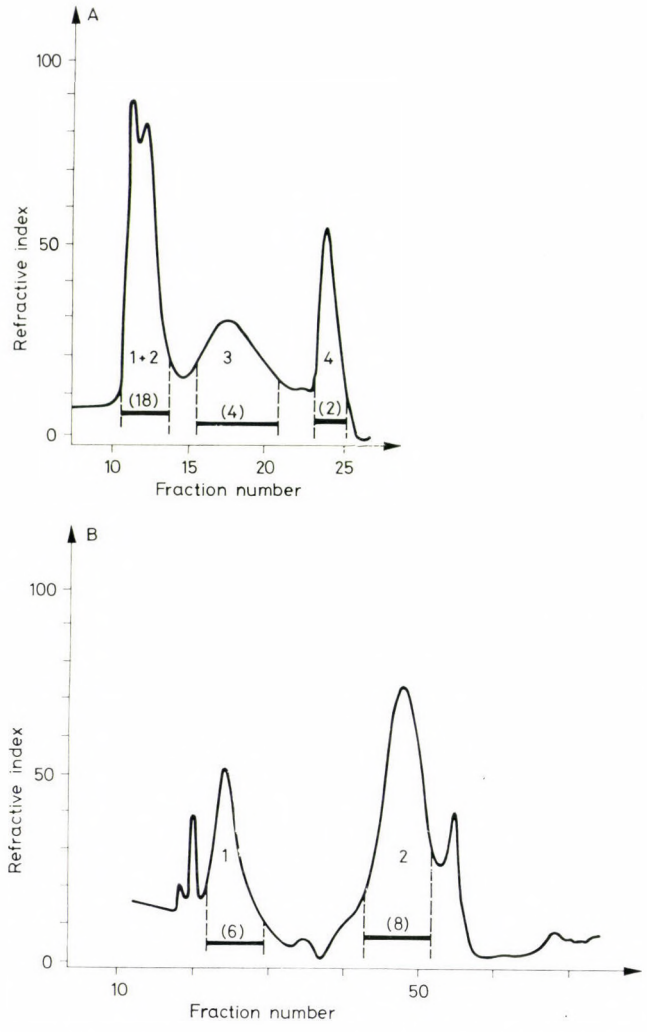

Fig. 2. Separation of glycopeptides on a Dowex 50× 2 column. A) The column (9× 750 mm 200—400 mesh) was equilibrated with 2 M pyridine-acetic acid buffer, pH 3.1. The glycopeptide mixture (30 mg) was applied to the column and eluted with the same buffer. The flow rate was 40 ml/h and 4 ml fractions were collected. Detection by differential refractometry, sensitivity 32×. B) Rechromatography of fraction 1 + 2 (from run A) on a Dowex 50× 2 column (11× 2200 mm, 200—400 mesh) in 2 M pyridine acetic acid buffer, pH 2.6. Conditions were the same as in run A. The weights of the glycopeptides are given in brackets

homogeneity by repeating the ion exchange chromatography in the last buffer and by paper electrophoresis.

The results of the amino acid and carbohydrate analyses for glycopeptide 1 and 2 are shown in Table 1. It is interesting to note that their composition was identical except the sialic acid content. Glycopeptide 1 contained two moles,

Table 1

Composition of Glycopeptides

	Glycopeptide moles/mole	
	1	2
Val	0.80	0.84
Glu	1.81	1.84
Asp*	1.00	1.00
Lys	0.90	0.87
Man	3.00	3.00
Gal	0.80	0.76
Sialic acid	1.94	1.10

* Asp set equal to 1.00.

glycopeptide 2 one mole of sialic acid. Both glycopeptides had pyrrolidone carboxylyl amino terminal, which came from the N-terminal glutamine by cyclisation under the acidic conditions used for isolation of the glycopeptides. The blocked peptides were unable to react with the coupling reagents used in the Edman degradation. Therefore the pyrrolidone rings were opened with methanolic hydrochloric acid (Samu, Mészáros 1978). After this treatment the sequence determination worked smoothly and gave the following sequence:

Gln-Val-Glu-Asn-Lys

for both glycopeptides. This sequence is identical with that described for the fragment 49 – 53 of the γ-chain of human fibrinogen (Iwanaga et al., 1968).

The data of methylation analysis (Table 2) are in good agreement with those of the sialic acid analysis. Glycopeptide 1 contained no terminal galactose unit, glycopeptide 2 however, furnished one mole of tetra-O-methyl galactose. In a separate experiment both glycopeptides were desialysed by mild acid hydrolysis and after methylation no difference was found in their composition (Column 2 in Table 2). Both of them yielded 2 moles of tetra-O-methyl galactose. On the basis

Table 2

*Relative amounts of methylated alditol acetates from glycopeptides 1 and 2***

		Galactose		Mannose	
		2,3,4,6-tetra-O-methyl	2,3,4-tri-O-methyl	3,4,6-tri-O-methyl*	2,4-di-O-methyl
Glycopeptide	1	0.1	1.6	2.0	0.9
	2	0.9	0.8	2.0	1.2
Asialoglycopeptide	1	1.8	0.13	2.0	0.9
	2	1.7	0.08	2.0	0.8

* 3,4,6-tri-O-methyl-mannose set equal to 2.0.
** The glucosamine derivative was not estimated.

of these data it may be concluded that in glycopeptide 1 the galactose residues are in penultimate positions from the non-reducing termini, each holding a sialic acid residue on carbon atom 6, while glycopeptide 2 one galactose is in terminal position and the other one carries one mole of sialic acid on carbon atom 6.

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Influence of the Electrical Activity of Muscle upon the Excitability of Neighbouring Nerve

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Starting from studies concerned with the electrical interaction between adjacent nerve fibres, experiments were carried out to investigate the different subthreshold effects of the electrical activity of muscle upon the neighbouring nerve. The results obtained on frog's sciatic-gastrocnemius preparations show that electrical muscle activity has an influence upon the excitability of the nerve in contact with the muscle. The results support the assumption that electrical muscle activity could play an additional role in the regulatory mechanism of the excitatory processes in nerve and muscle functions.

Introduction

The subthreshold influences of the active nerve fibres upon the excitability of the inactive ones which can be attributed to the electrical interaction between adjacent nerve fibres have been demonstrated by Blair and Erlanger (1940), Katz and Schmitt (1940), Marrazzi, Lorente de Nó (1944) and Katalymov (1974). Mathematical studies concerning this type of interaction between nerve fibres have been carried out by Clark and Plonsey (1971) and Markin (1973).

Continuing our previous experiments (Biró, 1975, 1977a, 1977b; Biró, Vu-Duy-Thinh, 1977) in which we showed that under different conditions the excitation could be transmitted from the muscle to the nerve which was in contact with the active muscle, the present work focused on studying the different subthreshold effects of the electrical activity of muscle upon the neighbouring nerve.

Methods

The experiments were performed on sciatic-gastrocnemius preparations isolated from frogs (Rana esculenta). The preparations were connected to each other with the nerve of the second preparation lying on the surface of the muscle of the first preparation.

The responses of the preparations were elicited with two square-wave stimuli 0.1 ms in duration applied to the nerves through the platinum electrodes 1 mm in diameter. The first stimulus given to the first nerve was submaximal in all cases.

The strength of the second stimulus given to the second nerve was submaximal, threshold and subthreshold depending on the type of the experiment. The interval between the first and the second stimuli was varied in steps of 1 ms within the range of 1 to 12 ms. A separatory transformator made the stimuli independent from each other.

The action potentials were recorded by oscillographic method. Two pairs of 5 mm-wide platinum plates were used for holding the preparations and recording the action potentials of the muscles. The action potentials of the first and the second muscle respectively were registered by the upper and the middle beam of the oscilloscope. The lower beam was used for recording the stimuli as the points above the time markers of 1 ms.

The experiments were carried out at room temperature (22-24 °C). Care was taken to keep the preparations under wet condition.

Results

In the first series of the experiments the second preparation was excited by submaximal stimuli of constant amplitude applied to the nerve portion lying on the first muscle (Fig. 1A) or to the proximal portion of this nerve (Fig. 1B). With the increase of the interval between the first and second stimuli, the amplitude of the action potentials recorded from the second muscle has changed. The characteristic changes of these amplitudes are illustrated in the diagrams (Fig. 1). According to the diagrams the highest amplitude of action potential generated by the second muscle could be recorded in the case of the stimulus interval of 5 ms.

In the next series of the experiments we measured the threshold of the second nerve during the excitation of the first muscle. It was found that the threshold measured 5 ms after the first stimulus was significantly lower than before or after the excitation of the first muscle (Fig. 2). Statistical analysis of the results obtained in 10 experiments showed that the decrease in the threshold of the second nerve lying on the excited muscle proved to be 22 ± 5 (mean $\pm$S.E.) per cent.

In accordance with the previous results the phenomenon of subthreshold summation also demonstrated the influence of the electrical activity of muscle on the excitability of the neighbouring nerve. The oscillograms presented in Fig. 3 show that the submaximal excitation of the first muscle and the subthreshold stimulation of the second nerve separately did not result in action potential of the second muscle, but these two effects together were able to bring about the excitation of the second preparation.

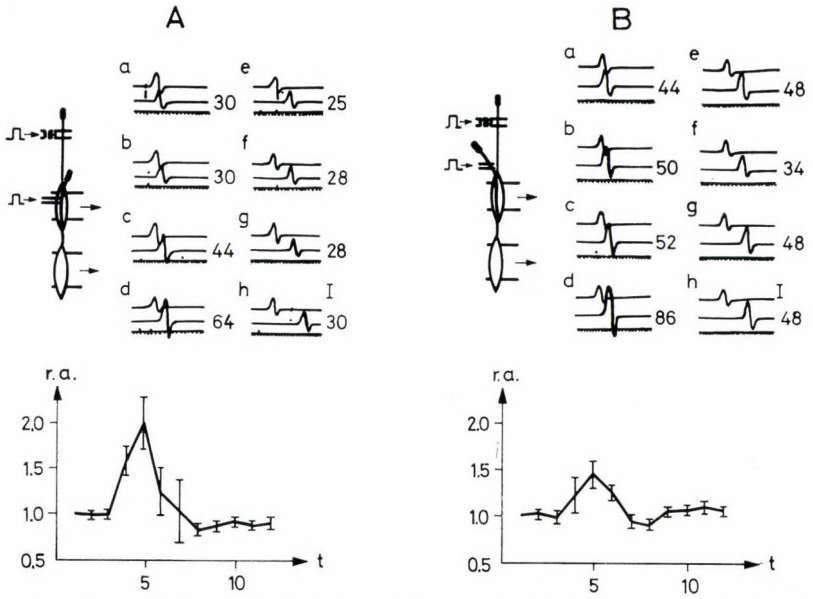

Fig. 1. The influence of the excitation generated by the first muscle on the amplitude of action potentials recorded from the second muscle. The second stimulus was applied to two different portions of the second nerve (A and B). The action potentials in the registrations a—h were obtained with increasing delay between the stimuli. The numbers at the oscillograms show the peak-to-peak amplitude of the second action potentials. Calibrations are 20 mV for the upper and the middle beams in part A as well as the middle beam in part B, and 50 mV for the upper beam in part B. The diagrams illustrate the changes in the relative amplitude (r.a.) of the second action potential as the function of the delay (t in ms) between the first and the second stimuli. Values represent means and ± S.E.

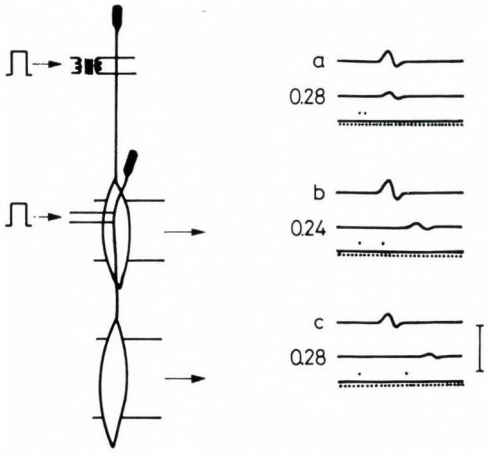

Fig. 2. Change in the threshold of the nerve lying on the excited muscle. The numbers at the oscillograms show the nerve thresholds in V before (a), during (b) and after (c) the excitation of the first muscle. Calibration is 100 mV and 20 mV for the upper and the middle beams, respectively

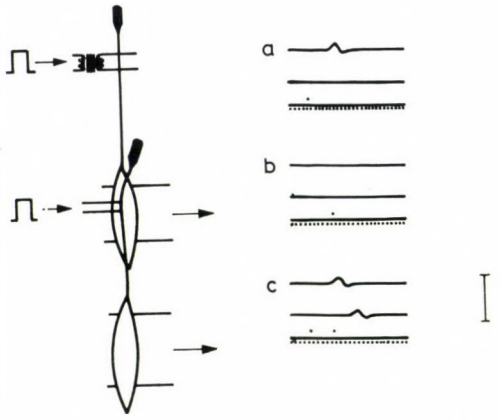

Fig. 3. The subthreshold summation of the electrical muscular activity and an electrical stimulus. a: action potential of the first muscle elicited by the first stimulus; b: there was no response when only the second nerve was excited with a subthreshold stimulus; c: the previous two stimuli together resulted in an action potential of the second muscle, too. Calibration is 100 mV and 20 mV for the upper and the middle beams, respectively

Discussion

The observation on the influence of the electrical muscular activity on the excitability of the nerve being in contact with a muscle has not yet been described in literature. The changes in nerve excitability brought about by the muscle action potential have been demonstrated indirectly by the results shown in Fig. 1. From the alterations in the amplitude of action potentials recorded from the second muscle, it follows that different number of fibres in the nerve lying on the first musle came into excitation. As this nerve was stimulated with constant electrical impulses, the changes in the number of active nerve fibres caused by the changes in excitability must have been produced by the action potential of the first muscle.

Moreover, the demonstration that the nerve threshold decreases (Fig. 2) and the phenomenon of subthreshold summation (Fig. 3) have also borne evidence of the influence of the electrical muscle activity upon the excitability of the neighbouring nerve. The possibility of this kind of influence exerted by the active muscle is supported by the finding that the medullary sheat is a poor or leaky insulator (Koide, 1975).

In addition to changing the threshold of the nerve fibres, the electrical muscle activity could modify the conduction velocity and the synchronization of nerve impulses similarly to the electrical interaction between adjacent nerve fibres (Katz, Schmitt, 1940; Arvanitaki, 1942).

The observations demonstrated in this paper support the assumption (Biró, 1975, 1977b; Biró, Vu-Duy-Thinh, 1977) that the electrical muscle activity could play an additional role in the regulatory mechanism of the excitatory processes in the nerve and muscle function.

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"Bound" Water as Solvent Water

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The misconception, which says that an extraneous material cannot be solved in "bound" water has reappeared especially in biological literature. In our experiments quantities of urea and glucose enough for saturation dissolved in the saturated solutions of NaCl and KCl, though a part of water forming the hydration envelopment of ions can surely be considered as "bound" for it migrates together with the ion in an electric field.

Introduction

When writing about the biologist's older view concerning: "the water in the cytoplasm is not free as in dilute salt solution but is bound", Ling (1972) mentions the "strong opposition" by Hill (1930) corroborated by Blanshard's (1940) review. Discussing the detailed history of this question in his paper, Ling states that the older "concept of cell protoplasm became all but extinct in the English-speaking world (in the years following . . . 1940)". The situation can be characterized among others, by the fact that Höber (1945) in contrast to his earlier (1914) standpoint, further Fenn (1945) and e.g. also Buchthal (1947) reverberated Hill's opinion. In general, this situation prevailed in international public opinion, though there appeared a paper from our Biophysical Institute (Ernst et al., 1950) describing three quite different methods and demonstrating unequivocally that from muscle and Ringer's fluid, in the course of continuous drying up, the former retained its water content much more than the latter.

In the next decade, however, the situation changed, in so far as there appeared two monographs by Ernst (1958, 1963) reviewing the experimental data concerning the "boundedness" of water and inorganic atoms in biological systems. Furthermore, Troshin published his detailed theory about the same questions in three editions (1956, 1958, 1966) and also Ling (1962) in his well known theory took up a similar position in respect of the problem. And, what is more, in contrast to the situation described by Ling and quoted above, there appeared three volumes edited by "The New York Academy of Sciences" (Saunders, Flynn, 1965; Hazlewood, 1973; Liboff, Rinaldi, 1974) dealing with these and similar problems and emphasizing the "boundedness" of water and inorganic ions in many papers quite decidedly.

In view of this situation it would not seem necessary to continue the debate described by Hazlewood (1977) as follows: "A. V. Hill (1930) published a report based on vapour pressure measurements that little, if any, water in blood or muscle is bound. This paper seems to have been taken as definitive even though Ernst (1934) adequately challenged this work". Notably, it is clear that Hill's standpoint (1930): "The free water fraction is defined as the weight of water in 1 g of fluid or tissue which can dissolve substances added to it" contains a basic mistake. The hydration water layer migrates with the ions of an aqueous solution in an electric field, thus this water surely can be considered to be "*bound*", notwithstanding it is certainly not settled that this water would necessarily have stopped "*free*" to solve other solutes. Dealing with the question whether "the bound water would also be nonsolvent water", Bateman (1945) emphasized "the latter property has been used, perhaps rather naively, as a . . . criterion of water binding".

Putting aside this debate we think that further discussion of the problem is necessary e.g. owing to a passage even in the very thorough work of physical chemistry (Erdey-Grúz, Schay, 1962) stating: (Vol. III. p. 22): "In the course of the increase in ion concentration, the hydration number per mol ion becomes so large that all molecules of water are bound in the hydrate envelope. The water content in solutions more concentrated than that can not be considered as solvent."

This statement is certainly not very clear-cut and that is why we performed a few experiments to demonstrate the real situation, according to which the "bound" water in the hydration shells of ions can act simultaneously as solvent water for certain solutes even in the quantity corresponding to saturation.

Methods

Saturated aqueous solutions of NaCl and KCl were examined in our experiments, after adding *quantities of glucose and urea appropriate for saturation in comparison with the quantity of water.*

I.1. In order to produce saturated NaCl solution 18.0 g of the analytically purest (a. lt.) NaCl was solved in 50 ml of bidistilled water (20 °C). After that glucose was added to one half of the series of solutions (a. lt., 49.5 g $C_6H_{12}O_6 \cdot H_2O$), and 54.7 g of urea ($H_2N \cdot CO \cdot NH_2$, a. lt.) was added to the other half. A solid precipitation occurred in the saturated solution of NaCl after the addition of glucose.

2. Saturated KCl was produced as follows: 17.0 g of KCl was added to 50 ml of bidistilled water; when it dissolved, quantities of glucose and urea appropriate for saturation were added, which entirely dissolved.

3. In the third group of our experiments KCl and NaCl was added to the saturated NaCl and KCl solutions, also in a degree of saturation. In this series

of experiments a certain quantity of solid substance was precipitated after the addition of the second quantity of material.

The experiments were performed at room-temperature (20–22 °C), which could have caused slight shifts in saturation.

II. Determination of inorganic and organic components of solution and precipitations:

1. Determination of solutions: 1 ml of solution was diluted to 1000 ml and the Na and K contents were measured with flame-photometer.

2. For control 0.25–0.50 ml of the solution was dried incinerated, and the NaCl and KCl content of the ash was determined in this way; the quantity of glucose and urea was determined from these data and the dry-matter content with gravimetry.

3. The inorganic and organic matter content of solid precipitations was also determined with gravimetry and with flame-photometry after dissolving.

Results

The following tables contain the results of our experiments.

Table 1 contains the data of NaCl + urea experiments. According to the data of the Table the saturated NaCl solution entirely dissolves the added quantity of urea appropriate for saturation.

Table 1

18.0 g NaCl + 54.7 g urea in 50 ml water

No.	Quantity altogether		Flame photometry				Gravimetry			Quantity altogether	
	solution, ml	remains g	sample ml	degree of dilution	Na altogether g	NaCl altogether g	Wet sample ml	Dry g	Ash NaCl mg	NaCl g	Urea g
90	98	0	1	1000	6.27	15.93	0.25	0.20	48	18.82	58.8
91	98	0	1	1000	6.27	15.93	0.25	0.19	46	18.03	56.8
98	98	0	1	1000	6.27	15.93	0.25	0.20	46	18.03	58.8
99	98	0	1	1000	6.27	15.93	0.25	0.19	45	17.64	56.5

Table 2 shows the data of the saturated NaCl + glucose experiments; the glucose which was added to the saturated NaCl solution in a degree of saturation did not dissolve entirely, but a significant quantity of solid precipitation occurred. This is about 1/5 to 1/6 part of the whole quantity of the added material, the proportion of NaCl and glucose in it is $\sim$ 1 : 5 in mols.

Table 2

18.0 g NaCl + 49.5 g glucose in 50 ml water

No.	Quantity altogether		Flame photometry			Gravimetry			
	solution ml	remains g	sample ml	degree of dilution	Na altogether g	NaCl altogether g	Wet sample ml	Dry g	Ash NaCl mg
44	82	13.09	1	1000	6.15	15.65	0.50	0.33	98
45	82	9.89	1	1000	6.15	15.65	0.50	0.34	97
46	81	11.80	1	1000	6.08	15.43	0.50	0.34	100
64	82	9.96	1	1000	5.99	15.21	0.50	0.33	96

Quantity altogether in solution		Gravimetry in remains		Quantity altogether remains		Quantity altogether solution + remains	
NaCl g	glucose g	Dry g	Ash NaCl mg	NaCl g	glucose g	NaCl g	glucose g
16.07	38.54	0.31	44	1.84	11.25	17.91	49.79
15.91	39.36	0.30	45	1.48	8.41	17.39	47.77
16.20	38.88	0.31	44	1.70	10.10	17.90	48.98
15.71	39.36	0.20	36	1.78	8.18	17.49	47.54

Table 3

17.0 g KCl + 49.5 g glucose in 50 ml water

No.	Solution altogether, ml	Flame photometry				Gravimetry			Quantity altogether	
		sample ml	degree of dilution	K altogether g	KCl altogether g	Wet sample ml	Dry g	Ash KCl mg	KCl g	Glucose g
74	90	1	1000	8.82	16.85	0.50	0.35	94	16.86	46.44
75	91	1	1000	9.28	17.73	0.50	0.35	95	17.28	46.96
76	90	1	1000	8.82	16.85	0.50	0.35	93	16.74	46.49
77	91	1	1000	8.74	16.69	0.50	0.36	96	17.54	47.32

Table 4

17.0 g KCl + 54.7 g urea in 50 ml water

No.	Solution altogether ml	Flame photometry				Gravimetry			Quantity altogether	
		sample ml	degree of dilution	K altogether g	KCl altogether g	Wet sample ml	Dry g	Ash KCl mg	KCl g	Urea g
94	98	1	1000	8.53	16.29	0.25	0.19	44	17.25	54.88
95	98	1	1000	8.53	16.29	0.25	0.18	43	16.86	54.48
96	98	1	1000	8.62	16.47	0.25	0.18	42	16.46	55.27
97	98	1	1000	8.62	16.47	0.25	0.18	42	16.46	55.27

Table 5

18.0 g NaCl + 17.0 g KCl or 17.0 g KCl + 18.0 g NaCl in 50 ml water

No.	Sequence of addition of materials	Quantity altogether		Flame photometry in solution			Flame photometry in remains			Quantity altogether g
		solution ml	remains g	sample ml	degree of dilution	altogether g	altogether g	sample g	solution ml	altogether g	
92	NaCl+KCl	50	15.43	1	1000	Na: 5.00 K: 3.75	NaCl: 12.70 KCl: 7.16	1.00	500	NaCl: 5.88 KCl: 9.55	NaCl: 18.58 KCl: 16.71
93	NaCl+KCl	50	14.53	1	1000	Na: 5.00 K: 3.75	NaCl: 12.70 KCl: 7.16	1.00	500	NaCl: 4.96 KCl: 9.57	NaCl: 17.66 KCl: 16.73
100	KCl+NaCl	50	14.53	1	1000	Na: 5.00 K: 3.75	NaCl: 12.70 KCl: 7.16	1.00	500	NaCl: 5.51 KCl: 8.96	NaCl: 18.21 KCl: 16.12
101	KCl+NaCl	50	13.92	1	1000	Na: 4.90 K: 3.70	NaCl: 12.45 KCl: 7.08	1.00	500	NaCl: 4.83 KCl: 9.09	NaCl: 17.28 KCl: 16.17

In order to control the experiments in Table 2 the solution was made in reverse order (glucose + NaCl); the data of these experiments approximately correspond to the results of Table 2.

Table 3 and 4 contain the results of the experiments performed with the saturated KCl solution.

Table 3 shows the data of experiments with KCl + glucose, Table 4 shows the data of those with KCl+urea. It can be seen from the data of the table, that the quantity for saturation both of glucose and of urea dissolves in the saturated KCl solution.

The reverse of the KCl + glucose experiment the glucose + KCl as a control showed a rough agreement with the data of Table 3.

Table 5 shows the experiments in which KCl was added to the solution saturated with NaCl, in a degree of saturation (or in reverse order).

The data of the table show that – as it is known – the inorganic substances which are added in a quantity appropriate for saturation are not completely dissolved in both series of experiments: the solid remains contain a relatively greater amount of KCl than of NaCl. The proportion of KCl and NaCl in mols is $\sim 5 : 4$.

Discussion

The present work examines the solubility of certain materials, not for producing analitically correct data, but in order to help in extinguishing the misconceptions connected with this problem in biological literature. The misconception mentioned in the preface, saying that *"bound" water cannot act as a solvent* seems unextinguishable not only from biological literature (e.g. Szent-Györgyi, 1957; Palmer, Gulati, 1976), but it is surviving also in physico-chemical and colloidical works (Tschapek, M., Wasowski, C., 1977). However, it is correct to pronounce only that water "bound" *in a certain way* can serve as a solvent *for certain materials* only *to a certain extent*. E.g. the "bound" water of $CuSO_4 . H_2O$ is not a solvent for any material; the water "bound" with the swelling of organic substance can be a solvent for every inorganic substance; but nothing definite can be said about the thin capillary "bound" water sticking to a glass plate. In the opinion stated in the present paper the hydration water "bound" to Na^+ and Cl^- ions act differently as a solvent for glucose and urea resp. (50 g water is $\sim$ 3 mol H_2O, 18 g NaCl – in a first approximation – supplies $\sim 2/3$ mol Na^+ and Cl^- ions; if each ion "binds" 6 molecules of water in the first approximation in its first hydration shell the quantity of urea appropriate for saturation was solved in this "bound" water).

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Study of Force Development in Resting and Activated Muscles during Velocity Dependent Stretching

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The time course in force development of gastrocnemius muscles isolated from frog *(Rana esculenta)* were investigated under the effect of stretching with different velocity and time characteristics. Time course of stretching were linear, parabolic and exponential. It was found that the changes in force development of both resting and activated muscles depend on the time characteristics of the stretching applied. Corresponding to data in literature, the length-tension curve of resting muscle in dynamic stretching differs from the length-tension curve measured in static circumstances, but the shape of the length-tension curve is not essentially affected by the change of the average rate of stretching with a duration of 0.1—0.4 s and constant size (0.2—0.25 l_0, l_0 is the reference length). The results can be interpreted with the aid of a muscle model as well. In the case of a contracted muscle, if the stretching and the stimulation of the muscle begin at the same time, the active force development reaches its maximum approximately at about half-length of maximum stretch.

Introduction

The study of force development in resting and activated muscle as a function of muscle length — especially in the case of quick stretching — is an important problem both from theoretical and practical point of view (physiology of work and sport). The results can contribute to the better understanding of contraction, the basic problem of muscular activity; and from a practical point of view they can help in achieving better sporting results (Ernst, 1963; Hettinger, 1966).

In the case of quick stretching the length-tension curve according to the experiences (Buchthal, 1942) — is not identical with the one of slow stretching. In the opinion of Buchthal the elastic tensions during stretching are delayed by internal friction because of the viscous character of the muscle, so a time factor is involved as a variable in the length-elastic tension curves of muscle (Buchthal et al., 1956). The length-tension curve taken with static and dynamic methods can differ from each other also because of the change in the degree of order of the protein under stretching (reorientation of myosin) (Ernst, 1963). The aim of the work was to perform experiments on isolated muscles modelling muscle activities in the body at quick movements. Muscle activity induced by stretching with different velocity and time characteristics greatly influence the power of muscle.

Methods

The experiments were carried out on fresh frog *(Rana esculenta)* gastrocnemius muscles. The muscles were kept in Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$ and 2.4 mM $NaHCO_3$ in 1000 ml bidistilled water) at 4 °C for about 30 min, before use. The measurements were performed at room temperature on resting length (l_0) of the muscles. The reference length of the muscles varied between 34 to 41 mm.

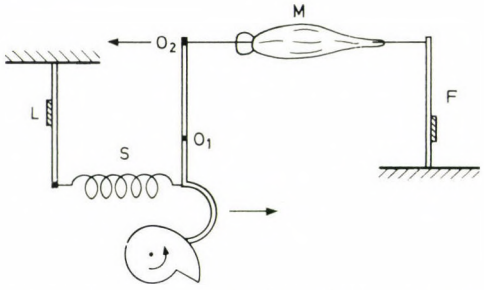

Fig. 1. Sketch of the measuring system. Symbols: M = muscle, F = force transducer, L = displacement transducer, S = linear spring, O_1 = axis of rotation of the lever with the arm ratio of 1 : 1, O_2 = axis of rotation of the hook fixing the muscle on the lever. The arrows show the direction of the movement of the disc and the lever respectively

A rough sketch of the experimental equipment is shown in Fig. 1. The isolated muscle was mounted to one side of a lever with a ratio of arms of 1 : 1, the other end of the muscle was hooked to a steel plate with a high modulus of elasticity. The strain gauge mounted on the steel plate gives the signal which is proportional to the force exerted by the muscle. The other end of the lever is connected with a spring the other of which is connected to a mechano-electric transducer working with a strain gauge. The signal of the transducer is proportional to the lengthening of muscle. The lengthening of the muscle is performed by the movement of the end of the lever on a special disc. The disc is placed on the axis of a high-moment motor. The discs are exchangeable. By shaping the profile of the discs a special time-dependent stretching can be generated (Bretz et al., 1978).

Three kinds of time-dependent stretching were applied in the experiments:

1. linear: $$l = l_0\left(l_1 + k\frac{t}{T}\right),$$

2. parabolic $$l = l_0\left(l_1 + k\frac{t^2}{T^2}\right),$$

3. exponential: $$l = l_0\left(l_1 + k\frac{t^2}{T^2}\exp\left|-2\left(1-\frac{t}{T}\right)\right|\right)$$

where l is the momentary length of muscle, l_0 is the reference length, t is the time, passed from the beginning of stretching, T is the duration of sliding (the duration of stretching), k is constant. Its value determines the maximum value of stretching, in our case $k \sim 0.25$. By changing the revolution number of the motor (operating voltage) the T value can be changed as well.

The output signals of the transducers were amplified on separate channels and displayed on an oscilloscop and recorded with a camera. An eccentric, on the shaft of the motor closes a switch at an appropriate moment of time, which ensures synchronization for photographing the signals. The muscles were directly stimulated with supermaximum square-pulses. The parameters of the applied impulses amplitude: 5 V, duration: 1 msec, frequency: 50 c/s, duration of stimulation: 1 sec.

Results

Figs 2, 3 and 4 show typical experiments on resting muscles. The upper curves show the stretching of the muscle, the lower ones show the force development. Fig. 2 shows muscle-responses for two stretchings of different durations besides the oscillogramme serving as the basis of the evaluation. For the sake of better comparability the force developments and the changes in length are normalized independently from the duration of stretching. The maximum value of force development varied between 6 to 15 N, depending on the cross-section and mass of the muscle, the change of length in the case of all the three time-dependent stretches was $\sim$ 8.5 mm, which means a relative stretching of 0.2 to 0.25 l_0; the whole duration of stretching varied between 0.1 to 0.4 s. A comparison of the figures shows, that the time course of force development depends on that of the applied stretching. The resting length tension curves calculated from the curves of the experimental results did not essentially differ from each other, but they differed from curve suggested by Hill's equation (1947) (Fig. 5):

$$F = F_0 \exp\left(\frac{l}{cl_0}\right),$$

where $c = 0.09$, while the value of F_0 was chosen as $7.47 \cdot 10^{-6}$ N, utilizing the fact, that the gastrocnemius muscle can be stretched from the excised length to the resting length by applying a force of about 0.5 N.

The change in the average velocity of muscle stretching does not essentially influence the time-dependent change on the force development of the muscle. The time, necessary for reaching the half of maximum force development is proportional to the T duration of stretching (Fig. 6). The values of proportion factors somewhat differ from each other in the case of discs generating different time-dependent stretching, although the differences are not significant.

The results of experiments performed on contracted muscles are shown in Fig. 7 in the case of stretching with linear time-dependence. The insert is a photo-

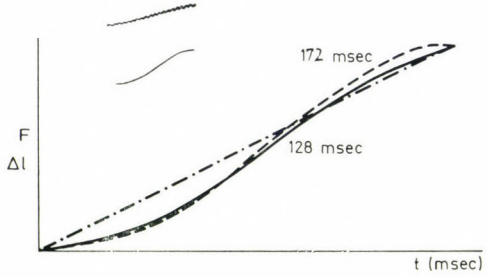

Fig. 2. Changes in the force development of resting muscle as a function of time in the case of a linearly changing stretch. Force development and change of length are normalized to the same value. Symbols: —— = force development with a stretching of 128 msec, --- = force development with a stretching of 172 msec, -·-·-· = change of stretching time. The insert shows the original oscillogramme in the case of a stretching of 128 msec. The upper curve shows the time dependence of stretching, the lower curve shows the force development of the muscle

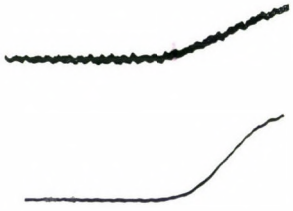

Fig. 3. Time course of force development during stretching of parabolic time dependence in the case of a resting muscle. The upper curve shows the time course change of stretching, the lower one shows the force development of muscle

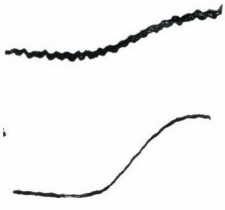

Fig. 4. Change in force development during stretching of exponential time-dependence in the case of a resting muscle. The upper curve shows the change of stretching in time, the lower one — the force development of muscle

graph taken of the original oscillograms. The results of the experiments performed on resting and contracted muscles are also indicated in the figure. Fig. 8 shows the length-tension curve of contracted muscle calculated from the curves. The curves deviate from the length-tension curves determined with classic measurements (Ernst, 1963; Buchthal, 1951). The cause of the deviation is, that the stimu-

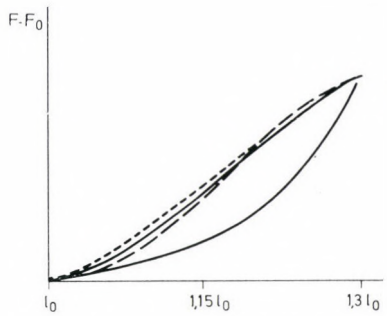

Fig. 5. Length-tension curve of resting muscle on the basis of experimental data. Symbols: —— linear stretching, ---- parabolic stretching, ····· exponential stretching. The lower continuous curve is the length-tension curve calculated on the basis of Hill's (1947) work. F_0 means the force, which stretches the muscle from excised length to reference length

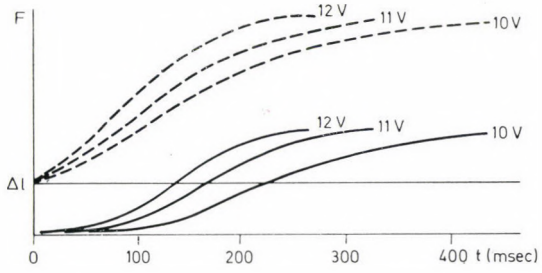

Fig. 6. Time course of force development during exponential stretchings of different average velocities but equal size on resting gastrocnemius muscle. Symbols: —— force development, ---- stretch. The values on the curves mean the values voltage used at the operation of the high-moment motor

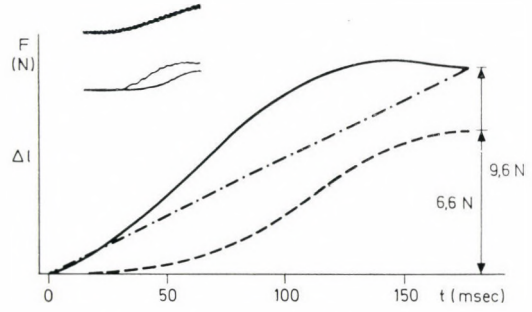

Fig. 7. Time course curve of force development of contracted gastrocnemius muscle during linearly changing stretch. Symbols: —— force development of activated muscle, ---- force development of resting muscle, ····· stretch. The value of stretching (0.25 l_0) in the figure is represented arbitrarily as being of the same size as the tension at the end of stretching. The insert shows the original oscillogramme, the upper curve meaning stretching, the lower one the force development of activated and resting muscle

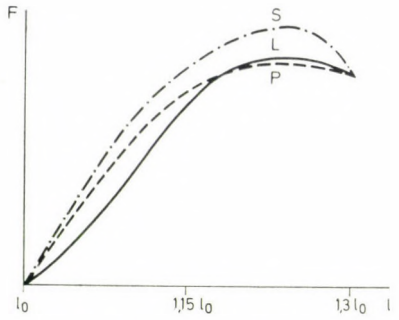

Fig. 8. Length-tension curve of contracted gastrocnemius muscle. Symbols: —— linearly changing stretch, ---- parabolically changing stretch, ·-·-·-· exponential stretching. For the sake of comparison the values of force development were normalized at the end of stretching. In the case of curves used for the length-tension function of activated muscle, the stretching-time varied between 0.1 to 0.22 s

lation and the stretching of the muscle begins at the same time; muscle needs a time of about 120 msec at room temperature to reach the maximum tension during contraction (Belágyi, Örkényi, 1967).

Discussion

Stretching experiments performed on resting muscles can be interpreted on the basis of Hill's three-element model (Hill, 1952;, Pringle, 1960; Bendall, 1969). Stretches of $0.2-0.25\ l_0$, but of different time-dependence and average velocity (0.02 – 0.08 m/s) – independently of their average velocity and time-dependence – result in an almost equal length-tension curve, which however differs from the length-tension curve measured under static experimental circumstances. During a stretching lasting for 0.1 – 0.4 s the relaxation time of the stress relaxation is great comparing to the duration of stretching owing to the characteristics of muscle-structure so the momentary tension is always greater than the force development calculated from the static length-tension relationship at a given length (Bozler, 1957).

It is characteristic of the behaviour of the muscle as a complicated mechanical system, that its lengthening under the effect of force depends on the extent of the load, the time course of the force, as well as the velocity of the change. At the same time the curves of tension and release differ from each other (Garamvölgyi, Belágyi, 1968). If the muscle is modelled with a mechanical system built of a spring, elements for damping and friction, and the response of this system is studied under the effect of stretching changing in time in a linear and parabolic way, the following is experienced:

1. The response to linear stretching force development consists of a linear and an exponential component, but because of the characteristics of the system

(a great modulus of elasticity) the contribution of the exponential component is slight, so the linear component is prevalent in the response.

2. In the case of a parabolic stretching the output takes temporarily a parabolic character because of the characteristics of the system.

The model outlined above well characterizes the dynamic changes in agreement with the experimental results, but it does not give a correct picture of the static condition.

In the case of a contracted muscle the experiments are determined by the fact, that the stimulation and stretching of the muscle begin at the same time. The time necessary for reaching the maximum tension at a given length during tetanic stimulation at room temperature is about 120 msec, so the tension of the muscle is smaller in the first period of stretching ($t < 120$ msec) than the maximum force-development at the given length. But in the second period of stretching ($t > 120$ ms) the active force development of the muscle decreases because of the increased length muscle. As a result of the two processes the muscle reaches the maximum of its active force not near l_0 (Gordon et al., 1966), but at a length greater than l_0. As the experiences have shown the active force development reaches its maximum at a range of $1.10-1.15\ l_0$ length. The maximum of force development shifts towards the shorter muscle-lengths in the case of stretching of longer duration. Stretching of different time-dependence does not essentially influence the time course of force-development. A similar statement is valid also for the dependence of force development on average velocity in the given period. One explanation of the experimental results can be, that the arrangement of muscle-proteins (reorientation of myosin) does not yet become complete and determinative in force development under short-time stretching (0.1–0.4 sec).

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Studies on the Biological Effect of Fast Neutrons. II. Variation of Total Nucleic Acid Content and Ultrastructure in Barley Leaves vs. Dose

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The effect of fast neutrons on the ultrastructure and nucleic acid content of leaves of barley seedlings was studied. Irradiation led to chloroplast degeneration. The walls of the cells containing degenerated plastides were abnormally thickened. Irradiation reduced the water content of the leaves. Variations in the RNA and DNA content of the leaves, expressed on a dry weight basis, could not be written as a simple function of the dose. Their content have a minimum at the semi-lethal dose.

Introduction

In a previous work the effect of fast neutrons on the growth and chlorophyll content of barley seedlings was reported (Kovács et al., 1977). On the basis of the phenotype of seedlings from irradiated seeds, appearance of a genetic anomaly was assumed already in the first generation upon fast neutron irradiation. Comparing the biological effects of various radiation types and chemical mutagens, Scarascia-Mugnozza (1967) suggested that fast neutrons were responsible for the majority morphological mutants. Mikaelsen (1968) showed that reduced growth of seedlings from seeds irradiated by fast neutrons, reduces respiration, and inhibition of protein synthesis was the result of the effect of irradiation on DNA and RNA synthesis. Therefore it seemed interesting to study the changes, induced by fast neutrons, in the morphology of chloroplasts and in the nucleic acid content of seedlings from irradiated seeds.

Materials and methods

Barley seeds (Táplán, spring type) were irradiated in the active zone of the Atomic Reactor of the Technical University, Budapest. Irradiation and germination conditions had been described earlier (Kovács et al., 1977). On primary leaves of seven-day-old seedlings originating from irradiated seeds the following experiments were carried out:

1. electron microscopy
2. determination of fresh and dry weights
3. determination of the DNA and RNA content.

1. Electron microscopy

Leaf pieces of seedlings from irradiated and control seeds were fixed in 1 per cent $KMnO_4$ solution (0.1 M veronal acetate buffer, pH 7.4) at room temperature for 2 hours. Dehydration was done in ethanol series, embedding in Durcupan ACM (Fluka). Sections made with a Porter-Blum ultramicrotome were contrasted with lead citrate and then examined in a KEM-1 type electron microscope.

2. Simultaneous determination of DNA and RNA content

Simultaneous determination of DNA and RNA was made by the method of Andersen (1971) based on a procedure by Smillie and Krotkov (1960). The extraction media were, in the order of application: 1. methanol; 2. methanol containing 0.2 per cent formic acid; 3.5 per cent $HClO_4$; 4. 96 per cent methanol; 5. 1 : 1 mixture of absolute ethanol and ether; 6. ether; 7. 70 per cent $HClO_4$ and 8. 0.3 M KOH. The leaves of seedlings were homogenized in 10 ml methanol, with a Teflon tissue grinder at 0 °C. After 10 min centrifugation ($3000 \times g$) the supernatant liquid was discarded and 10 ml methanol containing 0.2 per cent formic acid was added to the precipitate. The suspension was again centrifuged at $3000 \times g$ for 10 min. Then 10 ml of 5 per cent $HClO_4$ was added to the sediment and centrifugation was repeated. The precipitate was treated with ethanol, a 1 : 1 mixture of ethanol and ether, and ether with centrifugations in between. After the addition of 0.3 N KOH to the dried precipitate, the mixture was incubated at 37 °C for 15 to 20 hr. Thereafter the suspension was cooled to 0 °C and centrifuged. During cooling and stirring, the supernatant was supplemented with 4 ml of 5 per cent $HClO_4$ and then with another 4 ml $HClO_4$. After centrifugation, the supernatant liquid contained the hydrolyzed RNA and the precipitate the DNA. The DNA was hydrolyzed with 5 per cent $HClO_4$ at 90 °C for 15 min. After cooling and centrifugation, the supernatant liquid contained the hydrolyzed DNA. RNA and DNA were estimated spectrophotometrically at 260 nm by assuming that

$D_{260}^{1\,cm} = 1.000$, corresponds to 32.5 μg RNA/ml and
$D_{260}^{1\,cm} = 1.000$, corresponds to 35.0 μg DNA/ml

The results given in the Tables and Figures are the averages of three parallel measuring series.

Results and discussion

Leaves of seedlings grown in the light from seeds irradiated by a semi-lethal dose (1130 rad) exhibited macroscopic, pale, densely located irregular bands. Electron microscopy revealed that this pattern was due to an intermingling of both normal chloroplasts (Fig. 1b), similar to the control (Fig. 1a), and abnormal chloroplasts (Figs 2a, b and 3a). These abnormal plastids may exhibit different

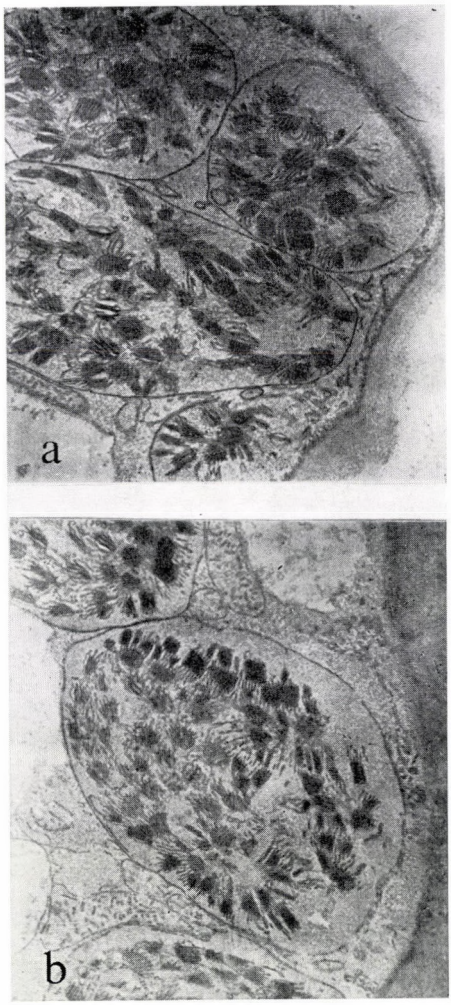

Fig. 1. Electron micrographs of chloroplasts in 8-day-old control and irradiated barley seedlings grown in the light. 1a. Chloroplasts of control seedlings. Magnification 13 200×
1b. Normal chloroplasts of seedlings from seeds irradiated with a semi-lethal dose. Magnification 13 200×

deficiencies but they are smaller and contain fewer inner membranes than the control. Also the walls of the cells containing irregular plastids are seen to be much thickened (Figs 2a, b and 3a, b).

The effect of fast neutrons on the fresh and dry weight of barley seedlings is shown in Fig. 4.

The fresh of seedlings decreased lineraly with increasing dose. Beyond the semi-lethal dose (31 sec) the curve was no longer linear. For the highest dose applied (57 sec) the weight of the irradiated leaves was only 18.3 per cent of the control.

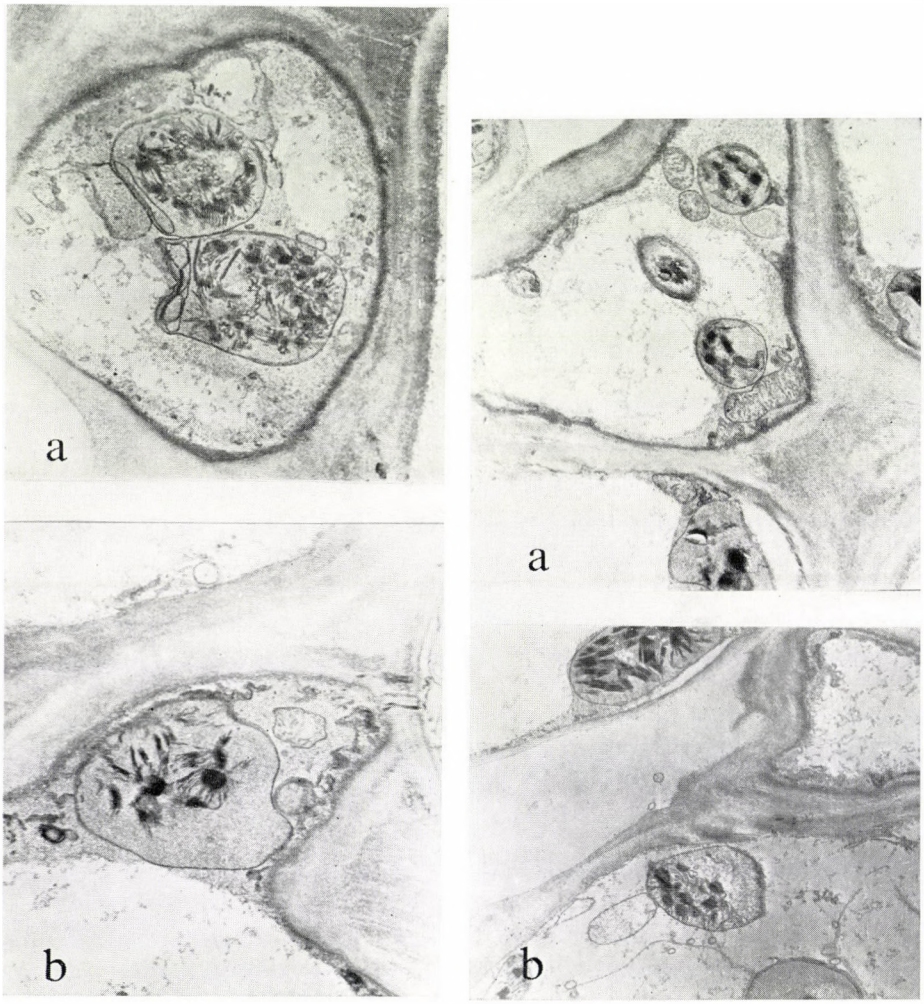

Fig. 2a, b and Fig. 3a, b. Electron micrographs of plastids form irradiated plants, degenerated to different degrees. Magnification 13 200× (Fig. 2) and 8000× (Fig. 3)

The dry weight curve is less steep. This difference in slope can be ascribed to a loss of moisture in the seedlings due to irradiation (Table 1).

Changes in RNA and DNA content of the leaves vs. dose are shown in Fig. 5.

RNA and DNA curves have a similar slope, except for a short section after LD 50 where the RNA curve is steeper. Decrease of nucleic acid content reflects inhibited leaf growth.

RNA and DNA contents calculated on a dry weight basis are shown as a function of dose in Table 2.

The RNA and DNA contents expressed on a dry weight basis show a little variation. While at the semi-lethal dose RNA content expressed on a dry weight basis shows no significant deviation, the DNA content has a minimum.

The results presented suggest the existence of a marked heterogeneity in the chloroplast population of seedlings derived from irradiated seeds with respect to both size and lamella structure. The experimental lay-out does not permit to decide whether the alterations observed are due to primary or secondary destruction. It is interesting to note that the walls of the cells containing degenerated plastids are quite thickened – a hint to serious troubles in the control of carbohydrats metabolism.

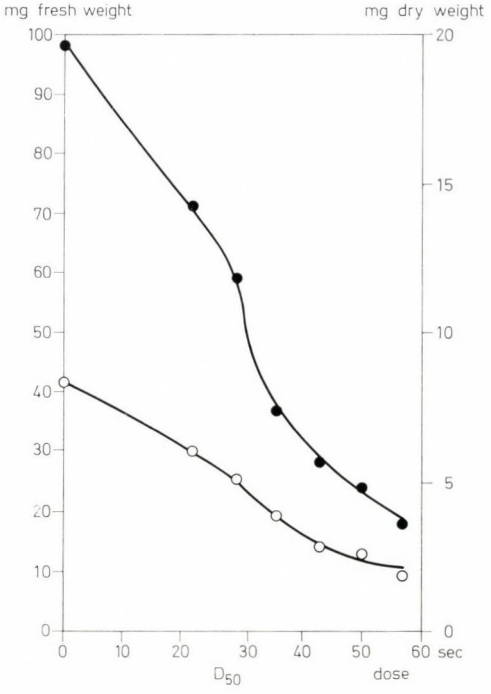

Fig. 4. Weight of leaves of 8-day-old barley seedlings grown in the light vs. dose (mg/leaf) —●—●— fresh weight; —○—○— dry weight

Table 1

Water content of leaves of barley seedlings vs. dose

Irradiation time (sec)	Relative water content of leaves (%)
Control	91.65
22	91.43
29	91.01
36	89.60
43	89.70
50	89.00
57	87.80

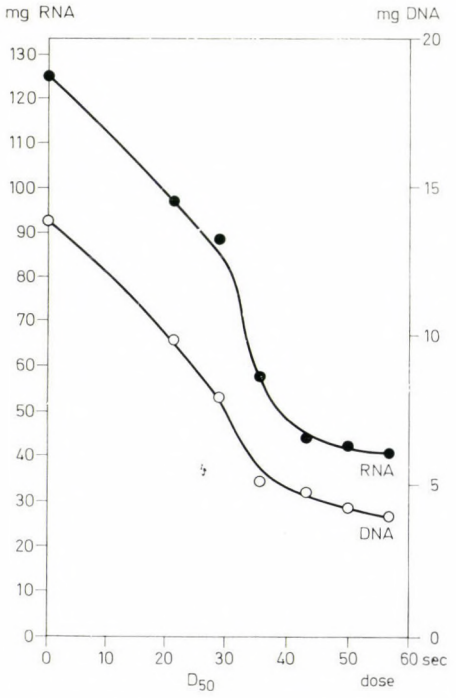

Fig. 5. DNA and RNA content of a leaf vs. dose (μg/leaf). —●—●— RNA; —○—○— DNA

Table 2

DNA and RNA content per unit weight of leaves vs. dose (μg/mg leaf tissue)

Irradiation time (sec)	RNA	DNA
	μg/mg dry w.	μg/mg dry w.
Control	15.2±1.52	1.80±0.18
22	16.1±1.63	1.60±0.19
29	17.0±1.53	1.55±0.16
36	14.5±2.32	1.44±0.19
43	15.1±1.80	1.71±0.20
50	16.8±4.00	1.79±0.29
57	18.6±5.56	1.90±0.38

Irradiation also couse a change in water content. This may reflect an overall metabolic disturbance. Since decrease in water content is linear with the dose of irradiation, the amounts of nucleic acids can be evaluated only with reference to dry weight. It was interesting to see that DNA content decreased parallel with increasing dose until the semi-lethal level was reached and then it increased. It is an empirical relationship, generally characteristic of irradiated organisms, that at semi-lethal doses the reaction of the organism differs from that observed at extreme (sublethal and lethal) doses. The standard deviations show parallel increases with dose, indicating the necrotic effect of lethal doses. The slight effect of the fast neutrons on nucleic acid content in leaves indicates the rather mutational than metabolic effect of radiations.

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Book Reviews

Pathobiochemie. Perspektiven und Entwicklungstendenzen für die klinische Chemie und Laboratoriumsdiagnostik, by Haschen R. and Wagenknecht, C. Ergebnisse der experimentellen Medizin, Band 30., VEB Verlag Volk und Gesundheit, Berlin, 1978, 245 pages (in German)

The rapid development of clinical chemistry and clinical biochemistry in the last decade considerably broadened the possibilities and the tools of clinical chemistry laboratories in the diagnosis and prognosis of various diseases as well as in controlling the effectiveness of therapeutic interventions. This development made it necessary to bring up to date and in some cases to re-examine our earlier view on several important fields of laboratory diagnostics.

The aim of this volume is to take the arduous task of reviewing some of the most rapidly developing areas of clinical chemistry and to establish new trends with promising perspectives. As a general approach throughout the book the authors introduce the subject by reviewing the pathobiochemical background of the diseases or group of disease concerned. Then they provide a general survey of recent literature and draw conclusions supported by their own experience.

The first part of the book mainly concentrates on the role of clinical enzymology in the diagnosis of organ specific diseases. Although some of the methods used by the authors are not quite properly up-to-date, this part of the book includes excellent reviews on the significance of isoenzymes in the diagnosis of liver diseases, on the clinical chemistry of liver diseases, and on the perspectives of enzyme determinations in the urine. The chapter devoted to the importance of clinical chemistry in the diagnosis of chronic-ischemic heart diseases and their complications, however, does not cover the subject promised in the title and represents a conservative pathomorphological view rather than up-to-date clinico-chemical approach.

The second part of the book has only loose connections with the first one and deals with the pathological biochemistry of the perinatale period. In spite of the fact the second part also includes well shaped papers like those on carbohydrate, protein and lipid metabolism in the perinatale period, the idea of Squeezing the two deviating topics in one volume is questionable. The pathophysiology and clinical biochemistry of the perinatale period is an autonomous discipline and important enough to devote a separate volume to this subject.

Apart from the inadequacies mentioned above the chapters comprising valuable reviews provide a good orientation in the fields of clinical chemistry in progress and could be recommended for clinical chemists and scientists interested in various aspects of medical biochemistry. It is also useful for clinical practitioners who just would like to follow the main stream of clinical chemistry and get an idea about the kinds of new everyday diagnostic procedures expected to emerge in the near future.

L. MUSZBEK

Enzymatic Mechanisms in Pharmacodynamics, by Victor Voicu, MD. PhD. and Radu Olinescu PhD. Abacus Press, Tunbridge Wells, Óent, England 1977.

The study of enzymatic mechanisms is a new possibility in the research of the pharmacodynamic properties of drugs. This new branch of pharmacology, called biopharmacology, contributes to a better understanding of the mechanism of action of drugs.

This book summarizes this topic in a concise, easy to survey manner. It is divided into nine chapters over 305 pages with detailed literature references.

Chapter one deals with the fate of drugs penetrated into the organism. It gives details about the factors involved in drug absorption, discusses the conditions of distribution, the possibilities of drug transfer across membranes, and finally elimination.

Chapter two is about the metabolism of drugs moving within the organism, summarizes the role of microsomal and non-microsomal enzymatic systems and the possibilities of conjugation during metabolic transformations. Furthermore, it touches upon genetic, physiological and environmental factors affecting metabolism.

In chapter three and four the authors give a survey of cholinergic and adrenergic neurotransmission, respectively. The availability of a transmitter depends on several complex processes, interrelated in a definite succession: synthesis, transport, storage of the neurotransmitter substances (acetylcholine and catecholamines), their release, depolarization-release coupling, interaction with the specific pre- and postsynaptic receptors, and the ending of neuronal activity by enzymatic hydrolysis and/or different uptake processes. These steps of both systems are discussed, as well as the pharmacodynamically active substances interfering specifically with the links listed above.

The next chapter includes a discussion of different cyclic-AMP interrelations with pharmacological responses. The authors outline some of the multiple functional implications of the cAMP system: synaptic transmission; striated, myocardial and smooth muscle contraction; lipolytic pathways; the pharmacodynamic action of cAMP and its derivatives; the cAMP and the stimulus-pharmacodynamic effect coupling.

Chapter six summarizes the biochemical mechanisms involved in the action of neuropsychotropic compounds. Talking into consideration the biochemical particularities observed in the central nervous system in normal and pathological states and the great variety of the drugs acting at this level, the authors restrict their study to an analysis of the changes occurring in the metabolism of catecholamines and other amines in the CNS.

Two short chapters are devoted to the biochemical mechanisms in radioprotection and sensitization, and to some aspects of pharmacogenetics. Pharmacogenetics, which includes a study of the significant clinical consequences of hereditary differences following the administration of drugs has gained increasing importance because of dosage problems and unforeseen side effects.

The last chapter summarizes the structure, biosynthesis, enzymatic degradation, release and receptors of prostaglandins, as well as their pharmacological effects. The implication of prostaglandins in the therapeutic or adverse effects of some drugs and using some of them in different treatments emphasize the pharmacological interest in these compounds.

To sum up, this book gives a good, up-to-date survey of this new topic and promotes the orientation in this rapidly increasing field of pharmacology. A detailed subject index would have made it even easier to use this valuable book.

ANNA GESZTESI

Mitochondria 1977: Genetics and Biogenesis of Mitochondria. Proceedings of a Colloquium held at Schliersee, August 1977 by W. Bandlow, R. J. Schweyen, *K.* Wolf and F. Kaudewitz (eds.) Walter de Gruyter, Berlin—New York, 669 pp.

Genetics and biogenesis of mitochondria became a special and rewarding field of molecular biology. One of the reasons why it became so is clearly stated in the introduction of the first paper in this volume: "Current rapid developments in the field of mito-

chondrial genetics owe much to a trivial property of the yeast *Sacharomyces cerevisiae*. This is the fact, a matter of common experience in many laboratories, that it is relatively easy to isolate mutants coded in mitochondrial DNA. If this were not the case, progress in this area would have been greatly retarded . . ." Being, however, this the case, an impressive material has been presented in the volume.

The 45 papers were organized into 4 sections and an appendix. The sections are: 1. Mitochondrial DNA, mutations and genetic mapping, 2. Organization of the mitochondrial genome, transcription and translation products, 3. Mitochondrial components and assembly, and finally 4. Mitochondrial-nucleocytoplasmatic interactions. The appendix consists of a mitochondrial genetic map of *Sacharomyces cerevisiae:* a literature compilation towards a unique map.

Two of the papers are of a review character: the first on "Mitochondrial DNA and the heritable unit of the yeast mitochondrial genome" by D. H. Williamson et al. and the second on "The physical map of yeast mitochondrial DNA anno 1977" by P. Borst and collagues. The other papers are the result of the most recent research.

There are two outstanding merits to the volume. The first is that the reader gets really first hand presentation of recent experimental data, the statue of research in 1977. The second is that it also contains original and non-orthodox thoughts, speculations and ideas as well as models and I personally think that these latter are almost as useful as facts.

The published papers are of high value for the specialists of the field (to which the author of this review does not belong) but also for the non-specialists who want to find the up-to-date status of the problems. To read the book the biochemist nedds a good knowledge of classical and molecular genetics, but it is rewarding to discover how many problems of mitochondrial function were and can/could be cleared by genetic methods.

Fortunately, *Sacharomyces cerevisiae* has been found to be a suitable object for genetic work and the result is this highly interesting volume.

A. FONYÓ

Effects and Metabolism of Insulin and Cyclic Nucleotides in Ergebnisse der experimentellen Medizin (Vol. 28) by H. Frunder (ed.) VEB Verlag, Berlin, 1978

This volume of the Ergebnisse der experimentellen Medizin contains the main results of a joined symposium (held in 1975) of the All-Union Biochemical Society of the USSR and the Biochemical Society of the GDR. As such it became obviously a somewhat heterogeneous collection of papers. Nevertheless it is worth reading from many points of view. On the whole this book indicates that significant efforts and achievements have been made during the last ten years in both countries to clarify the mechanism of action of insulin and cyclic nucleotides at the level of cellular metabolism. In some articles we find basically new approaches and results concerning both topics. Let us cite only a few of them.

Staroselzeva provides important data on the involvement of two enzymes in the transformation of proinsulin to insulin. Fiedler and coworkers report an interesting relationship between diet and insulin sensitivity of glucose metabolism. Ilyin et al. have demonstrated that insulin interacts with hexokinase and other enzymes and that in these interactions the SH groups of the respective enzymes are involved. Although insulin interacts primarily with cell surface protein receptors, SH groups are also involved. Therefore the study of hexokinase-insulin interaction may prove to be a good model to clarify some aspects of the interaction of insulin with its receptor.

Probably the best results are presented in the third part of the book which deals with cyclic nucleotides. The action of catecholamines on intracellular events is likely to involve both an enhancement of cyclic AMP formation and an increase in calcium translocation inside the cell. Pertseva and coworkers made an important contribution to clarify how cyclic AMP molecules and calcium ions work together as messengersof catecholamine action. Closely related to this are the findings of Krause that cyclic AMP increases the phosphorylation of a cardiac cell surface protein and that this process is accompanied by an increase in the binding of calcium to the cell membrane.

Since in most cases cyclic AMP seems to act, through proteinkinase, it is highly important to study the structure and function of this enzyme. Severin and his group made an outstanding work in characterizing a histone kinase (which is a sort of proteinkinase). This work includes the purification and investigation of the structure of both the regulatory and catalytic subunits of the histone kinase, as well as the elucidation of the mechanism of histone kinase dissociation.

Still in the third part there is a promising attempt to localize cytochemically the adenylate cyclase enzyme (Schulze and coworkers).

The book also contains some articles, that are related neither to insulin nor to cyclic nucleotides, but make a good service to keep our memory fresh about the complexicity of the intracellular machinery.

Finally it should be emphasized that this book does good service to those who wish to know more about the present stage of research on these important fields in the USSR and the GDR.

Z. KISS

Mitosis — Facts and Questions. By Little, M., Paweletz, N., Petzelt, C., Ponstingl, H., Schroeter, D. and Zimmermann, H. P. (eds.). Springer Verlag, Berlin—Heidelberg—New York, 1977. 253 pages, 55 figures.

To separate "hard" facts about cell proliferation control from "soft" ones — this was the purpose of the symposium organized in April, 1977 at the German Cancer Research Center in Heidelberg. Upon reading the lectures and the discussions presented in this book it becomes clear that for the moment the hard to soft ratio is rather low. Contrary to this, or perhaps just therefore, the volume includes important and interesting facts and questions to be answered experimentally.

The main topics discussed were as follows: timing of the cell cycle (Mitchison), surface signals in proliferation (Edelman et al.), mitotic ultrastructure (Fuge), microtubules (Borisy and Gould), nontubulin molecules in the spindle (Sanger), correlation between division, differentiation and cancer (Duspiva), chromosome movement (Nicklas), models of the mitotic apparatus (McIntosh) and the future of mitosis research (Mazia).

Introductory lectures on the main topics were followed by extremely active discussions. These discussions indicate that mitotic control constitutes a complex molecular-cytological problem. The conciliation of views and results in the field of biochemistry, cytology and oncology proved to be exciting and useful.

Rapidity of publication and quality of the editorial work praise the high level of coordination between editors and the publisher.

A. BALÁZS

Radiation Chemistry (ed. P. Hedvig, and R. Schiller), Akadémiai Kiadó, Budapest, 1977.

The field of application of radioisotopes and radiation has developed significantly in the recent years — just like other fields of science. This is proved by the volume containing the proceedings of the 4th symposion in Tihany.

This review contains (in English) all the lectures held during the "4th Symposion of Tihany" organized jointly by the Association of Hungarian Chemists and the International Agency of Atomic Energy (Keszthely, 1 to 6 June, 1976).

The 4th Symposion continued the traditions of the meetings in Tihany, with the difference that the extent of the subjects lectured and discussed was greatly amplified. This can be seen as compared with the former meetings first of all in the fact that besides problems of radiation-chemistry connected with biophysics and biochemistry other significant problems were also included. Radiation chemistry in the problems of utilization of radiations in technics and even in industry took an important place in the programme of the 4th Symposion. The editor and the organizers of the Symposion carefully selected the lectures dealing with the radiation-resistence of combinations of wood, plastics and microorganisms.

Experts interested in different fields of radiation-chemistry can get useful information from the lectures.

When selecting the lectures about the processes examined in organic compounds, polymers and aqueous solutions the authors chose 33 lectures on organic materials (p. 29—313), 41 about polymers (p. 313—673), 33 about aqueous solutions (673—939) and 15 on miscellaneous subjects.

From practical view point the list of the 173 authors from 22 countries can be also considered as valuable, for it is necessary in forming direct connections.

The conference is a significant contribution to the peaceful uses of atomic energy; the utilization of isotopes in agriculture and industrial researches came powerfully and emphatically into prominence. The significant result is their technological application which has proved unambiguously in the different fields that the results of traditional methods are surpassed by these of the new technology. The lectures and subjects show that the characteristics and peculiarities of unknown polimers of coal-coal connections produced by man during history can be formed further by ionizing radiations. The problems of sterilization of medical instruments and materials by radiation mean a kind of practical application and offer a qualitative and quantitative possibility for introducing once-usable plastic instruments in large number. It is a very significant thing that with the expectable increase of utilization of nucleic energy a larger quantity of radiating material gained as by-product will be at our disposal. The application of radioisotopes as sources of radiation is beyond the experimental stage also in agriculture. The problems of storing and accumulation of products and deworming will be solved safely with this technology. The aversion and the completely unfounded views beginning to seem unscientific, but evidently strongly effecting public opinion will change due to the widening of the field of application of irradiation technology as a result of the conference and the facts proved by basic researches.

The Publishing House of Acad. Sci. Hung. published the well constructed volume in a clear and attractive form. I am convinced that this volume is not only a significant scientific material in the library, but it is also a great help in daily work, because it is essential to have the lectures at our disposal for more detailed analyses after hearing them and taking part in discussions about them. Starting from the programme of the symposion we can establish, that "Radiation Chemistry" can claim a wide interest even with its present results. As a review and summarization, the volume is definitely valuable.

F. ANTONI

Biophysikalische Aspekte der Struktur, Dynamik und Biosynthese der Eiweissmoleküle (Vol. 2. p. 151) and *Biophysikalische Aspekte der multimolekularen Eiweiss-strukturen. Koazervate, Membrane, Fasern* (Vol. 3. p. 124) J. Segal, and A. Kalaidjiew. VEB Georg Thieme Verlag, Leipzig, 1977.

As it can be seen from the title the two authors published again their particular and special conception about the structure of proteins and protein complexes, the dynamic characteristics of the structure of proteins and their synthesis. The first part of the series dealt with immune-proteins. The first chapter of the second volume deals with the criteria of the dynamic structural model of protein molecules. After that the authors come to the treatment of their hypothesis: Faltentrommel model. They try to interpret the characteristic biological features arising from the structure of proteins with this model. The model itself is not new; the authors already reported their hypothesis more than ten years ago (in 1966). It is important to note that no other authors succeeded in demonstrating anything similar to this even with the most up-to-date methods. In the following chapter the authors use this model for the interpretation of some problems of molecular biophysics in connection with proteins. From the chemical point of view — as it can be seen in chapter 3 — they refer to diketopiperazine structure, because this is the view which corresponds to the Faltentrommel model. It is necessary to characterize the model more deeply because the authors try to interpret some protein related basic phenomena of biology on the

basis of this model in the following chapters. In this way they consider it to be resolvable to interpret the structural bases of immune reaction in chapter 5, taking little care of the results of research in this area in our days. The following four chapters deal with the biosynthesis of proteins, the phenomenon of keto-enol tautometry — and in connection with this with the structural interpretation of enzyme activity on five pages — with the optical examination of proteins (ORD, CD), and at last with allosteric structural changes.

In the last ten or fifteen years several papers have been published about the above mentioned problems; owing to the results quite a few problems have been satisfactorily clarified, which is considered by the scientific public opinion to be acceptable at the present level of our knowledge, and even they think it successfully applicable in practice. The authors raise original, but not grounded ideas, the practical consequences of which are uncertain either in scientific practice, or in the everyday employment of the results. Assertion of false theories is permissible in science if they are provocative and induce the experts to find *the right standpoint* due to their disapproval.

The third volume of the series deals with the structure and functions of molecular complexes of proteins. This group of problems stands in the centre of examinations in several areas of biological investigation in our days. It can rightly be expected, that getting acquainted with the complexes brings us nearer to the interpretation of several complicated phenomena on a molecular basis.

The first chapter gives a short summary of the interactions between solved proteins and the solvent. The following seven chapters are about coacervates, the authors include here also cytoplasm and carioplasm.

The following five chapters give a detailed treatment — as compared with the other chapters — of the structure of biological membranes, membrane models, the change of structure characteristic of the membrane and its significance as well as hypotheses about membrane transport. These chapters of the two volumes are the nearest to the "standard" method of treatment according to which the handbooks treating proteins and protein complexes in general approach this sphere of problems. The last two chapters deal with "thread-forming spheroproteins". It is typical of the peculiar view of the treatment, that the properties of actine are not mentioned, but tubuline and the others, as well as fibrine are considered as the most characteristic representatives of this group. In the last chapter, where the inclination of proteins to self-arrangement (macromolecules) is dealt with, Faltentrommel hypothesis as the only possible explanation of arrangement is back again.

It is evident that the authors were inspired to write the two volumes by the necessity to extent their theory, which had already been published in a volume in 1966, and to prove that it is suitable for explaining the basis of several phenomena in biology. References to the works of X-ray men are only occasionally found in the list of references of the two volumes. In the work reported here the method of the authors is to build the data — selected on the basis of a peculiar sorting (including several that are the results of "biophysical" examination) — into their own theory. The price of the plain-looking two volumes is 25.50 and 21.00 M resp.

P. ELŐDI

Walter Heiligenberg: *Principles of electrolocation and jamming avoidance in electric fish.* Studies of Brain Function, Vol. 1., Coord. Ed.: V. Braitenberg; Eds: H. B. Barlow, E. Florey, O.-J. Grüsser, H. van der Loos; Springer Verlag, Berlin, Heidelberg, New York, 1977. 85 pp

The author of this small monograph has to be complimented for finding and utilizing such an excellent object — the electric fish — for a combined behavioral and neurophysiological approach to the perception of complex stimulus patterns. Several species of the African order (Mormyriformes) and the South American suborder (Gymnotoidei) possess electric organs to generate, and receptors capable of monitoring the intensity and distortion of electric fields around the animal. Species of both groups fall into two subgroups according to the function of the electric organ: "pulse" and "wave" species.

Accordingly, the electroreceptors are different in the two kinds of electric fish.

The histology of the electric organ and electroreceptors, as well as the connectivity of nervous pathways transmitting the signals of the receptors to rhombencephalic and mesencephalic nuclei are relatively well known.

The distortion of the electric field generated by the electric organ discharges is perceived by the animals and signals an object in the near environment. This is called electrolocation. The performance of the animals in the electrolocation is determined by several factors, like the size of the fish, the proportion between tail (the location of the electric organ) and body (the highest concentration of receptors), resistance of the body wall and that of the surrounding water. Some animals improve their performance by active movements around the object to be explored. A comparison made by the author between electrolocation and vision, in various aspects, is most illuminating.

Electrolocation is potentially disturbed or completely blocked in the presence of electric noise originating under natural circumstances from other species of or from conspecific electric fish. "Pulse" and "Wave" species use different ways of protecting their electrolocation ability by raising or lowering the rate and by shifting the frequency of the electric organ discharge respectively. This behavior is called jamming avoidance response. The histological and connectivity substrate of the jamming avoidance response is discussed in detail offering appropriate feedback circuit diagram.

J. SZENTÁGOTHAI

Grundlagen der Datenverarbeitung by Dvoratschek, S. Walter de Gruyter, Berlin, New York, 1977. 538 pages.

The book is the sixth, completely revised and amplified edition of the author's work entitled "Introduction into data processing".

The quick development of natural and social sciences the careful analysis and evaluation of the results, the systematization of the accumulated material of knowledge, optimalization of economic decisions, modelling of processes, problems of simulation all call for a better utilization of data-processing with computers. The literature concerning data-processing is closely dependent on the time of publication as a consequence of the quick theoretical and technical development; several are very specialized and complicated owing to the wide spectrum. Anyone interested in data-processing with computers either just to get aquainted with a special profession or already familiar with a special profession, first of all wants to get a general view of the wide range of data-processing with computers; but the repeated renewal of general knowledge is useful also for experts with a good knowledge of data-processing to make a progress in their profession.

The book, composed with great experience and excellent sense of pedagogy is appropriate from every point of view as an introductory work, which aims at the clearly arranged treatment of the wide field of data-processing with computers. It enables a thorough reader to get a better view of special literature. A table can also be found at the end of the book, which is a useful help for readers interested in special literature. The collection of exercises for each chapter helps the reader to acquire thoroughly more the material treated in the book. The book is also an aid for those who want to get acquainted with the problems of data-processing more deeply, and also for those who get into connection with experts at home in data-processing because of the special problems of their profession.

The book is constituted in six chapters:

I. functional knowledge

II. mathematical basic knowledge

III. basic knowledge of information theory and logics

IV. technical basic knowledge

V. basic knowledge of operation-organization

VI. basic knowledge of application-organization

The first chapter — after a short historical introduction — deals with analogous and digital computers, then with the structure of digital computer, tasks of each unit, the notions connected with them. The second chapter comprises mathematical elements and numerical systems, their conversions. No high-level mathematical konwledge is expected of

the reader, the chapter can be easily understood with medium- level mathematical knowledge. The same goes for the third chapter, which contains basic knowledge connected with information-theory and coding, as well as elements of mathematical logics (switching algebra). The fourth chapter deals with the technical description and way of working of the most important units of computers. Its chief aim is to clarify the functional role of each unit and to outline the most important characteristics. The fifth chapter treats organization of data, programming, the ways of working of computers as well as system-software. Chapter six includes the expounding of two problems: 1) data-processing as a working-process 2) fields of application. This latter comprizes some examples, like data-processing in medical science, in teaching.

The book consists of 538 pages, including a detailed table of contents, list of references, subject index, English equivalents of technical expressions as well as 59 photographs connected with the treated material. 208 figures and 212 exercises make it easier for the reader to study the material. Typographically the book is a well edited one, though some misprints are disturbing.

J. BELÁGYI

Heinz David: *Ortho- and Pathomorphology of Human and Animal Cells in Drawings, Diagrams, and Constructions.* VEB Georg Thieme, Leipzig, 1978

H. David's book is rather unusual among scientific publications. The author attempted to show human and animal cells, on the electron microscopic level, without any electron microscopic pictures. He collected from the literature almost 1000 schematic drawings, which are the abstractions of many thousands of electron microscopic observations. At the first glance, one would think that a book, like that cannot have a great scientific value. Instead, this could be used only for teaching purposes. Undoubtedly, this large volume is an excellent handbook for students, but it can also be a good guide book for scientists, especially those who are at the beginning of their electron microscopic work. The drawings may help in the identification of tissue elements, and subcellular particles in the electron micrographs.

The constructions, and diagrams give a short, but good summary of the function of cells, and an abstraction of structure at the molecular level. Most of the drawings from other authors, slightly are modified. The designer successfully coped with the difficult task of giving uniform style to the many different drawings. In some cases, the original pictures are nicer than the redesigned ones, but the information they containe is not altered.

The book is divided into three main parts. In the first part the whole cell is shown in normal, and in pathological conditions. All constituents of the cell (nucleus and nucleoli, endoplasmic reticulum and ribosomes, mitochondria, Golgi apparatus, lysosomes, microbodies, microtubules, glycogen bodies, plasma membranes and their specializations) are represented. The second part is very short. Here the structure of extracellular substances — collagen, fibrils, basement membranes, and the amyloid — are shown. This part is not too useful. In the third part the normal and pathological states of tissues and organs are summarized. The main paragraphs are as follows: liver, exocrine pancreas, respiratory system, digestive system, urinary system, nervous system, sensory organs, skin and connected glands, haemopoetic system, endocrine glands, reproductive system, connective tissue, bone, cartilage, and teeth. The main paragraphs are finished with a list of references. The abundance of the cited literature makes the book a very good source of references.

The quality of the paper and printing is good. On the large pages (21 × 30 cm) several drawings could be placed. This is very advantageous when the sequence of changes in normal, and in pathological conditions has to be shown. The book is recommended to graduates and postgraduates who want to achieve comprehensive knowledge of biological structure. It is also useful for clinicians who are interested in submicroscopic changes of organs during diseases, and for scientists who want to keep at hand a great deal of results of electron microscopic investigations collected in one volume, and showed in a simplified form.

G. LÁZÁR

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Detection of Maple Syrup Urine Disease on Resin Coated Chromatosheets

(Short Communication)

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(Received March 13, 1979)

Thin layer ion exchange chromatography on resin coated chromatosheets for the separation of amino acids in biological materials (Dévényi, 1970; Dévényi et al., 1972) has found wide application in clinical practice as well as in the early detection of various aminoacidopathies (Kovács, 1973; Kovács, Kiss, 1978).

Recently a 3-year old girl was admitted to our ward on account of MSUD*. The first routine blood test was performed as usual (Kovács, 1973; Kovács, Kiss, 1978), using sodium citrate buffer, pH 5.2, on 10 × 10 cm resin coated chromatosheets (Fixion 50 × 8, Chinoin-Nagytétény, Budapest, Hungary; Ionex 25 SA-NA, Macherey, Nagel + Co., Düren, GFR).

In three of the blood samples dried on filter paper, strikingly intensified spots in the region of leucine and isoleucine were observed (Fig. 1a). Sample 1 belonged to a patient with recognized MSUD. Samples 2 and 3 in Fig. 1a originated from a family investigated because of suspicion of having some hereditary disease.

Since the separation of branched chain amino acids is incomplete in citrate buffer, pH 5.2, to achieve better results, the blood and urine samples from the patient with recognized MSUD were applied to 10 × 10 cm resin-coated chromato-

Table 1

The branched chain amino acid level of blood sera
Amino acid analysis were carried out according to the single-column method of Dévényi (1969)

	Patient with MSUD	Family suspected of being afflicted with MSUD			Normal values (Liappis)
		child	father	mother	
Leucine μmol/l	1200	168	203	152	106
Isoleucine μmol/l	452	93	93	53	47
Valine μmol/l	1240	360	396	308	211

* *Abbreviation:* MSUD, maple syrup urine disease

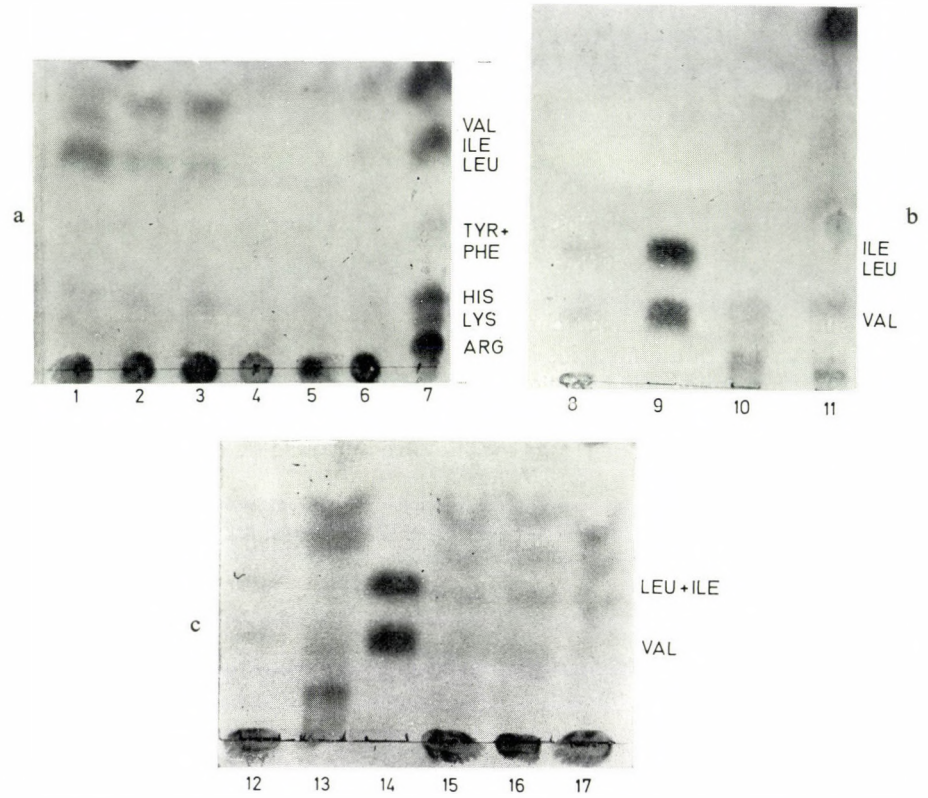

Fig. 1. Thin-layer ion-exchange chromatography of blood and urine samples on strongly acidic resin-coated chromatosheets. a: Blood-samples: 1 = patient with recognized MSUD, 2, 3 and 4 = family members investigated on genetic disorder, 5 and 6 = normal sera, 7 = control amino acid mixture. Eluting buffer: sodium citrate pH 5.2; b: Blood-samples: 8 = Blood-sample from patient with MSUD *after* dietary restriction of branched-chain amino acids, 9 = control mixture of valine, isoleucine and leucine, 10 = urine sample from the same patient, 11 = normal urine. Eluting buffer: sodium citrate pH 3.3; c: Blood-samples: 12 = as 8 in Fig. 1/b, 13 = as 10 in Fig. 1/b, 14 = control amino acid mixture of valine, isoleucine and leucine, 15, 16 and 17 = blood samples of the family members investigated on suspicion of MSUD. Eluting buffer: sodium citrate pH 3.3

sheets and eluted with sodium citrate buffer, pH 3.3, at 45 °C (Dévényi et al., 1971, Vargáné, 1976), without previous equilibration of the chromatosheets. A typical chromatogram is shown in Fig. 1b.

A quantitative amino acid analysis of the blood serum amino acid levels confirmed the diagnosis (Table 1).

The chromatographic test was repeated with the family members mentioned above. As shown in Fig. 1c the serum level of branched chain amino acids was found to be fairly high. The data of quantitative analyses are given in Table 1.

Our results show that to make a sure diagnosis of MSUD and its variants, the use of ion exchange thin layer chromatography on strongly acidic resin coated chromatosheets applying citrate buffer at pH 3.3 is a very reliable procedure.

Thanks are due to Mrs Judit Báti for the performance of quantitative amino acid analyses and to Mrs Ibolya Midrák for technical assistance.

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Determination of Enzyme Activity by Chromatography and Videodensitometry

I. Microassay of Amino Acid Transforming Enzymes in Human Tissue Homogenates

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(Received March 5, 1979)

A chromatographic-videodensitometric assay was found to be appropriate for measuring the activity of glutamate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, ornithine-2-oxoacid aminotransferase and histidine ammonia-lyase in human tissue homogenates.

From the assay mixtures containing substrate(s), cofactor(s), buffer and tissue extract, five or ten μl samples were taken at different time intervals and chromatographed on Dowex 50×8 type resin-coated chromatosheets. On each chromatoplate 50 nmoles of the amino acid to be measured were separately run as a reference for videodensitometric evaluation. By comparing the density of the reference amino acid to that of the individual samples the molar amount of amino acids formed or consumed in the reaction could be calculated.

The present findings suggest that the chromatographic-videodensitometric microassay* (CV-technique) is suitable for measuring the activity of amino acid transforming enzymes in minute amounts of tissue extracts.

Introduction

The quantitative determination of amino acids by ion-exchange column chromatography was developed by Moore and Stein (1951). Several attempts have been made since to combine the favourable properties of the column technique, e.g. excellent resolving power, with the rapidity and economy of paper or thin layer chromatography. The introduction of a chromatosheet coated with a thin layer of Dowex 50×8 type ion-exchange resin (Dévényi, 1970; Dévényi et al., 1971) has partly met the expectations, since it made it possible to resolve a mixture of 16 or 20 amino acids reliably in about two hours. This method was succesfully applied for the detection of inborn errors of amino acid metabolism both in infants and adults (Kovács, 1973; Tasnády et al., 1977).

The quantitative evaluation of thin layer ion-exchange chromatograms, electropherograms and autoradiograms by videodensitometry (Dévényi, 1976) has offered new possibilities in the analytical chemistry of amino acids. It was found that even 3–5 nmol specimens of amino acids could be determined by videodensitometry in a few seconds.

* *Abbreviation:* CV, chromatographic-videodensitometric microassay

The assay of amino acid transforming enzymes in homogenates and cell extracts from minute amount of tissue in amino acid consuming, producing or transforming reactions can be carried out by the combination of thin layer ion-exchange chromatography with videodensitometry. The present paper describes the chromatographic-videodensitometric determination (CV-technique) of glutamate dehydrogenase, aspartate, alanine and ornithine-2-oxoacid aminotransferase activities in human liver as well as the assay of histidine ammonia-lyase in human skin.

Materials and methods

Liver extracts

10–20 mg samples of fresh liver were obtained by biopsy after laparatomy of patients without any clinical and laboratory evidence of liver damage. In control experiments liver samples from cadavers were used, which were taken not later than four hours after death in accident, and were stored on ice for usually less than 30 min before homogenization. No liver damage was observed in the latter cases either. Liver samples were homogenized with 6 volumes of 0.1% cetyltrimethyl ammonium bromide. The homogenates were allowed to settle in capillary tubes and, when it was necessary, the supernatants, which contained 16 to 20 mg protein per ml, were diluted with 0.9% sodium chloride. Protein concentration was determined by Lowry's method (Lowry et al., 1951).

Skin extracts

A 20–25 mg sample of epidermis stratum corneum was cut into small fragments, then it was homogenized with 6 volumes of 30 mM sodium pyrophosphate buffer, pH 9.2, containing 1 mM dithiothreitol and 1 mM ethylenediamine tetraacetate. The clear supernatants, which contained 4 to 7 mg protein per ml, were added to the assay mixtures to determine histidine ammonia-lyase activity. Samples were obtained from 10 healthy children and from 20 adults comprising 10 males and 10 females. Both liver and skin extracts were kept at 5 °C for not more than an hour before the assay.

Assay mixtures

The final volume of the assay mixture containing substrates, cofactors, buffer and tissue extract, was 60 μl. The amount of the tissue extract was chosen such that during the incubation period the change in amino acid concentration of the assay mixture should be about 50 nmoles. Usually five samples, 5 or 10 μl each, were applied onto the chromatoplates on which 5 or 10 μl of 10% trichloroacetic acid had been previously spotted.

Chromatography was performed on cationic resin coated chromatosheets (Fixion-50 × 8, Chinoin, Budapest, Hungary, or Ionex 25-SA-Na, Macherey-

Nagel and Co., Düren, GFR). Chromatosheets were used either in Na^+ or in Li^+ form. The latter was prepared from chromatosheets commercially available in Na^+ form by prechromatography in 1.0 M LiCl for 24 hours (Váradi, 1975).

The products of glutamate dehydrogenase, ornithine-2-oxoacid aminotransferase and histidase activities were chromatographed on chromatosheets in Na^+ form at room temperature in sodium citrate buffer, pH 4.25 (0.1 M citric acid, 0.4 M sodium hydroxyde, adjusted to pH 4.25 with HCl). Aspartate and alanine aminotransferase activities were determined on chromatosheets in Li^+ form,

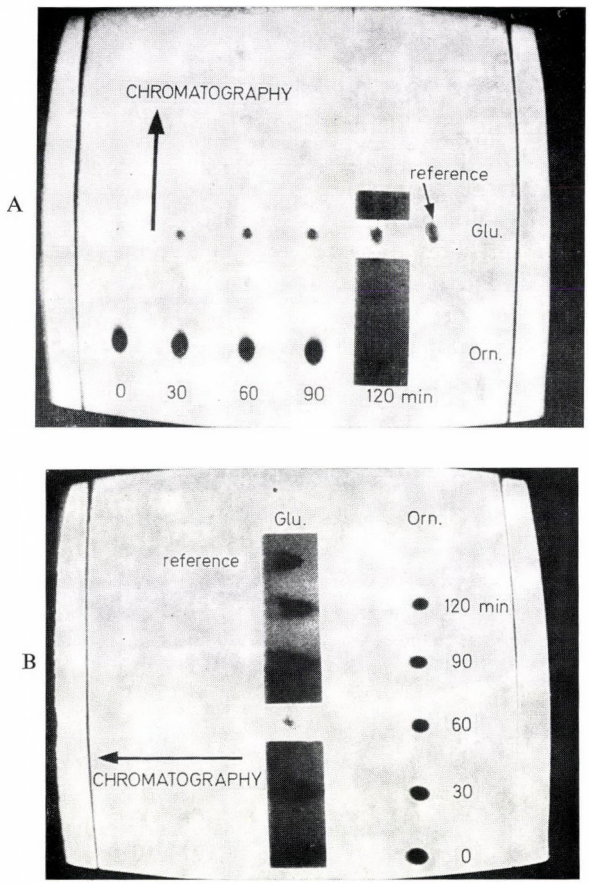

Fig. 1. Videodensitometric measurement of glutamate formed by ornithine-2-oxoacid aminotransferase (for details see Results). Samples were taken from the assay mixture at the time intervals indicated. *A*. The black window covers the 120 min sample while a white one is seen at the place corresponding to glutamate. *B*. Same chromatogram placed into the videodensitometer perpendicular to the direction of chromatography. The spots of glutamate in different samples and 50 nmole glutamate reference are measured as total density. The arrows indicate the direction of chromatography

in lithium citrate buffer, pH 4.1 (0.1 M citric acid and 0.3 M lithium hydroxyde, adjusted to pH 4.1 with HCl). The amino acids were stained with cadmium-ninhydrin spray reagent as described by Dévényi (1970).

Quantitative determination of amino acids by videodensitometry

A Telechrom high-speed videodensitometer (Euro-Lab, Munich, GFR) was used for the determination of amino acids on the chromatograms. By this method geometrically uniform chromatograms can be evaluated. One vertical column represents the total amount of colored material in a chromatogram and this is measured first as total *density*. Then the amount of individual amino acids, i.e. the *single spot density* is measured as the percentage of the total as shown in Fig. 1a.

For the determination of the activity of amino acid transforming enzymes in tissue extracts, the molar concentration of only one particular amino acid (either the substrate or the product) is usually to be measured. Therefore, the chromatosheets stained with ninhydrin were placed into the videodensitometer perpendicular to the direction of chromatography. Thus, the column formed by spots of identical amino acids of different samples are taken as total density (Fig. 1b). On each chromatosheet 50 nmol of the measured amino acid was run separately as reference. By comparing the density of the reference amino acid to that of the experimental samples the molar amount of formed or consumed amino acid(s) can be calculated.

It is important that the amount of the product to be measured in individual samples should be in the 2 to 200 nmol range. In this case the error of determina-

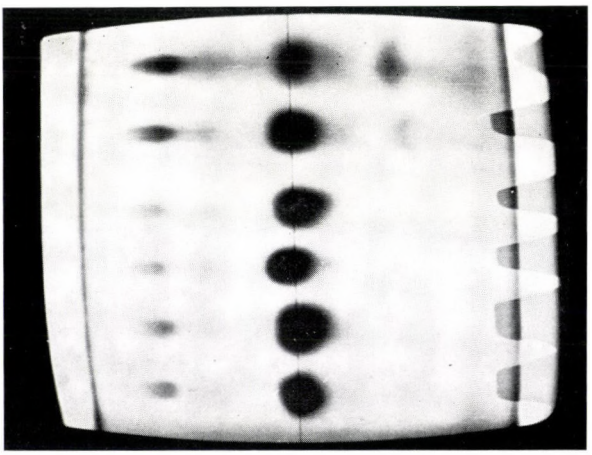

Fig. 2. Sensitivity control of the videodensitometer. The perpendicular base-line is positioned in the middle of the amino acid spots. The densitogram is displayed on the left half of the monitor screen. The spots in this case are too dark, the peaks in the densitogram are cut off indicating that the densitometer is overloaded

tions by the CV-technique is less than $\pm 5\%$. When selecting the area to be measured it is recommended to position the perpendicular base-line in the central part of the chromatographic run. The densitogram is then displayed on the left half of the monitor, and one can see wether or not the components of the selected sample are within the measuring range. If a spot is too dark, the peak of the densitogram is cut off (Fig. 2) indicating that the Vidicon tube is overloaded and no linear function between concentration and density can be expected.

Results and discussion

Glutamate dehydrogenase (EC 1.4.1.3)

The enzyme catalyzes reversibly the following reaction:

$$\text{2-oxoglutarate} + NH_3 + \text{NADH (or NADPH)} + H^+ \rightleftarrows$$
$$\rightleftarrows \text{glutamate} + NAD^+ \text{ (or } NADP^+\text{)}$$

For activity measurements the assay mixture contained 40 mM 2-oxoglutarate, 200 mM ammonium acetate, 30 mM NADH and 20 mM EDTA in 50 mM triethanolamine-HCl buffer, pH 8.0. The liver homogenate was diluted with 0.9% sodium chloride solution and was added to a final concentration corresponding to 0.3 mg wet tissue in a 60 μl assay mixture.

Enzyme activity was determined with samples from the livers of twelve cadavers both by optical test (Schmidt, Schmidt, 1962) and by the CV-technique (Fig. 3). Data obtained by the two methods were in good agreement (Table 1) and the results correlated well also with those published elsewhere (Schmidt, Schmidt, 1963a, b, c). The activity of glutamate dehydrogenase in liver samples obtained by biopsy was somewhat higher than that in samples taken from nec-

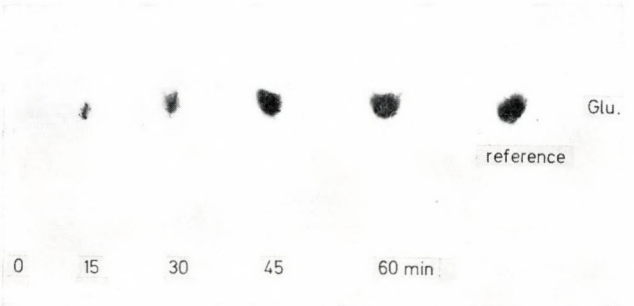

Fig. 3. Determination of glutamate dehydrogenase activity by the CV-technique (for details see Results). Activity is measured as glutamate production. The assay mixture contained liver extract corresponding to only 0.3 mg of wet tissue, thus the endogeneous amino acids are not seen

Table 1

Glutamate dehydrogenase activity in human liver homogenate
Activity is expressed as μmol glutamate formed $\times$ hour$^{-1} \times$ g^{-1} wet tissue

	Source of liver		
	Necropsy, 12 cases		Biopsy, 8 cases
	Photometry	CV-technique	CV-technique
Activity	1505.12	1487.16	1732.32
$\pm$ SD*	57.55	57.96	58.18
Range	1410—1720	1380—1690	1585—1930

* SD = standard deviation.

ropsy (Table 1). Similar observations with other enzymes from liver have been described by other authors, too (Miller, McLean, 1967).

In clinical diagnostics glutamate dehydrogenase activity in serum is tested routinely (Fillips, 1965). The investigation of liver samples obtained by biopsy has shown that the altered activity of this enzyme in the liver is characteristic of some diseases (Schmidt, Schmidt, 1963a, b, c; Bär et al., 1963).

Ornithine-2-oxoacid aminotransferase (EC 2.6.1.13)

The enzyme catalyzes the following reaction:

ornithine + 2-oxoglutarate $\rightleftharpoons$ glutamate-γ-semialdehyde + glutamate.

The determination of this enzyme in human liver samples obtained by biopsy has recently assumed diagnostic importance since it is suitable for the detection of hyperornithinemias (Bickel et al., 1968; Kekomäki et al., 1969). The activity of this enzyme is usually determined by measuring the color of dihydroquinazolium formed through the condensation of the 5-aminobenzaldehyde and pyrroline-5'-carboxylate. The latter compound is a product of spontaneous cyclization of glutamate-γ-semialdehyde formed in the reaction (Strecker, 1957, 1965).

The 60 μl assay mixture contained 60 mM ornithine-HCl, 30 mM 2-oxoglutarate and 500 μM pyridoxal phosphate in 50 mM potassium phosphate buffer, pH 8.0, and also the liver extract corresponding to about 1 mg wet liver tissue. Fig. 1a and b show the videodensitometric determination of ornithine-2-oxoacid aminotransferase. The activity can be more precisely determined by measuring the change in glutamate concentration rather than that of ornithine present in excess in the assay mixture (Fig. 4). Both glutamate-γ-semialdehyde and pyrroline-5'-carboxylate, which are also formed in the assay mixture, give poorly visible spots with ninhydrin.

In 10 samples taken from necropsy an activity of 152 $\pm$ 44.5 μmol glutamate $\times$ hour$^{-1} \times$ g^{-1} wet tissue was found (range 82 to 210 μmol h^{-1} g^{-1}),

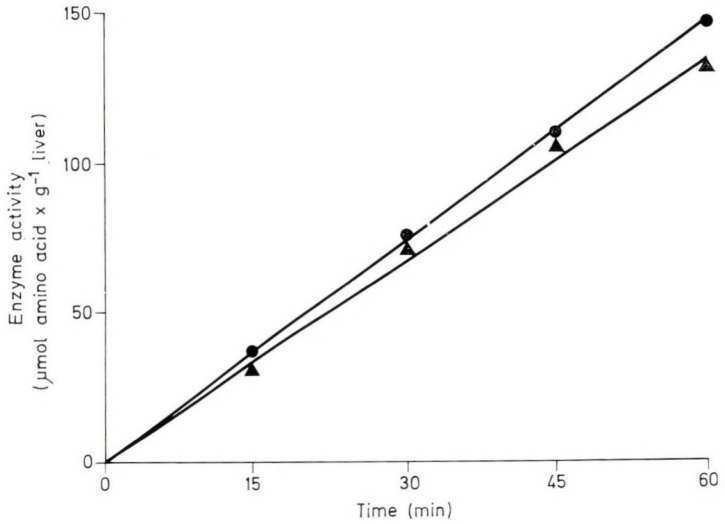

Fig. 4. Determination of ornithine-2-oxoacid aminotransferase activity by the CV-technique. Activity was measured as μmole glutamate formed per hour per gram of wet tissue (— ● — ● —) and as μmole ornithine consumed per hour per g liver (— ▲ — ▲ —). For details see Results

whereas in those obtained by biopsy an activity of 169 $\pm$ 45.1 μmol glutamate $\times$ hour$^{-1} \times$g^{-1} wet tissue was calculated (range 96 to 224 μmol). The relatively great deviation may be due to different reasons, e.g. state of nourishment (Räihä, Kekomäki, 1968), individual differences, time passed after death, etc.

Aspartate aminotransferase (EC 2.6.1.1) and *alanine aminotransferase* (EC 2.6.1.2) catalyze the reversible transfer of an amino-group between amino acids and 2-oxoacids:

aspartate + 2-oxoglutarate $\rightleftharpoons$ oxaloacetate + glutamate

alanine + 2-oxoglutarate $\rightleftharpoons$ pyruvate + glutamate

The reaction mixture contained 40 mM 2-oxoglutarate and either 60 mM aspartate or 60 mM alanine in 100 mM phosphate buffer, pH 7.4, and liver extract corresponding to about 0.3 mg wet liver tissue, in a final volume of 60 μl. The activity of both enzymes was calculated from the amount of glutamate formed in the assay mixture. In the case of aspartate aminotransferase an activity of 2200 $\pm$ 36 μmol glutamate $\times$ hour$^{-1} \times$g^{-1} wet tissue (range 1680 to 2500) was found in 10 liver samples taken from necropsy. The activity of alanine aminotransferase was 1380 $\pm$ 21 μmol glutamate $\times$ hour$^{-1} \times$g^{-1} wet tissue (range 1270 to 1620). These values are somewhat lower than those found by Schmidt and Schmidt (1963a, b, c). The data of these authors, however, were obtained

with an optical method by measuring NADH consumption during the reduction of oxoacids.

The method of Rowsel (1962) can also be applied for the determination of aminotransferase activity. In this procedure amino acids are separated by chromatography, developed with ninhydrin, then eluted and measured by photometry. In the videodensitometric evaluation of the chromatograms, however, elution becomes unnecessary.

The chromatographic-videodensitometric method permits the direct determination of the ratio of aminotransferase and glutamate dehydrogenase in liver tissue, that is frequently needed in the enzyme-diagnostics of serum (Schmidt, Schmidt, 1962). Only a 2–3 mg specimen of fresh liver tissue is required when the CV-method is applied.

Histidine ammonia-lyase (histidase, EC 4.3.1.3)

The irreversible transformation of histidine to urocanate is catalyzed by this enzyme:

$$\text{histidine} \rightarrow \text{urocanate} + NH_3$$

As described by Zannoni and La Du (1963), the normal human epidermis contains histidase in high concentration. By measuring enzyme activity in the stratum corneum, histidinemia, i.e. histidase deficiency can be discovered. Histidase activity is usually determined by measuring the ultraviolet absorption of urocanate. Urocanate has to be removed from the skin homogenate by dialysis for several hours (Tabor, Mehler, 1955) before measuring histidase activity. Since the urocanate content of the epidermis does not interfere with the determination

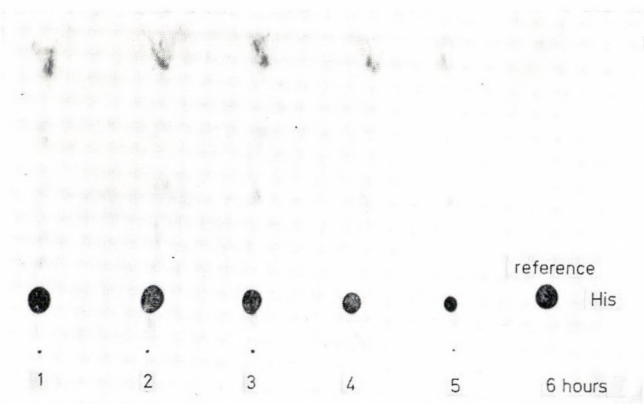

Fig. 5. Determination of histidase activity in skin by the CV-technique (for details see Results). Activity is measured from the amount of histidine consumed. The assay mixture contained skin extract corresponding to about 5 mg wet tissue, therefore endogeneous amino acids are faintly seen

Table 2

Histidine ammonia-lyase activity in human skin extract
Activity is expressed as $\mu mol \times hour^{-1} \times g^{-1}$ epidermis

	Histidine consumed, by CV-technique		Urocanate formed by photometry*	
	mean	range	mean	range
Children	5.2	4.6—6.3	5.4	4.2—6.6
Adults				
Male	3.4	2.3—4.2	3.6	1.9—5.3
Female	3.2	2.5—3.8	3.0	1.8—5.3

* Data of Zannoni and La Du (1963).

of histidine, it is not necessary to remove by dialysis when the CV-technique is used.

The reaction mixture for histidase assay was composed of 20 μl of 30 mM histidine-HCl, pH 9.2, and 40 μl of skin extract corresponding to 5—6 mg of epidermis stratum corneum. 10 μl samples taken at one-hour intervals were applied onto the chromatoplates.

Enzyme activity was calculated from the decrease of histidine concentration. The videodensitometric evaluation of a chromatogram is shown in Fig. 4. The results obtained for histidase activity both in samples from adults and children (Table 2) correlate well with those published by Zannoni and La Du (1963). In agreement with their observations we also found that the activity of skin histidase is slightly higher in males than in females.

The authors are indebted to Drs L. Buris and S. Dobi for supplying the human liver samples and to Dr É. Ladányi for obtaining the skin specimens. The excellent technical assistance of Mrs Ilona Lőrinczi is gratefully acknowledged.

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Determination of Enzyme Activity by Chromatography and Videodensitometry

II. Urea Cycle Enzymes in Tissue Homogenates

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(Received March 5, 1979)

Methods are described for the determination of the activity of urea cycle enzymes in human and rat tissues by chromatography and videodensitometry* (CV-technique). With specific substrates carbamoyl-phosphate synthetase and ornithine carbamoyltransferase activities were determined as the amounts of citrulline formed. Argininosuccinate synthetase, argininosuccinate lyase and arginase activities were measured from the changes in ornithine concentration. For measuring the activity of five enzymes 5 to 10 mg wet weight of tissue was sufficient. The CV-technique could be conveniently applied for the investigation of enzyme content in samples from human biopsy.

Introduction

In the mammalian organism ammonia, a product of metabolism but poisonous for the cells is removed by the urea cycle functioning in the liver. If the function of any of the five enzymes in the cycle is damaged, hyperammonemia may develop. Anomalously high ammonia concentrations in body fluids, e.g. in blood, liquor or urine, may indicate the inadequate functioning of the urea cycle (Shih, Efron, 1972). The cause of malfunctioning, however, can be revealed only if the defective enzyme is identified with specific methods (Levin, 1971).

Individual enzymes of the cycle may occur in other tissues than liver, e.g. in the brain, the kidney, the testicles, the intestine, amniotic cells and red blood cells, but the whole functioning cycle can be found only in the liver. In addition, the activity of these enzymes is by orders of magnitude higher in the liver than in other tissues.

A technique which requires less liver tissue for diagnosis than others such as spectrophotometry, reduces the risk of manipulation in biopsy. Thus it may be useful both for clinicians and pathologists.

In the accompanying paper (Karsai, Előd i, 1979) a micromethod was described for the investigation of amino acid transforming enzymes in human tissues by CV-technique. The present paper describes the determination of the

* *Abbreviation:* CV, chromatographic videodensitometric microassay

activity of five enzymes functioning in the urea cycle. For this method only a small amount, about 5 to 10 mg of liver tissue is needed. The method was tested with human liver obtained both by biopsy and from necropsy, with different other human tissues, such as brain, intestine, kidney, erythrocytes and amniotic fluid cells as well as with rat liver.

Materials and methods

Tissue extract

The activity of urea cycle enzymes was determined both in human and rat livers. Female CFY rats of about 200 g weight were anesthetized with ether and were exsanguinated. The livers were removed and washed with ice-cold distilled water. Liver tissue was homogenized with six volumes of 0.1% cetyltrimethyl ammonium bromide solution. The homogenate was then centrifuged and the clear supernatant was used for enzyme assay. The protein concentration of the rat liver extract varied between 18 and 30 mg per ml.

Human liver tissue was obtained both from biopsy and necropsy. The liver extracts were prepared as described in the preceding paper (Karsai and Elődi, 1979). The protein concentration of different human liver extracts varied between 10 and 20 mg per ml. Tissue extracts were prepared with cetyltrimethyl ammonium bromide solution also from human kidney, brain, intestine and their enzyme content was also measured. Tissue samples were kept in ice for less than an hour before homogenization.

The protein content of tissue extracts was determined according to Lowry et al. (1951) using bovine serum albumin as reference.

Erythrocytes

Blood samples of normal human subjects were taken from a finger prick. 50 μl of blood was mixed with 450 μl of hemolyzation solution containing 20 mM $MnCl_2$ and 0.4 mg/ml heparin (Azizi et al., 1970). Hemoglobin was determined by the hemoglobin-cyanide method (van Kampen, Zijlstra, 1961).

Amniotic fluid cells

Amniotic fluid specimens, usually 5 to 10 ml, were obtained from normal, uneventful pregnancies by transabdominal or transvaginal amniocentesis (Ember et al., 1977) performed before legal abortions. Specimens contaminated with blood or vaginal mucus were excluded. The samples were immediately centrifuged at 500 g for 5 min and pellets were washed twice with cold 0.9% sodium chloride. Finally, amniotic cells were resuspended in 0.5 ml of distilled water then the samples were quickly frozen and thawed three times.

Assay of enzyme activities

Usually assay mixtures of 60 μl were prepared. They contained the substrates, cofactors and the tissue extract (see Results for details). Enzyme activities were determined at 37 °C.

Onto a 20×20 cm chromatosheet 5 or 10 μl 10% trichloroacetic acid droplets were applied at distances of about 2 cm, then 5 or 10 μl samples withdrawn from the assay mixture at different time intervals were added to the trichloroacetic acid on the chromatosheet.

For the separation of the amino acids which were formed in the assay mixtures, chromatosheets covered with a thin layer of cation exchange resin (Fixion 50×8, Chinoin, Budapest, Hungary or Ionex SA-25, Macherey-Nagel Co. Düren, GFR) were used in sodium form. Chromatography was carried out at room temperature in citrate buffer, pH 4.25. The buffer was composed of 0.4 M sodium hydroxyde and 0.1 M citric acid and was adjusted with hydrochloric acid to pH 4.25. Ascending chromatography under the conditions described here takes about two hours. The chromatosheets were dried with hot air and stained with cadmium-ninhydrin reagent (Dévényi, 1970).

The amounts of amino acids were determined by measuring the intensity of ninhydrin spots with a Telechrom high speed videodensitometer (Euro-Lab, Munich, GFR) according to Dévényi (1976). Other details are described in the previous paper (Karsai, Előd i, 1979).

Results

Since argionosuccinate synthetase is the rate limiting enzyme in the urea cycle, the activity of the first two enzymes, i. e. carbamoyl-phosphate synthetase, and ornithine carbamoyltransferase, can be determined by measuring the amount of citrulline formed in the assay mixture. The activity of the other three enzymes, argininosuccinate synthetase, argininosuccinate lyase and arginase, can be assayed by determining the changes in ornithine concentration (Fig. 1) in the assay mixtures containing specific substrates.

The assay mixtures were made up according to Brown and Cohen (1959) and Schimke (1962). Slight modifications of the substrate and cofactor concentrations were introduced to fulfill the requirements of the CV-technique.

If the change in amino acid concentration during the incubation period was about 60 nmole in 60 μl assay mixture, the enzyme activity could be conveniently determined with the CV-technique by withdrawing for chromatography 5 or 10 μl aliquots from the assay mixture at different time intervals. It has to be pointed out that in accordance with other authors (Schepartz, 1973) we have found great differences in the activity of various enzymes of the cycle. For example, the activity of arginase in liver is about fivehundred to thousand times as much as that of argininosuccinate synthetase. Therefore, different amounts of tissue extract

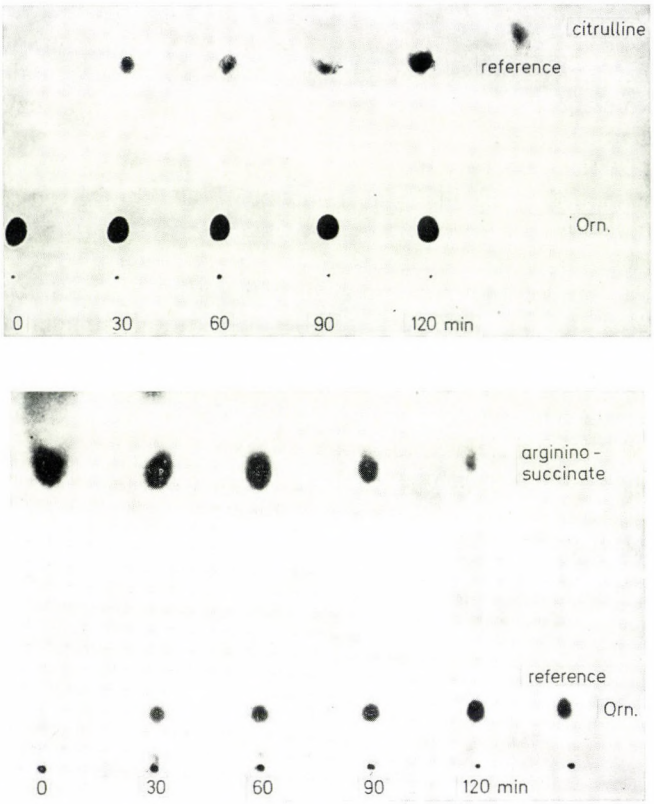

Fig. 1. Determination of enzyme activities by the CV-technique. (*A*) Carbamoyl-phosphate synthetase activity was measured as the amount of citrulline formed by a coupled reaction of carbamoyl-phosphate synthetase and ornithine carbamoyl-transferase; (*B*) Arginino-succinate lyase activity was estimated from the amount of ornithine formed in a coupled reaction with arginase (for details see Results)

(see Results) were added to the assay mixtures to obtain similar changes with various enzymes with respect to the formation of the products.

The conditions for the determination of the activity of the individual enzymes of the urea cycle by the CV-technique are the followings.

Carbamoyl-phosphate synthetase (ammonia) (EC 2.7.2.5)

The enzyme catalyzes the formation of carbamoyl-phosphate

$$2\,ATP + NH_4^+ + HCO_3^- + H_2O \xrightarrow{N\text{—}Ac\text{—}Glu,\ Mg^{2+}} \rightarrow \text{carbamoyl-phosphate} + 2\,ADP + P_i$$

Carbamoyl-phosphate formed in the reaction cannot be directly determined by the CV-technique, because it does not react with ninhydrin. However, when the assay mixture contains ornithine, the ornithine carbamoyltransferase, which is present in liver homogenates in about a fiftyfold excess to carbamoyl-phosphate synthetase (Schepartz, 1973), immediately converts the product to citrulline

carbamoyl-phosphate + ornithine → citrulline + P_i.

Therefore, the activity of carbamoyl-phosphate synthetase in the presence of ornithine can be conveniently determined by measuring the amount of citrulline formed by the enzymes in the liver extract.

The assay mixture contained 25 mM ATP, 50 mM magnesium sulfate, 20 mM N-acetyl glutamate, 200 mM ammonium hydrogen carbonate saturated with carbon dioxide and tissue homogenate corresponding to about 1 mg fresh liver tissue in 60 μl final volume. Samples were taken at 15 min intervals and were added to trichloroacetic acid drops on the chromatoplates. From a separate spot citrulline (50 nmoles) was run as reference.

Normal liver tissues contain ornithine carbamoyltransferase in sufficient amounts, therefore it is not rate limiting in citrulline formation when ornithine is present. The addition of purified ornithine carbamoyltransferase to the assay system is necessary only if in a previous determination no formation of citrulline could be detected due to a lack of carbamoyl-phosphate synthetase or ornithine carbamoyltransferase in the tissue extract.

The activity of glutamine-dependent carbamoyl-phosphate synthetase (EC 2.7.2.9), which participates in pyrimidine synthesis, can also be determined similarly. In this case, however, instead of N-acetyl glutamate and ammonium hydrogen carbonate the assay mixture should contain glutamine at 100 mM concentration.

Ornithine carbamoyltransferase (EC 2.1.3.3)

As it has been mentioned above, the activity of this enzyme can be conveniently determined by measuring the amount of citrulline formed in the assay mixture. The reaction mixture contained 10 mM ornithine and 15 mM carbamoyl-phosphate lithium salt in 20 mM triethanolamine-hydrochloric acid buffer, pH 7.7. Liver extract corresponding to about 0.03 mg fresh liver tissue was added to initiate citrulline formation in the assay system. 10 μl samples were withdrawn at 15 min intervals from the assay mixture and were applied onto a chromatosheet previously treated with trichloroacetic acid. As reference citrulline (50 nmoles) was also chromatographed separately.

The activity of aspartate carbamoyltransferase (EC 2.1.3.2), which is involved in pyrimidine synthesis can be determined in a similar way, but the assay mixture in this case contains aspartate instead of ornithine.

K_M values for both substrates of ornithine carbamoyltransferase were determined by the CV-technique using liver extract from human necropsy as enzyme source. In the first set of experiments the concentration of ornithine was

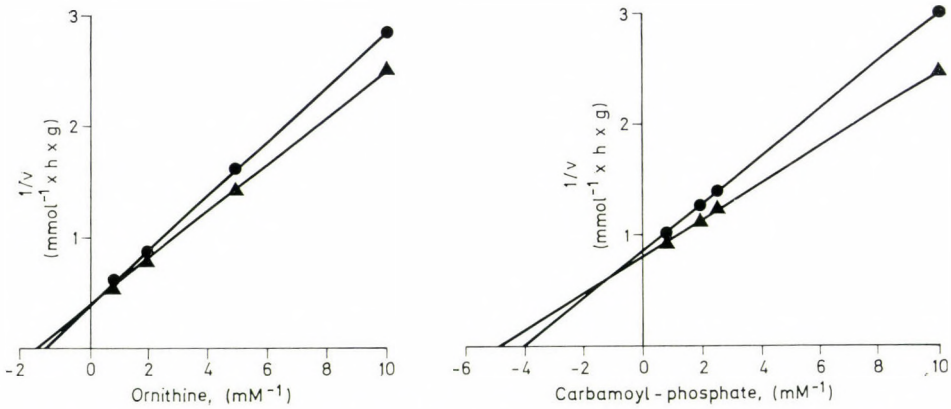

Fig. 2. Determination of K_M values of ornithine carbamoyltransferase (*A*) for ornithine in the presence of 7.5 mM carbamoyl-phosphate and (*B*) for carbamoyl-phosphate (ornithine concentration was 5 mM) in 50 mM triethanolamine -HCl buffer, pH 7.7, at 37°C. —▲—▲— by the CV-technique, —●—●— by spectrophotometry

kept constant at 5 mM and that of carbamoyl-phosphate changed between 50 μM and 4.0 mM. In the other series the assay mixture contained 7.5 mM of carbamoyl-phosphate and the concentration of ornithine varied between 100 μM and 5.0 mM. In a separate series the activity measurements were carried out by spectrophotometry as well (Ohshita et al., 1976) to compare the data with those found with the CV-technique. As seen in Fig. 2 there is a good agreement between the K_M values obtained by the two methods. The values calculated for ornithine and carbamoyl-phosphate were 550 μM and 210 μM, respectively, similar to those found by other authors (Snodgrass, 1968; Pierson et al., 1977).

Argininosuccinate synthetase (EC 6.3.4.5)

This enzyme is rate limiting in the urea cycle. It catalyzes the formation of argininosuccinate from citrulline and aspartate in the presence of magnesium ions and ATP:

$$\text{citrulline} + \text{aspartate} + \text{ATP} \xrightarrow{Mg^{2+}} \text{argininosuccinate} + \text{AMP} + PP_i.$$

The formation of argininosuccinate, however, can be followed only with the purified enzyme, since the liver extract contains five times as much argininosuccinate lyase activity as synthetase and an about fivehundredfold excess of arginase activity (Schepartz, 1973). Therefore, argininosuccinate synthetized by the enzyme is rapidly transformed into arginine and fumarate by the lyase, then into ornithine and urea by arginase. In the equilibrium mixture we found about 95% ornithine and about 2 to 3% argininosuccinate and arginine. This fact suggested that the activity of argininousuccinate synthetase can actually be determined by measuring the amount of ornithine formed in the reaction mixture.

The assay mixture contained aspartate, citrulline and ATP, each at 25 mM concentration in 50 mM phosphate buffer, pH 7.0. The mixture also contained liver homogenate equivalent to about 1.2 mg fresh liver tissue. 10 μl samples were withdrawn at 30 min intervals and were applied onto the chromatosheet. From a separate spot 50 nmoles of ornithine or 50 nmoles of ornithine and 5 nmoles of arginine were run, as reference, on the same sheet as the assay samples.

Argininosuccinate lyase (EC 4.3.2.1)

This enzyme splits argininosuccinate to arginine and fumarate:

$$\text{argininosuccinate} \rightarrow \text{arginine} + \text{fumarate}.$$

The activity of the enzyme can be determined either by measuring the decrease of argininosuccinate concentration or the increase in ornithine concentration. Ornithine is formed from arginine by arginase present in excess in the liver extracts. The reaction mixture contained in 50 mM phosphate buffer, pH 7.7, 20 mM argininosuccinate and tissue extract corresponding to about 1 mg of fresh liver tissue. 10 μl samples were taken at 30 min intervals and were chromatographed together with 50 nmoles of ornithine and/or 50 nmoles of argininosuccinate as reference.

Arginase (EC 3.5.3.1)

Arginase is the terminal enzyme in the urea cycle. It catalyzes the formation of urea and ornithine from arginine in the presence of manganese ions:

$$\text{arginine} + H_2O \xrightarrow{Mn^{2+}} \rightarrow \text{ornithine} + \text{urea}.$$

The activity of the enzyme can be measured from the increase in ornithine concentration. The reaction mixture contained 60 mM arginine in 50 mM glycine-sodium hydroxide buffer, pH 9.5, and liver extract corresponding to about 0.01 mg fresh liver tissue. The pH of the arginine stock solution was adjusted to 9.5 before addition to the assay mixture. The activity of arginase in the liver extract was very high, thus it has to be diluted before use with 50 mM Tris-hydrochloric acid buffer, pH 7.8, containing 5 mM manganese chloride. The diluted extract was incubated for 10 min at 37 °C to activate arginase with Mn^{2+} ions. 5 μl samples were taken from the assay mixture at 10 min intervals and were applied onto the chromatoplate. Ornithine (50 nmoles) was chromatographed as reference.

Activity of urea cycle enzymes in different tissues

Table 1 summarizes the data obtained with different liver samples. The activity of each enzyme was determined from at least twelve different individuals. The relatively wide range of enzyme activities may reflect both individual differences in the enzyme activities and the quality of liver samples, i.e. amount of

Table 1

Activity of urea cycle enzymes determined by the CV-technique
1 unit being 1 μmole citrulline or ornithine × hour^{-1} × g^{-1} wet tissue

Enzymes	Source of enzymes		
	Human liver		Rat liver units ± SD*
	from biopsy units ± SD*	from necropsy units ± SD*	
Carbamoyl-phosphate synthetase	264 ± 54	192 ± 82	625 ± 134
Ornithine carbamoyltransferase	6 178 ± 1 634	3 758 ± 1 076	13 573 ± 2 483
Argininosuccinate synthetase	87 ± 18	39 ± 10	225 ± 28
Argininosuccinate lyase	216 ± 32	98 ± 21	323 ± 63
Arginase	83 000 ± 13 000	35 000 ± 7 850	127 000 ± 23 000

* SD = standard deviation.

Table 2

Activity of urea cycle enzymes in human tissues and cells
1 unit being 1 μmole citrulline or ornithine × hour^{-1} × g^{-1} wet tissue

	Brain units ± SD***	Jejunum units ± SD	Kidney units ± SD	Erythrocytes* units ± SD	Amniotic** fluids cells units ± SD
Carbamoyl-phosphate synthetase	5.2 ± 1.3	—	33.6 ± 3.7	—	260 ± 183
Ornithine carbamoyl-transferase	6.6 ± 1.1	1.0 ± 0.3	28.7 ± 2.6	—	9557 ± 5836
Argininosuccinate synthetase	92.5 ± 12.8	—	22.7 ± 2.8	1.9 ± 0.7	140 ± 103
Argininosuccinate lyase	21.1 ± 3.6	0.8 ± 0.3	14.5 ± 2.0	12.8 ± 5.3	846 ± 349
Arginase	420 ± 39	2.2 ± 1.0	438 ± 21	1250 ± 445	44437 ± 12532

* activity in μmole ornithine × hour^{-1} × g^{-1} hemoglobine
** activity in μmole ornithine or citrulline × hour^{-1} × g^{-1} soluble protein
*** SD = standard deviation

connective tissue in the sample, etc. The activities are expressed in units corresponding to 1.0 μmole of the product formed per hour per g fresh tissue.

The activities of urea cycle enzymes in brain, intestine and kidney taken from necropsy and that of erythrocytes and amniotic fluid cells are shown in Table 2. In these tissues either some of the enzymes were absent or their activity was less than what could be detected by the CV-technique.

Discussion

A CV-technique described in the present paper for the determination of urea cycle enzymes enabled us to determine the activity of five enzymes by using not more than about 5 to 10 mg liver tissue. Only 60 μl assay mixture is needed to measure the changes either in product formation or substrate consumption by each enzyme of the cycle at five different time intervals, whereas for an assay of the same enzymes by spectrophotometry at least 50 mg liver tissue is necessary (Levin, 1971). Other experience suggests (Karsai, Előd, 1979) that the CV-technique is suitable for the investigation of other amino acid transforming enzymes, too, and with slight modifications it can be employed for biological materials other than mammalian tissue extracts.

The kinetic properties of these enzymes can also be conveniently determined by this method. Usually, the accuracy of activity determinations were satisfactory when the changes in the concentration of the amino acid to be measured were at least 80–100 nmole per hour per g wet tissue. One may expect to measure smaller amounts of enzyme by increasing the length of incubation if the enzyme is stable enough i.e. its activity remains the same during the assay period. In the case of the urea cycle enzymes just the rate limiting argininosuccinate synthetase becomes inactive within a few hours at 37 °C (Levin, 1971). The amounts of the individual enzymes in Tables 1 and 2 are given in units, i.e. μmole of substrate consumed or product formed per hour per g wet tissue.

The protein content of liver tissue extracts was also measured and in ten different liver samples the following values were found for 1.0 g fresh tissue. Human liver extract obtained from biopsy or necropsy and rat liver extract contained 142 $\pm$ 3.6 mg, 131 $\pm$ 5.1 mg and 198 $\pm$ 5.2 mg protein, respectively. In rat liver the activity of argininosuccinate synthetase was about three times, and that of carbamoyl-phosphate synthetase and ornithine carbamoyltransferase more than two times as high as the activity of the corresponding enzymes in human liver extract, whereas the protein concentration was only 40% higher in rat liver than in human liver. Human liver samples from necropsy contained about 10% less protein than those obtained by biopsy. The enzyme activities, however, were 30–60% lower in the samples from necropsy indicating that these enzymes are more sensitive to the decomposition of the liver tissue than other proteins. Similar changes in the enzyme activities of human tissues were found by Nuzum and Snodgrass (1971) as well as by Glick et al. (1976).

The activity of argininosuccinate synthetase in human skin fibroblast cultures has been demonstrated by Tedesco and Mellman (1967). These authors detected also an increased K_M of synthetase in cultured fibroblasts from patients with citrullinemia. Using the technique of amniocentesis it is possible to culture amniotic fluid cells, and to diagnose antenatally several inborn errors of metabolism (Nadler, Gerbie, 1970). Prenatal diagnosis of urea cycle disorders has been carried out by Goodman et al. (1970). They demonstrated the lack of argininosuccinate lyase activity in cultured amniotic fluid cells.

Our method presented here makes it possible to demonstrate the activity of the urea cycle enzymes in amniotic fluid cells directly, without any culturing. In this manner, the CV-technique may be suitable for the detection of an enzyme damage, i.e. the antenatal diagnosis of urea cycle enzymopathies, using minute amounts of amniotic fluid specimens obtained by amniocentesis at early pregnancy.

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Simple Method for the Purification of Bacterial Plasmids on Hydroxyapatite

(Short Communication)

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Bacterial plasmids are widely used as cloning vehicles in recombinant DNA technology. Caesium chloride-ethidium bromide centrifugation is the most generally applied method for the purification of these covalently closed circular supercoiled DNA molecules (Clewell, Helinski, 1969). Although this technique provides a high degree of purification, the lengthy centrifugation is expensive and time consuming, especially if large-scale preparation is needed. The demand for a rapid and inexpensive plasmid purification method is clearly indicated by the fact that several new procedures have been described recently (Ohlsson et al., 1978; Kozlov et al., 1978; Zasloff et al., 1978). In this paper we present a simple method which is based on two formerly known DNA purification steps, i.e. monomolecular reannealing of supercoiled DNA and separation of single stranded and double stranded DNA on hydroxyapatite. This procedure is especially suitable for large-scale purification of bacterial plasmid DNA.

The E. coli strain HB 101 carrying plasmid pBR 313 was obtained from Dr H. W. Boyer. The recombinant plasmid 2/12 carrying a bacterial ribosomal RNA gene inserted in the Bam HI site of pBR 313 was constructed in our laboratory (Kiss et al., 1978). Endonuclease Eco RI and T_4 phage-induced DNA ligase was purified and applied as described earlier (Kiss et al., 1978). Plasmid DNA was prepared from late exponential phase cultures treated with chloramphenicol (170 μg/ml) overnight to amplify plasmid DNA. Cleared lysates were prepared according to Clewell and Helinski with the modification that 0.1 per cent Triton X 100 was used as detergent. In order to remove the contaminating RNA and proteins the cleared lysate was incubated at 37 °C with pancreatic RNase (20 μg/ml, 1 hour) and subsequently with Proteinase K (50 μg/ml, 2 hours). DNA was precipitated with two volumes of cold ethanol in the presence of 2 per cent potassium acetate and allowed to stand at -20 °C for 5–6 hours. The precipitate was collected by centrifugation, dissolved in 0.01 M sodium phosphate buffer,* pH 6.8 and diluted with the same buffer to $OD_{260} = 10$. At this stage of purification fragmented bacterial chromosomal DNA is the only contaminating macromolecular component. Further purification is based on the monomolecular reannealing property of the plasmid DNA in contrast to chromosomal DNA

* *Abbreviation:* SPB, sodium phosphate buffer

(Helinski, Clewell, 1971). The solution was divided into 5 ml portions in test tubes, quickly denatured in a boiling water bath for 3 min and immediately cooled in ice-water. Denatured chromosomal DNA is separated from the rapidly reannealed supercoiled plasmid DNA by hydroxyapatite chromatography. The column was equilibrated at 60 °C with 0.15 M SPB. The cold DNA solution was

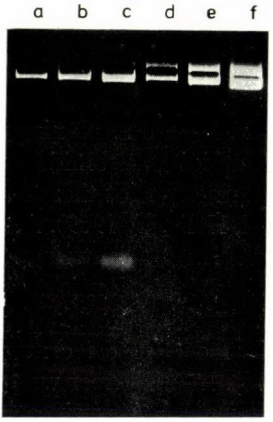

Fig. 1. Purity of plasmid 2/12 prepared on hydroxyapatite. Increasing quantity of cleared lysate (slot a, 0.4 μg; slot b, 0.8 μg; slot c, 1.2 μg) and purified plasmid (slot d, 0.4 μg; slot e, 1.5 μg; slot f, 3 μg) was subjected to electrophoresis in 1 per cent agarose in the presence of 0.5 μg/ml ethidium bromide

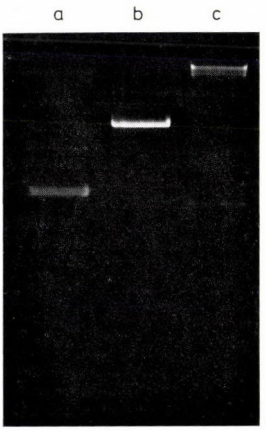

Fig. 2. Integrity of plasmid pBR 313 purified by hydroxyapatite chromatography. pBR 313 DNA was digested with endonuclease Eco RI and religated with T_4 ligase. Slot a, pBR 313 purified on hydroxyapatite; slot b, the same DNA after Eco RI digestion; slot c, the same as in slot b after ligation with T_4 ligase

adjusted with 2 M SPB to a final concentration of 0.15 M and applied on to the column with 4 ml/min flow rate. This high flow rate was necessary to minimize the reassociation of the chromosomal DNA at 60 °C in 0.15 M SPB. Granulated hydroxyapatite with excellent flow properties (Mazin et al., 1974) is especially suitable for this purpose. The column was washed with 0.15 M SPB until the OD_{260} was less than 0.03 in the flow-through. No circular DNA was detected in the flow-through. Double stranded plasmid DNA was eluted with 0.3 M SPB. Peak fractions were pooled, dialysed against 0.01 M NaCl, 0.01 M tris-HCl, pH 7.5, 1 mM EDTA and concentrated by ethanol precipitation. 2 mg plasmid DNA may be purified on a 2×3 cm column.

In order to compare the efficiency of this purification with the conventionally used CsCl-ethidium bromide centrifugation technique 4 l cleared lysate of E. coli

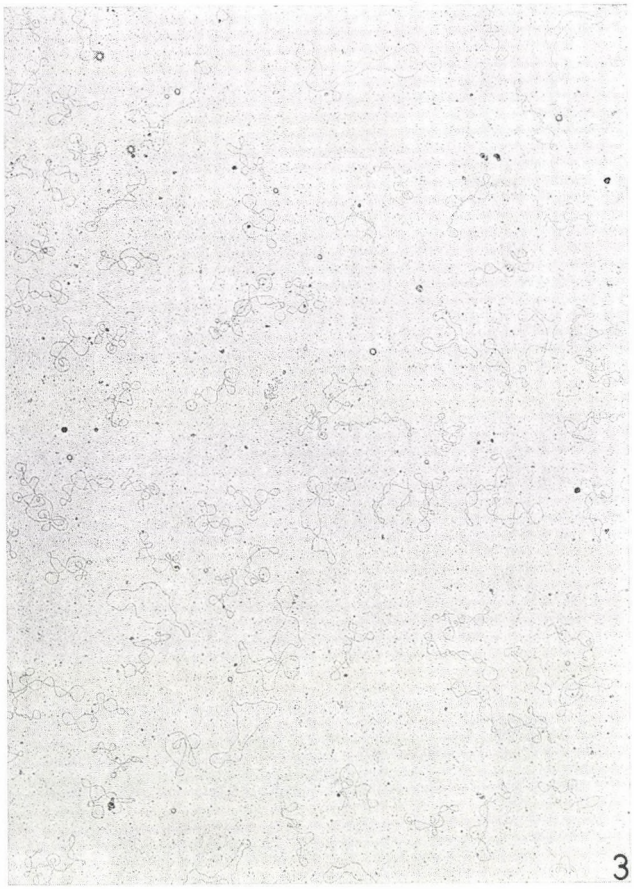

Fig. 3. Purity of plasmid pBR 313 prepared on hydroxyapatite. Electron microscopy was made according to Davis et al. 1971

carrying the plasmid 2/12 (M. W. 10.4×10^6) was divided into two equal parts. One part was purified on hydroxyapatite as described above, the other part on CsCl-ethidium bromide equilibrium gradient. The yield of purification on hydroxyapatite was 61 per cent of the CsCl method. No detectable chromosomal DNA or RNA impurity was found on 1 per cent agarose gel, even after serious overloading (Fig. 1).

The integrity of hydroxyapatite-purified plasmids for molecular cloning experiments was checked by digesting pBR 313 with endonuclease Eco RI and religating the linearized plasmid DNA with T_4 ligase. As shown in Fig. 2 the linear plasmid DNA is quantitatively recircularized.

The purity was further checked by electron microscopy. Not more than 1 linear DNA was seen out of 100 circular molecules of which 80 per cent were found to be supercoiled (Fig. 3).

It is known that the recovery of DNA from hydroxyapatite depends on the molecular weight of DNA fragments. In our case the recovery was 61 per cent for a plasmid of molecular weight 10^7. No attempt was made to determine the upper molecular weight limit of economic purification, but with smaller plasmids the recovery is always better. Thus the procedure described above is especially suitable for large-scale purification of small and medium size plasmids with excellent purity and integrity.

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Myofibrillar Proteins, Enzyme Activity and Fibre Types in Human Skeletal Muscle

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The myofibrillar proteins and relative LC-3 content of myofibrils prepared from five different, left and right muscles of the human forearm were studied by electrophoresis in 10% SDS-polyacrylamide gels followed by densitometry. The specific K-activated ATPase activity of the myofibrillar myosin of the myofibrils was determined and the distribution of the fibre types was analyzed by histochemical methods. The qualitative pattern of the myofibrillar proteins was found to be identical in the different muscles; in the other parameters, however, a variation in a narrow range was observed. The relative LC-3 content, the ATPase activity and the type II fibre content of the right side muscles were always higher than those of the corresponding left side muscles.

Introduction

The myofibrillar proteins of human striated muscles have been the subject of relatively few studies (Samaha, Gergely, 1968; Taylor et al., Romero-Herrera et al., 1976; Sugita, 1976; Bailin, 1977). The light chains of mixed type human muscles have also been only very little studied, therefore very few data are available concerning the classification of human muscles on the basis of the myofibrillar proteins or the light chains of myosin (Bailin, 1976; Sreter et al., 1976). The light chains of myosin offer a sound basis for such a classification, since – as is known from experiments on animal muscles – LC-3 is present in the fast white skeletal muscles while it is absent from the slow red ones (Sarkar et al., 1971). These light chains are present also in human skeletal muscle (Bailin, 1976; Sreter et al., 1976); thus it can be supposed that the amount of LC-3 in the mixed type muscles might be proportional to the quantity of the fast fibres. It is also well-known that the ATPase activity of fast muscles is higher than that of slow muscles (Bárány et al., 1965; Bárány et al., 1967), and therefore the measurement of enzyme activity offers another possibility for the classification of muscles.

The aim of our present work was the investigation of some human muscles by the above methods, completed by the histochemical study of the muscle tissue. In the course of our studies five different muscles of the forearm and also the identical muscles of the left and right forearms were compared.

Abbreviations: LC-1, LC-2 and LC-3, myosin light chain 1, myosin light chain 2 and myosin light chain 3; DTNB, 5-5'-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; S. E., standard error; SDH, succinate dehydrogenase; TCA, trichloroacetic acid; Tris, tris-(hydroxymethyl)aminomethane.

Materials and methods

Muscle Samples. Samples were taken from the forearm muscles of both sides of 9 men and 1 woman deceased in accidents, aged 19–53 years (average age 32 years). The persons studied were all right-handed. Samples were taken only from those persons who did not exhibit any pathological alteration besides those caused by the accident and in whose case samples could be taken within 12 hours after the time of clinical death. After longer times, proteolysis interferes (Vándor, Józsa, 1978). The forearm muscle samples taken from the left and right sides are the following: M. flexor pollicis longus, M. flexor carpi radialis, M. flexor digitorum superficialis, M. extensor pollicis longus, M. extensor digitorum. From part of the muscle samples myofibrils were prepared and another part was subjected to histochemical analysis.

Preparation of myofibrils. Myofibrils were prepared according to Perry (1952) with minor modifications (Vándor, 1977). The protein content of the myofibrils was determined by the biuret reaction (Gornall, 1949).

Measurement of the K-activated ATPase Activity of Myofibrillar Myosin. The reaction was carried out in a medium containing 10 mM EDTA, 600 mM KCl, 2.5 mM ATP and 25 mM Tris-HCl buffer, pH 7.6, at 25 °C. Protein concentration was 1 mg/ml and the final volume of the reaction mixture was 2 ml. The reaction was stopped by the addition of 0.5 ml of 20% TCA. The amount of the liberated inorganic phosphorus was determined according to Lohmann and Jendrassik (1926). Specific activities are expressed as μmoles of P/mg protein/minute.

SDS-polyacrylamide Gel Electrophoresis of Myofibrillar Proteins. After the solubilization of myofibrillar samples (Pinset-Härström, Ehrlich, 1973), gel electrophoresis was carried out in gels containing 10% acrylamide (Weber, Osborn, 1969). To one gel column 70–80 μg of protein were applied. The individual

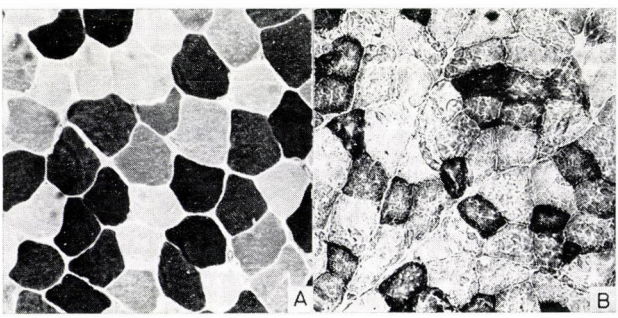

Fig. 1. The type II fibres of the muscle tissue of the forearm give a positive myofibrillar ATPase reaction (pH 9.4) measured according to Padykula and Herman (1955). Picture *A*, dark-coloured cells. Magnification: 100×. M. flexor carpi radialis, right side. The type I fibres of the same muscle give a positive SDH reaction carried out according to Nachlass et al. (1957), picture *B*, dark-coloured cells. ×70

proteins were identified on the basis of their apparent molecular weights calculated from their mobilities, with the help of reference proteins (Weber, Osborn, 1969).

Densitometry. The gels were stained in Coomassie Brilliant Blue and the densitometric curves were recorded in an ERI 65 m type densitometer (Carl Zeiss, Jena) using a 560 nm filter, in transmitted light. Peak areas were determined by gravimetry (Potter, 1974).

Histochemical Studies. For the histochemical classification of skeletal muscles, two basic groups were considered: type I fibres, i.e. slow twitch, oxidative, red fibres and type II fibres, i.e. fast twitch, glycolytic, white fibres. In type I fibres the histochemical SDH reaction is highly positive, while the ATPase reaction carried out after preincubation at pH 9.4 is very weak. In type II fibres the intensities of these two reactions are reversed, as shown in Fig. 1. A series of unfixed slices were made of each muscle sample right after sampling. In these sections the SDH reaction was carried out according to Nachlass et al. (1957) and myofibrillar ATPase (pH 9.4) reaction performed as described by Padykula and Herman (1955). From each muscle sample 5 sections were prepared which were stained by the two above methods separately; then in 300 fibres of each section the number of positive and negative fibres was determined and the results were statistically evaluated. The determination of fibre type by the SDH reaction and that by the ATPase reaction gave identical results in 98.6% of the cases.

Results

The myofibrillar proteins of human muscle, separated on 10% SDS-polyacrylamide gels corresponded to the myofibrillar proteins observed in rabbit muscle on the basis of their mobility, their molecular weight and their position

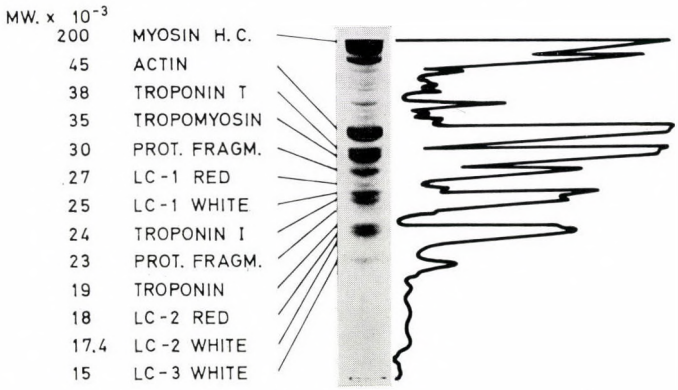

Fig. 2. The myofibrillar proteins of right side M. flexor carpi radialis separated in a gel containing 10% acrylamide and 0.1% SDS and the densitogram of the separated proteins. The figures on the left side of the gels indicate the molecular weight of the proteins X 10. Tank buffer: 0.05 M sodium phosphate buffer, pH 7.1, containing 0.1% SDS. Current applied: 5 mA/tube

in the gel column. On this basis, the following proteins could be identified among the myofibrillar components of every human muscle studied: actin, 45 000 daltons; troponin T, 38 000 daltons; tropomyosin, 35 000 daltons; a breakdown product, 30 000 daltons; red LC-1, 27 000 daltons; white LC-1, 25 000 daltons; troponin I, 24 000 daltons; a breakdown product, 23 000 daltons; troponin C, 19 000 daltons; red LC-2, 18 000 daltons; white LC-2, 17 400 daltons and white LC-3, 15 000 daltons. The myofibrillar proteins of a right side M. flexor carpi radialis, separate on 10% SDS-polyacrylamide gel and the corresponding densitogram are shown in Fig. 2.

As can be seen in Fig. 2, red LC-1, white LC-1 and troponin I, furthermore troponin C, red LC-2 and white LC-2 show very close banding; therefore their separate evaluation could not be carried out appropriately owing to the high degree of overlapping in the densitometric tracing. For this reason, it was not the proportion of the myosin light chains as compared to each other that we used in our calculations; instead – assuming that the actin content of myofibrils is practically constant (Sugita et al., 1973) – we calculated the relative amount of

Table 1

Relative LC-3 content and ATPase activity of the myofibrils of forearm muscles
Actin and LC-3 were quantitated densitometrically after the SDS-polyacrylamide gel electrophoresis of the myofibrillar samples and the staining of the gels by Coomassie Brilliant Blue. The specific K-activated ATPase activity of myofibrillar myosin is expressed as μmoles of P_i/mg protein/minute. Reaction conditions: 10 mM EDTA, 600 mM KCl, 25 ml Tris-HCl buffer, pH 7.6, 2.5 mM ATP, 1 mg/ml protein, 25 °C, in a final volume of 2 ml. The difference between the identical right and left muscles marked □ is significant, $p < 0.05$. The difference between the different muscles of the same side marked x, o and + is significant, $p < 0.05$.
*: the number of cases studied

Muscle sample	Relative LC-3 content ± S. E.		K-activated ATPase activity of myofibrillar myosin ± S. E.	
	right (10)*	left (10)*	right (10)*	left (10)*
Fl. pollicis longus	11.5 ± 0.8□×	9.5 ± 0.5□+	0.352 ± 0.021□	0.297 ± 0.018□
Fl. carpi radialis	10.6 ± 1.0	9.9 ± 0.6°	0.359 ± 0.024	0.321 ± 0.025
Fl. digitorum superficialis	9.8 ± 0.8	8.8 ± 0.7	0.327 ± 0.024	0.309 ± 0.025
Ext. pollicis longus	9.4 ± 0.9	8.7 ± 0.6	0.330 ± 0.021	0.305 ± 0.023
Ext. digitorum communis	9.3 ± 0.5□×	8.0 ± 0.4□°+	0.324 ± 0.018□	0.268 ± 0.015□

Table 2

Percentage of type II fibres in the muscle tissue of the forearm as determined histochemically

The fibre type was determined on the basis of the histochemical SDH reaction (Nachlass et al., 1957) and of the myofibrillar ATPase preincubated at pH 9.4 (Padykula, Herman, 1955). For each muscle, 1500 fibres were counted. The difference between the corresponding left and right muscles is significant in every case, $p < 0.05$. *: the number of cases studied

Muscle sample	Type II fibre % ± S. E.	
	Right (10)*	Left (10)*
Fl. pollicis longus	52.7 ± 1.9	46.8 ± 1.7
Fl. carpi radialis	51.9 ± 1.3	46.1 ± 1.8
Fl. digitorum superficialis	54.1 ± 2.1	45.2 ± 1.3
Ext. pollicis longus	53.5 ± 1.5	44.9 ± 1.9
Ext. digitorum communis	53.2 ± 2.4	45.5 ± 2.0

LC-3 expressed as the percentage of actin, i.e. the so-called relative content of LC−3. The relative LC-3 content of myofibrils prepared from the various muscles is shown in Table 1.

Table 1 shows that the relative LC-3 content of the individual muscles exhibited only a slight variation; at the same time, however, the LC-3 content of the right side muscles was always higher than that of the corresponding left side ones. The K-activated myofibrillar ATPase activities also showed only a slight variation in the five muscles studied, and here too, the activities of the right side muscles were always higher than those of the corresponding left side ones (Table 1).

The results of the histochemical analysis of the forearm muscle tissues are summarized in Table 2.

The data in Table 2 reveal that the fibre composition of the different muscles varied within a very narrow range. On the other hand, we observed that the fibre composition of the identical left and right muscles was different. In the right side muscles, type II fibres predominated while in the left side ones the majority of the fibres belonged to type I. This difference was statistically significant in every case.

The relative LC-3 content, the K-activated myofibrillar ATPase activity and the number of type II fibres of the left side muscles, expressed as a percentage of the same values for the corresponding right side muscles, are summarized in Table 3.

It is seen from Table 3 that the parameters studied of the left side muscles are on the average more than 10% lower than those of the corresponding right side muscles.

Discussion

The protein components of human myofibrils, separated by gel electrophoresis are essentially similar to those identified in rabbit skeletal muscle on the basis of their position in the gel and their molecular weight. Besides these proteins, the presence of two proteolytic products of molecular weights of 30 000 and 23 000 daltons was also demonstrated. The former one of these is probably a breakdown product of troponin T, formed in the course of endogeneous proteolysis (Drabikowsky et al., 1973; Vándor, Józsa, 1978), and the latter one is also produced by way of endogeneous proteolysis (Vándor, Józsa, 1978).

According to the studies of Bailin (1976), human skeletal muscle contains at least three types of myosin light chains, including white LC-3. Sreter et al. (1976) observed that in healthy human skeletal muscle all of the five (three white and two red) myosin light chains are present and that the "faster" is the muscle, the higher is the amount of white LC-3. Thus, at the molecular level the LC-3 content of muscles is characteristic of the proportion of the fast twich, white component. Our observations also support this assumption; the relative LC-3 contents were in accord with the results of the enzymatic and histochemical studies. In the right side muscles of the forearm, the amount of LC-3, the ATPase activity and the number of type II fibres were always higher than in the corresponding left side muscles (see Table 3).

In the case of the persons studied, the higher LC-3 content of the dominant right side muscles is presumably related to the different function of the more "skilful" hand. It is well-known that the function and composition of the muscle fibres is also affected by innervation, which is convincingly supported by cross-reinnervation and electric stimulation experiments (Buller et al., 1960; Sreter et al., 1974; Pette et al., 1976). Therefore it can be supposed that the different innervation of the muscles performing a very complex function may play a role

Table 3

Relative LC-3 content, ATPase activity and type II fibre content of the left side muscles expressed as the percentage of the corresponding right side muscles

Muscle sample	Relative LC-3 content %	ATPase activity %	Type II fibre %
Fl. pollicis longus	82.6	84.4	88.8
Fl. carpi radialis	93.4	89.4	88.8
Fl. digitorum superficialis	89.8	94.5	83.6
Ext. pollicis longus	92.6	92.4	84.0
Ext. digitorum communis	86.0	82.7	85.5
Average value for the five muscles	88.9	88.7	86.1

in bringing about the differences described above. However, the difference in physical strain may also be of importance. Training of long duration may alter the ATPase activity of actomyosin (Baldwin et al., 1975). In the cardiac muscle of rats exposed to prolonged physical strain, the LC-1 : LC-2 ratio was also shifted; in their skeletal muscles, the amount of LC-3 was increased as compared to the control animals, and the ATPase activity of myosin and actomyosin increased (Medugorac, 1975; Yamaguchi et al., 1977). Among the very scattered data on the fibre composition of human muscles, only one was available about a hand muscle, M. abductor pollicis longus, not studied by us (Johnson et al., 1973). The muscles studied by us contained type I and II fibres in a ratio varying between 45 and 55%. Changes in the amount of these two fibre types, parallel to function, were observed in rat soleus (Jaweed et al., 1977): in animals performing intensive work, the quantity of type II fibres increased or decreased according to the animals' being exposed to static or dynamic strain.

To sum up, it seems probable that the cause of the differences between the muscles of the right and left forearms, observed in the relative amount of LC-3, in the ATPase activity and in fibre composition lies in the fact the right hand performs a more complicated, differentiated and physically harder work than the left hand does. At present, however, it cannot be decided whether these differences are genetically determined or rather subsequently induced by the different physical strain.

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Effect of a Polyanion Macromolecule on Mitochondrial DNA Synthesis in Isolated Rat Liver Mitochondria

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The incorporation of (^{3}H) thymidine into isolated rat liver mitochondria in the resence of 0—0.5 mM ATP was stimulated by a polyanion with an average molecular weight of 10 000 daltons (at 50—200 μg/ml concentration) and containing in a proportion of 1 : 2 : 3 methacrylate, maleate and styrene. The above concentrations of the polyanion inhibit the incorporation of (^{3}H) thymidine in the presence of 2 mM ATP and have no substantial effect on (^{14}C)isoleucine incorporation *in vitro*. The mtDNA synthesis stimulatory effect of the polyanion is independent of its inhibitory effect on adenylate translocase. The polyanion does not enhance the nucleotide transport across the inner membrane of the mitochondria and the changes of thymidine phosphorylating activity are not involved in the increased mtDNA synthesis.

It is supposed that the polyanion attached to the inner membrane of the mitochondria alters the binding of mtDNA to the inner membrane of the mitochondria. Thus, the polyanion might be an effective tool in studying the functional significance of mtDNA-membrane association in mtDNA replication.

Introduction

It is known that polyanions with different chemical structures can speciifically bind to biological membranes and thus alter their functions (Ogata, Kondo, 1972; Just et al., 1974; König et al., 1977). It has been shown that depending on their molecular weight and charge distribution polyanions increase DNA synthesis in isolated nuclei (Kraemer, Coffey, 1970). König et al. (1977) have found an inhibitory effect of a synthetic polyanion (a copolymer of methacrylate, maleate, and styrene in a proportion of 1 : 2 : 3 with an average molecular weight of 10 000 daltons on energetic functions of isolated rat liver mitochondria. They have suggested that specific binding of the polyanion to the outer surface of the inner membrane of the mitochondria results in an inhibition of mitochondrial adenylate translocase.

The above findings led us to study the effect of an inner membrane-bound polyanion macromolecule on mtDNA replication. It may be postulated that the polyanion bound to the inner membrane of mitochondria could influence mtDNA synthesis because similarly to prokaryotic systems (Firshein, 1972; Siegel, Schaechter, 1973; Leblanck, Singer, 1974) a functional significance is attributed

Abbreviations: Tricine, N-tris-hydroxymethylglycine; EGTA, ethyleneglycol-bis-(2-aminoethyl)-tetraacetate; dNTP, deoxyribonucleoside triphosphates.

to the membrane association of mtDNA in the regulation of mtDNA replication (Van Tuyle, Kalf, 1972; Attardi et al., 1976).

In the present paper it is shown that the polyanion macromolecule synthesized from the mixture of methacrylate/maleate/styrene in a 1 : 2 : 3 ratio (simply referred to as polyanion in the following), stimulates mtDNA synthesis in isolated rat liver mitochondria at low intra-mitochondrial adenine nucleotide concentrations. This effect is independent of the inhibitory effect of the same polyanion on mitochondrial adenylate translocase.

Part of this work has been published in an abstract form (Szabados et al., 1978).

Materials and methods

In our experiments male Wistar rats weighing 150–180 g were used. Liver mitochondria were prepared by the differential centrifugation technique of Schneider (1948) under semisterile conditions in a medium containing 0.25 M sucrose, 1.0 mM EGTA and 5.0 mM Tris-HCl buffer (pH 7.4) and washed three times with the same medium. For testing the functional integrity of the mitochondrial preparations, the value of respiratory control ratio was estimated from the O_2 uptake according to Chance and Williams (1956) using a Clark-type oxygen electrode. In studies of thymidine incorporation the respiratory control index in the presence of glutamate-malate was higher than six.

Bacterial contamination of the incubation mixture was negligible.

Assay of thymidine incorporation

The standard incorporation medium contained, except where otherwise stated, in a final volume of 0.5 ml: 4 mM KCl, 30 mM Tricine-HCl (pH 7.4), 5 mM $MgCl_2$, 10 mM KH_2PO_4, 2 mM ATP, 2 mM malate, 10 mM glutamate, 100 mM sucrose, 200 U penicillin and 30 μM dATP, dGTP, dCTP in the presence of 0.25 μCi (6-^{3}H) thymidine (specific activity: 18.0 Ci/mmol). The mitochondrial suspension containing 1–2 mg protein was incubated in the above reaction mixture at 30 °C for 60 min in a metabolic shaker. The reaction was stopped by adding 0.5 ml of ice-cold trichloroacetic acid to the reaction mixture. The precipitate was collected on a Sartorius membranefilter, washed and dissolved in 0.4 ml of formic acid. Two ml of absolute ethanol and 10 ml of toluene based scincillation fluid were subsequently added to each vial and radioactivity was measured in a Beckman LS 255 liquid scintillation counter. Counting efficiency was determined with an external standard by the channels ratio method. Mitochondria, precipitated with trichloroacetic acid and incubated under the same conditions served as control in the thymidine incorporation assay. The difference in radioactivity between the duplicate or triplicate determinations was less than 10%. Data shown in the tables and figures represent the average values of a representative experiment.

Assay of thymidine-phosphorylating activity

Isolated mitochondria were incubated in 100 μl standard incorporation mixture under the conditions employed for thymidine incorporation. As radioactive marker 1 μCi/ml (2-^{14}C) thymidine (specific activity 43 mCi/mmole) was used. Incubation was followed by spotting 40 μl of the reaction mixture on Whatman DE 81 paper, which was washed with 1 mM ammonium formate (pH 7.4) then water and ethanol. Radioactivity was assayed in toluene based scintillation liquid without solubilization.

Assay of amino acid incorporation

The reaction mixture contained 30 mM Tricine-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 10 mM KH_2PO_4, 2 mM malate, 10 mM glutamate, 100 mM sucrose, 200 U penicillin and 0.25 μCi (U-^{14}C) isoleucine (specific activity 185 mCi/mmol). Other experimental conditions were the same as in the thymidine incorporation assay.

Protein was determined by a modified method of Lowry described by Schacterle, Pollack (1973) with bovine serum albumin as standard. The polyanion was synthesized from 0.25 mol methacrylic acid, 0.5 mol maleic acid anhydride and 0.75 mol styrene according to Völker (1962). The average molecular weight of the polyanion determined with a Knauer electronic osmometer was found 10 000 dalton. One hundred μg of polyanion is equivalent to 0.83 μequivalent carboxyl group.

In our experiments commercially available compounds of analytical grade were used. Deoxyribonucleoside triphosphates were products of Boehringer (Mannheim, FRG). Tricine and atractyloside came from Calbiochem (USA), EGTA was obtained from Fluka (Switzerland), (6-^{3}H) thymidine, (2-^{14}C) thymidine and (U-^{14}C) isoleucine were purchased from UVVVR (Czechoslovakia). Other chemicals were the products of Reanal (Budapest).

Results

The rate of (^{3}H) thymidine incorporation into isolated mitochondria is markedly influenced by the presence of ATP. The incorporation of (^{3}H) thymidine was about 60% lower in the absence of ATP as compared to that in the presence of 2 mM ATP, found to be the optimal ATP concentration for thymidine incorporation (Figs. 1, 2). An even more pronounced effect in the absence of ATP was observed by Mitra, Bernstein (1970) and Koike, Kobayashi (1973).

Antimycin and 2,4-dinitrophenol caused a marked inhibition of (^{3}H) thymidine incorporation (Table 1). Incorporation of thymidine into mitochondria was very sensitive to ethidium bromide. The radioactivity of acid-insoluble product was diminished in a concentration dependent manner by unlabelled dTTP

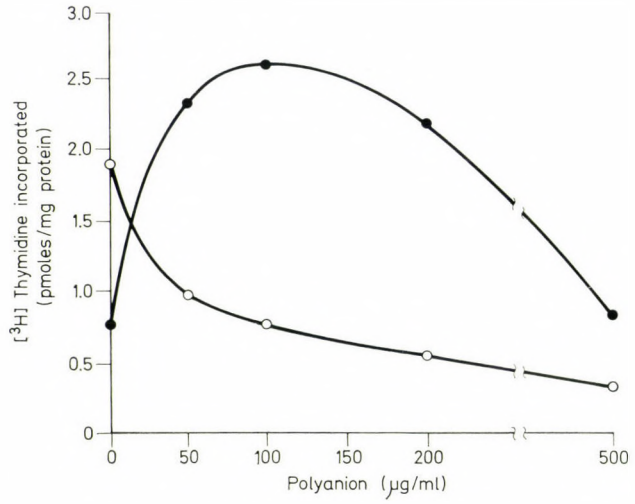

Fig. 1. Polyanion concentration dependence of (^{3}H) thymidine incorporation. Mitochondrial suspension containing 1.6 mg protein was incubated under the conditions described in Materials and methods. Radioactivity of acide-precipitable products after incubation of mitochondria ●—●—● in the absence and ○—○—○ in the presence of 2 mM ATP

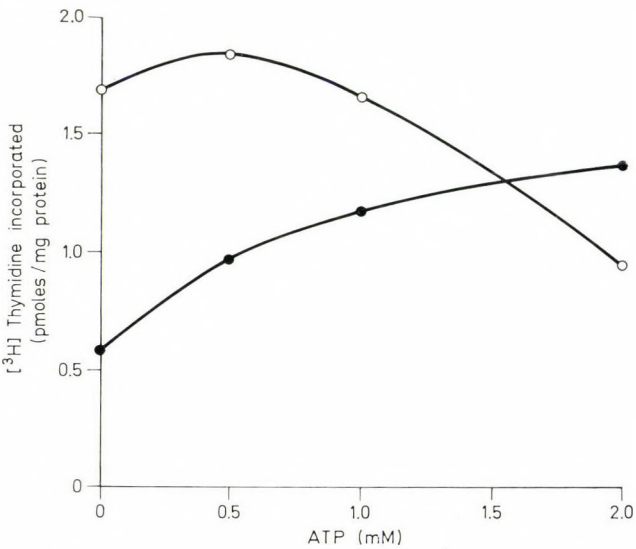

Fig. 2. Effect of ATP on mtDNA synthesis in the presence of the polyanion. Incubation of mitochondria (1.8 mg protein) was performed under the conditions described in Materials and methods in the presence of 50 μg/ml polyanion (○—○—○) or its absence (●—●—●). Radioactivity was measured in the acid-insoluble material

(Table 1). The above results, in accordance with the findings of others (Mitra, Bernstein, 1970; Koike, Kobayashi, 1973; Borst, 1972; Gause et al., 1973), suggest that under our experimental conditions the observed incorporation of (^{3}H) thymidine is attributed to mtDNA replication.

The rate of thymidine incorporation is markedly influenced by not only the presence or absence of dTTP but also by the absence of the other unlabeled deoxyribonucleoside triphosphates (Table 2). This observation indicates that the

Table 1

Effect of inhibitors and dTTP on (^{3}H) thymidine incorporation into isolated rat liver mitochondria

Incubation was carried out under standard experimental conditions (see Materials and and methods). The reaction mixture contained 1.6 mg mitochondrial protein

Additions	(^{3}H) thymidine incorporated into acid-insoluble product pmoles/mg mt. protein/h	Inhibition %
—	1.57	—
Antimycin A (0.05 μg/mg mt. prot.)	0.13	92
2,4-dinitrophenol (5×10^{-5} M)	0.17	89
Ethidium bromide (10 μg/ml)	0.08	95
dTTP (2 μM)	0.88	44
dTTP (4 μM)	0.47	70
dTTP (10 μM)	0.21	87
dTTP (20 μM)	0.09	94

Table 2

Effect of deoxyribonucleotides on (^{3}H) thymidine incorporation into mitochondria

Incubation was carried out under standard experimental conditions (see Materials and methods). The reaction mixture contained 1.8 mg mitochondrial protein

Omission of dTNPs	(^{3}H) thymidine incorporated into acid-insoluble product pmoles/mg mt. protein/h
none	1.31
dCTP	1.12
dATP	0.70
dGTP	0.30
dATP, dGTP	0.22
dCTP, dGTP	0.32
dATP, dCTP	0.67

low deoxyribonucleotide pool (Borst, Kroon, 1969) of the mitochondria fails to ensure optimal conditions for thymidine incorporation *in vitro*. It seems that the concentration of deoxyguanosine nucleotide is the rate limiting factor of (^{3}H) thymidine incorporation into isolated rat liver mitochondria and it is hardly influenced by the presence or absence of dCTP.

The rate of (^{3}H) thymidine incorporation in the presence of the polyanion was linear during the 1 hour incubation and it was also proportional with the protein content of the reaction mixture within the range of 1 – 6 mg mitochondrial protein/ml (unpublished data). The incorporation of thymidine into DNA depending on the availability of ATP was influenced by the polyanion. In the absence of ATP 50 – 200 μg/ml polyanion caused an at least two to three-fold increase in thymidine incorporation (Fig. 1). Under these experimental conditions the extent of thymidine incorporation was higher than that in the presence of 2 mM ATP without the polyanion (Figs. 1, 2). The mtDNA synthesis was stimulated by the polyanion in the presence of up to about 1 mM ATP. In the presence of 2 mM ATP (^{3}H) thymidine incorporation was diminished by 50 – 500 μg/ml polyanion in a concentration dependent manner (Fig. 1).

In Fig. 3 the effect of the polyanion on mitochondrial protein synthesis and on (^{3}H) thymidine incorporation is compared. The polyanion, contrary to its effect on mtDNA synthesis, is substantially ineffective on (^{14}C) isoleucine incorporation in the presence of 2 mM ATP. In the absence of ATP a very low level of protein synthesis was observed and the activity was not influenced by the polyanion.

Kőnig et al. (1977) have established that the polyanion inhibits adenylate carrier of the mithochondria, thus, an interaction may be postulated between the

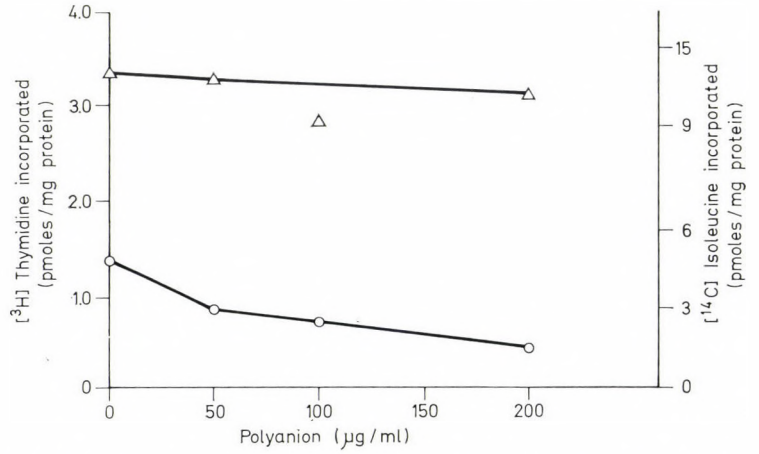

Fig. 3. Effect of the polyanion on mitochondrial amino acid incorporation. The incorporation mixture contained 1.7 mg mitochondrial protein. Conditions of incubation are described in Materials and methods. Incorporation of (^{14}C) isoleucine, △—△—△; and (^{3}H)thymidine, ○—○—○ into acid-precipitable product

inhibition of adenylate carrier and alteration of mtDNA synthesis under the influence of the polyanion. To test this assumption the effect on mitochondrial thymidine incorporation of a known adenine nucleotide carrier inhibitor, atractyloside (Klingenberg, Pfaff, 1966; Vignais, 1976), was investigated.

The effect of atractyloside and that of the polyanion on thymidine incorporation are different. The rate of incorporation is unaffected by 7.5 – 75 μM atractyloside in the absence of ATP (Table 3). In the presence of 0.5 – 1.5 mM ATP thymidine incorporation was dependent on the atractyloside/ATP ratio: at low ATP concentration even as low as 7.5 μM atractyloside, whereas at higher one, only 75 μM atractyloside exerted a marked inhibitory effect. No inhibition of thymidine incorporation was observed by as high as 75 μM atractyloside concentration in the presence of 2 mM ATP. The results obtained may be explained by the known competitive inhibitory effect of atractyloside on the adenine nucleotide carrier (Vignais, 1976; Pfaff, Klingenberg, 1968). The difference between the effects of atractyloside and polyanion on (^{3}H) thymidine incorporation means that the influence of the polyanion on mtDNA synthesis cannot be attributed to its adenylate carrier inhibition.

In Figure 4 the effect of the polyanion on mitochondrial thymidine phosphorylating activity is demonstrated. In a concentration range of 50 – 200 μg/ml the polyanion has a strong inhibitory effect depending on the ATP concentration. In the absence of ATP it only slightly alters thymidine phosphorylation.

Discussion

Our experimental results, the deoxyribonucleoside triphosphate dependence of (^{3}H) thymidine incorporation (Table 2), the effect of inhibitory agents (Table 1) and the radioactivity of mtDNA *in vitro* labeled with (^{3}H) thymidine and isolated on neutral sucrose gradient (unpublished experiments) in agreement with previous reports (Mitra, Bernstein, 1970; Koike, Kobayashi, 1973; Borst, 1972; Gause

Table 3

Effect of actractyloside on (^{3}H) thymidine incorporation at different ATP concentrations
Incubation was carried out under standard experimental conditions (see Materials and methods). The reaction mixture contained 1.8 mg mitochondrial protein

Added ATP	(^{3}H) thymidine incorporation pmoles/mg mt. protein/h			
	in the absence of atractyloside	in the presence of 7.5 μM atractyloside	in the presence of 15 μM atractyloside	in the presence of 75 μM atractyloside
none	0.45	0.44	0.42	0.47
0.5	1.35	0.78	0.45	0.45
1.0	1.42	1.09	1.08	0.87
1.5	1.42	1.61	1.14	0.89
2.0	1.43	1.63	1.56	1.40

et al., 1973; Borst, Kroon, 1969) indicate that incorporation of (^{3}H) thymidine into isolated mitochondria reflects specific mtDNA synthesis. These results suggest that the increased radioactivity detectable in the acid-insoluble fraction in the presence of the polyanion corresponds to the enhancement of DNA replication in isolated mitochondria (Figs 1, 2). In the presence of the polyanion at low intramitochondrial adenine nucleotide concentration the intensity of mtDNA synthesis is considerably higher than that in the absence of the polyanion even at 2 mM ATP concentration, optimal for thymidine incorporation.

Enhanced mtDNA synthesis in isolated liver mitochondria upon changes in physiological conditions or treatment of the animals has been reported by several authors, in agreement with our finding. In mitochondria isolated from the liver of new-born rats mtDNA synthesis is higher than in those from adults (Koike, Kobayashi, 1973). Albring et al. (1973) have demonstrated an increased DNA synthesis in the liver mitochondria isolated from cuprizon treated mice. A high rated mtDNA synthesis was observed by D'Agostino et al. (1975) in rat liver mitochondria under the influence of a protein isolated from regenerating liver cytoplasm. These mechanisms of stimulation of mtDNA synthesis are not fully understood.

It is unlikely that the adenylate carrier inhibitory effect of the polyanion (König et al., 1977) is involved in the stimulation of mtDNA synthesis. First, we failed to demonstrate a similar mtDNA synthesis stimulating effect demonstrable by the polyanion with atractyloside which specifically inhibits adenylate translocase (Vignais, 1976). In addition, contrary to the effect of the polyanion, the inhibition of thymidine incorporation by atractyloside can competitively be released by ATP (Table 3).

(^{3}H) thymidine incorporation can be stimulated in isolated mitochondria by addition of dNTPs (Table 2). It is improbable, however, that in the presence of the polyanion there is an increased dNTP transport across the inner membrane of the mitochondria. First of all, according to König et al. (1977) it is the polyanion that inhibits the adenylate carrier which is responsible for transport of dATP as well (Mitra, Bernstein, 1970). Secondly, elevated concentrations of dNTPs in the incubation mixture do not result in increased thymidine incorporation (data not shown).

The rate of (^{3}H) thymidine incorporation may also be limited by the thymidine phosphorylating activity of mitochondria. Thus, the possibility can also be considered that the changes in thymidine phosphorylating activity are responsible for the increased mtDNA synthesis. The observation, however, that in the absence of ATP the polyanion at 50–200 μg/ml concentration does not affect thymidine phosphorylation in isolated mitochondria (Fig. 4) contradicts this assumption.

It is difficult to explain the inhibition of mtDNA synthesis by the polyanion in the presence of 2 mM ATP. The observation that both thymidine incorporation and thymidine phosphorylating activity of mitochondria were diminished in the above circumstances may indicate a polyanion-caused inhibition of thymidine

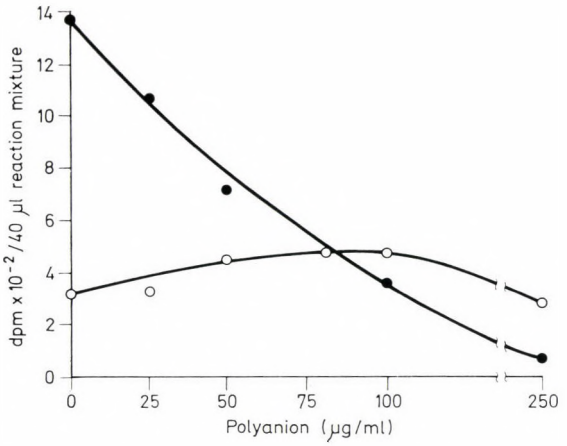

Fig. 4. Thymidine phosphorylating activity of mitochondria in the presence of the polyanion. Standard incorporation mixtures containing 0.35 mg mitochondrial protein were incubated under the conditions described in Materials and methods in the presence of ATP (2 mM) ●—●—●, or its absence ○—○—○. Radioactivity of 40 µl aliquots of the incubation mixture washed on Whatman DE 81 paper was measured

phosphorylation, however, that in the presence of the polyanion and of 2 mM ATP the thymidine phosphorylating activity was considerably higher than in a system containing only polyanion, and that in spite of the lower thymidine phosphorylating activity an increased mtDNA replication could be detected under the influence of the polyanion in the absence of ATP, are against this assumption.

All the above findings indicate that the increased mtDNA synthesis is not due to changes in some respiratory parameters of the mitochondria resulting from the adenylate carrier inhibitory effect of the polyanion. It seems to be unlikely that either the alteration of intra-mitochondrial deoxyribonucleotide concentration or thymidine phosphorylating activity may play a role in the increased mitochondrial DNA synthesis.

Since the half-life of rat liver mtDNA is approximately 8 days (Neubert et al., 1968) and the time of mtDNA replication is 120 min (Shearman, Kalf, 1977), it is obvious that only a very low percentage of the DNA content of the mitochondrial preparation actually takes part in the replication process. In the course of replication, mtDNA is attached to the inner membrane of the mitochondria at or near the site of initiation of the replication (Attardi et al., 1976). Similarly to prokaryotic systems a regulatory role is attributed to DNA-membrane association (Firshein, 1972; Siegel, Schaechter, 1973; Leblanck, Singer, 1974) in mitochondrial genome synthesis (Attardi et al., 1976). On the basis of these observations the most likely explanation of the mtDNA stimulatory effect of the polyanion is that it causes alteration in the mtDNA-membrane association leading to an increased mtDNA synthesis.

Thus, the polyanion may serve as an effective tool for studying the role of mtDNA-membrane association in mtDNA replication.

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Binding of Exogenous DNA by Human Lymphocytes and by their Isolated Plasma Membranes

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The binding of labeled bacterial DNA to human tonsil lymphocytes and to plasma membranes isolated from these cells involved macromolecules mainly located at the cell surface, since plasma membrane preparations showed properties in common with those of viable cells. DNA binding to lymphocytes was a dissociable, saturable, time, temperature, pH and concentration dependent process. A double reciprocal plot of the data obtained from DNA saturation curves gave an apparent dissociation constant of about 4×10^{-10} M. The process of interaction of 3H-DNA with lymphocyte membranes gave a complex Scatchard plot, whose first slope yielded an apparent dissociation constant similar to that obtained for DNA-cell interaction.

Binding was shown to be specific for DNA because RNA did not displace the bound DNA. Double stranded DNA was preferentially bound relative to single stranded DNA. Temperatures greater than 55 °C abolished the binding capacity. Results of experiments in which pH and salt concentration were varied point to the importance of conformational changes of the nucleic acid in the binding process.

Introduction

Genetic transformation in bacteria mediated by somatic nucleic acid was described as early as 1944 (Avery et al., 1944). Interaction of foreign DNA with mammalian cells has been and still is the subject of intensive studies (Bhargava, Shanmugam 1971), biological effects, however, could be obtained only with DNA of viral origin. The low frequency of DNA integration into the chromosomes of the recipient cell could be explained by the variety and complexity of the events involved. Interaction between foreign DNA and the cells begins with the "binding" process. It has been shown in bacteria that competent and incompetent cells contained DNA binding sites (Seto et al., 1975), and in mammalian cells, most of the DNA taken up by the cells surface during the early phase (Ehrlich et al., 1976, Farber et al., 1975). Therefore, binding factors and their properties may be decisive for the fate of foreign DNA. Receptors, specific for different macromolecules, such as mitogens, anti-immunoglobulins, insulin, glucagon, have been found (Cuatrecasas, 1974). The kinetics of receptor macromolecule complex formation, as well as the number of receptors and dissociation constants, have been determined in several cases.

Abbreviations: MEM, minimal essential medium; PBS, phosphate buffered saline.

If binding of DNA is a fixed and specific property of the cell surface, the same parameters should characterize both cell-DNA and membrane-DNA associations, and the kinetic parameters used to characterize hormone-receptor interactions should be applicable to cell-DNA and membrane-DNA interactions as well. In the present study subcellular fractions were used for the investigation of DNA binding because in this relatively simple biological system interference by cellular functions other than binding can be avoided. The nearly identical nature of the interaction of foreign DNA with intact cells and with plasma membrane preparations indicated that the latter probably possess all the components of the biologically significant DNA binding sites.

Materials and methods

Human tonsil lymphocyte culture

Human lymphocytes obtained from tonsils surgically removed were prepared as described elsewhere (Ohlbaum et al., 1977). Viability of the cells was monitored by tripan blue dye exclusion.

Preparation of membranes

Preparation of membranes from lymphocytes was carried out according to Demus (1973). Fractions denominated as light plasma membrane and heavy plasma membrane, isolated from microsomes, were used. These preparations show a 10 times higher specific 5' nucleotidase activity than cell homogenates and it is free of cytoplasmic DNA dependent DNA polymerase activity. Protein was determined according to Lowry et al. (1951), with bovine serum albumin as standard.

Preparation of DNA and RNA from B. cereus

(^{3}H) labeled and nonlabeled DNA was prepared from *B. cereus* as described earlier (Ohlbaum et al., 1977). The molecular weight of isolated DNA was about 10^7 daltons as determined by ultracentrifugation analysis (Burgi, Hersey, 1963). The specific activity was 4 – 5000 cpm/nmol and the acid soluble fraction present was below 0,5%.

RNA from the same bacteria was prepared by a conventional phenol – detergent technique.

Assay of DNA-cell association

10^7 cells were freshly suspended in 0.5 ml of MEM or in 0.1 M acetate buffer, pH 4.5, containing 0.15 M NaCl, incubated at 37 °C with various concentrations of labeled DNA. DNase activity was inhibited by 10^{-2} M Na_2SO_4

(Bernardi, 1968). The reaction was stopped by dilution with 1 ml of cold MEM containing 500 μg of nonlabeled DNA and was centrifuged at 1000 $\times$ g for 10 min in the cold. The cells were resuspended in 0.5 ml of MEM and put onto Whatman GF/C filters, dried and digested in counting vials with 0.5 ml of Soluene 100 (Packard). Radioactivity was measured in a Beckman LS 355 scintillation counter in 10 ml toluene based scintillation fluid. All the samples were done in duplicate or triplicate and parallel samples were made in the presence of 500 μg of nonlabeled DNA to determine and correct for nonspecifically bound nucleic acid.

Assay of DNA-membrane binding

0.05 ml aliquots of membrane preparations at the indicated concentrations were incubated in 0.1 ml of PBS or 0.1 ml of 0.1 M acetate buffer, pH 4.5, containing 0.01 M $MgCl_2$ with 0.05 ml of labeled, *B. cereus* DNA at different concentrations. After incubation the samples were collected by filtration onto Whatman GF/C filters and washed under vacuum with 10 ml of PBS at room temperature. Control experiments showed that no more than 1% of the free labeled DNA was retained by the filters provided the filters had been wetted with PBS. Filtration and washing took no more than 30 sec. Radioactivity was measured similarly as with the cell samples. Each binding determination was performed in duplicate and for each one parallel samples were made in the presence of excess nonlabeled DNA (500 μg) to determine and correct for DNA bound nonspecifically to the membrane preparation.

Assay of DNase activity

To 0.05 ml sample 0.01 ml of 0.1 M acetate buffer, pH 4.5, and 0.05 ml labeled DNA were added and the mixture was incubated at 37 °C. The nucleolytic activity was stopped by addition of 0.11 ml cold 1 M perchloric acid. Acid soluble radioactivity was measured after centrifugation in a suitable amount of supernatant.

Results

(^3H) DNA binding as a function of concentration

The specific binding of foreign DNA to lymphocytes and membrane preparations with respect to DNA concentration is show in Fig. 1. Complex formation seems to be a reversible process and follows a regular enzyme saturation curve. In those enzymatic catalized reactions in which the rate constant of the product formation is negligible, $K_m \sim K_s$ (K_s is the substrate constant). The data of saturation experiments with cells (Fig. 1a) give an apparent dissociation constant of 4×10^{-10} M, when the specific binding was plotted in a double reciprocal form. The specific DNA binding of membrane preparation was also determined (Fig. 1b).

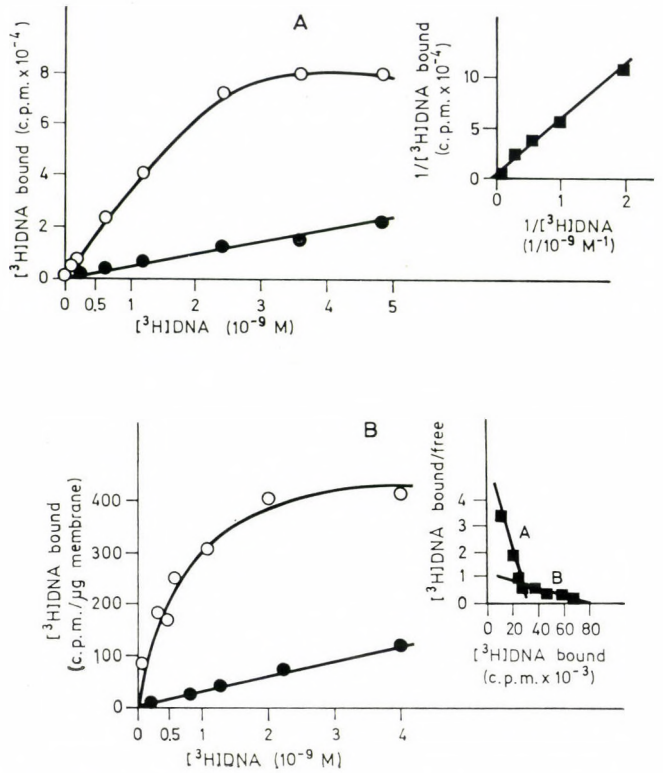

Fig. 1. Influence of the concentration of (^{3}H) DNA on the specific binding of DNA to lymphocytes and membrane preparations. (1a) 10^7 cells, suspended in 0.5 ml of MEM were incubated with 0.05 ml of *B. cereus* (^{3}H) DNA at the indicated concentrations for 10 min at 37 °C and processed as described in Materials and methods. For each DNA concentration tested, control incubation was performed in the presence of displacing amounts (500 μg) of nonlabeled DNA. Specific binding means the difference between the total amount of bound DNA and the amount of DNA bound non-specifically. ○——○ specific binding; ●——● nonspecific binding. Insert: Double reciprocal plot of the rate of DNA binding as a function of DNA concentration. (1b) 15 μg of a membrane fraction were suspended in 0.05 ml of 0.01 M Tris-HCl buffer, pH 7.4, and added to 0.1 ml of PBS and 0.05 ml of (^{3}H) DNA at increasing concentrations. After 10 min of incubation the complex was collected as described in Materials and methods. ○——○, specific binding; ●——●, nonspecific binding. Insert: Scatchard plot analysis. The amount of labelled DNA was plotted against the bound free ratio

When this data was subjected to Scatchard plot analysis (Inserted figure) (Scatchard 1949) a two orders of binding site were obtained. From the first (A) a dissociation constant of about 3.6^{-10} M, in agreement with data found for cells, was calculated. The second (B) slope probably represents the low order site of the DNA binding.

Temperature and time dependence of the binding of double stranded DNA

Fig. 2 illustrates both the time and temperature dependences of the association of DNA with cells and membrane preparations. Complex formations was a very fast event, reaching an equilibrium at 37 °C in 5 min for membranes and in 10 min for cells. The amount of DNA specifically bound to the lymphocytes and to the membrane was higher at 37 °C than at 0 °C throughout the observation period. The high and the heavy membrane preparations behaved in the same way.

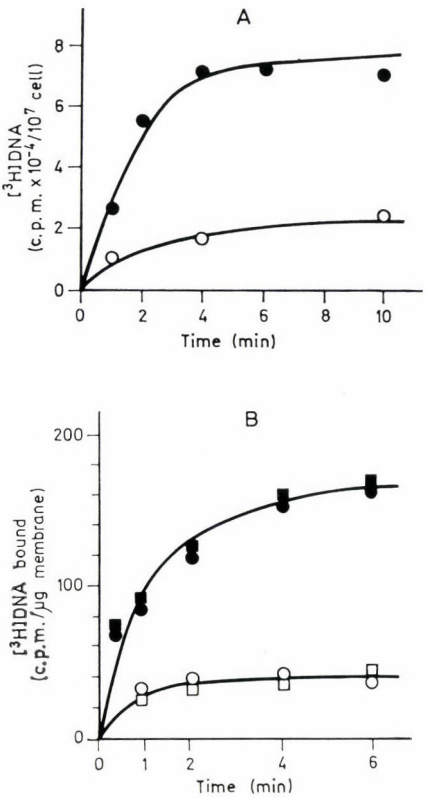

Fig. 2. Time and temperature dependence of DNA binding to lymphocytes and membrane preparations. (*A*) 10^7 cells suspended in 0.5 ml MEM were incubated with 14 μg of labelled DNA. After incubation for the times indicated at 0 °C or 37 °C the samples were diluted with 1 ml MEM containing 500 μg of nonlabelled DNA and processed as described in Materials and methods. Controls were made to obtain the nonspecific binding. o——o, DNA bound at 37 °C; ●——●, DNA bound at 0 °C. (*B*) 25 μg of light (o) or heavy (□) membrane preparation in 0.05 ml of 0.01 M Tris-HCl buffer, pH 7.4, were incubated with 1.2×10^{-9} M (^{3}H) DNA in 0.1 ml of PBS at 0 °C. At the times indicated the complex was collected as described in Materials and methods. ●——●, incubation at 37 °C; o——o, incubation at 0° C

Specificity of binding of double stranded DNA

Evidence for the specificity of the intreaction of DNA with cells and membranes was to obtained by showing that RNA molecules did not compete with DNA for binding sites even when present in a 50 to 1000 fold excess (Table 1).

The specificity for double stranded DNA was examined with both light and heavy membrane preparations. Twice as much native DNA was found to bind to the membrane preparations than heat denatured DNA.

Dissociation of the membrane-DNA complex

A characteristic property of the protein-nucleic acid interaction is its dependence on pH and ion concentration (Printz, Hippel, 1968). In order to investigate the effect of monovalent ions on the dissociation process, the binding of (^{3}H) DNA to membrane was allowed to reach steady state in PBS and then dissociation in PBS was followed in the absence or presence of NaCl at various concentrations. The membrane-DNA complex was readily dissociated by raising the ionic strength of the incubation medium. Fig. 3 shows that upon increasing the concentration of NaCl, the amount of the complex decreased concomitantly.

The non-linearity of the Scatchard plot suggests a negative cooperative event (De Meyts et al., 1976). Fig. 4 shows that different dissociation rates were obtained depending on whether the measurement was carried out in PBS alone or in PBS with 500 μg of nonlabeled DNA present.

Table 1

Effect of RNA on DNA-cell association

10^7 cells suspended in 0.5 ml MEM or 20 μg of membrane in 0.05 ml of 0.01 M Tris-HCl buffer, pH 7.4, plus 0.1 ml PBS were incubated for 10 min with the indicated amounts of labelled DNA in the presence or absence of 500 μg RNA. Radioactivities were measured as described in Materials and methods

(^{3}H) DNA added		(^{3}H) DNA bound c.p.m. $\times 10^{-3}$			
		cell		membrane	
μg	c.p.m. $\times 10^{-3}$	−RNA	+RNA	−RNA	+RNA
10	700	142	143	—	—
5	350	110	91	33	31
2.5	175	—	—	28	32
2	140	62	60	—	—
1.2	80	—	—	21	26
0.6	40	—	—	10	12
0.5	35	30	27	—	—

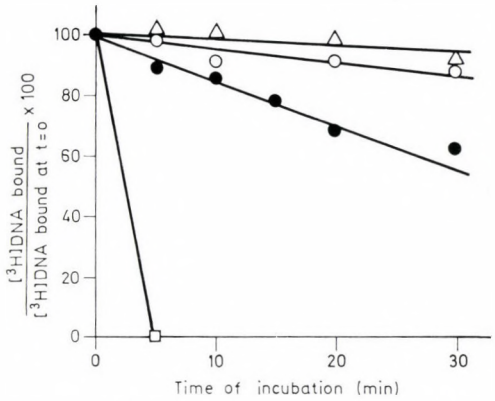

Fig. 3. Dissociation rate of the membrane-DNA complex. Effect of NaCl. 20 μg of a membrane preparation in 0.05 ml of 0.01 M Tris-HCl, pH 7.4, were added to 0.1 ml of PBS and 0.05 ml of 4×10^{-9} M (^{3}H) DNA. After 10 min of incubation at 37 °C the mixture was diluted with either 0.5 ml PBS or 0.5 ml PBS containing NaCl at increasing concentrations. Samples were taken at the times indicated. Filtration was carried out as described in Materials and methods. Dissociation in the presence of △——△, 0.05 M NaCl; ○——○, 0.15 M NaCl; ●——●, 0.3 M NaCl; and □——□, 0.6 M NaCl

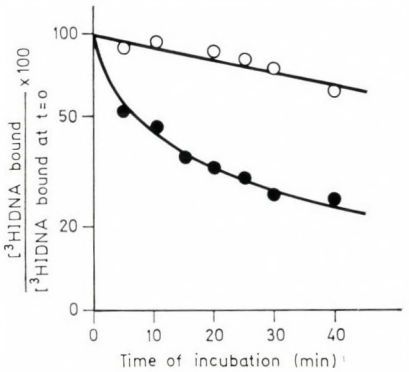

Fig. 4. Dissociation rate of the membrane-DNA complex. Effect of nonlabelled DNA 20 μg of a membrane preparation in 0.05 ml of 0.01 M Tris HCl, pH 7.4, were added to a mixture containing 0.1 ml of PBS and 0.05 ml of 4×10^{-9} M (^{3}H) DNA. After 10 min of incubation at 37 °C the mixture was diluted with either 0.5 ml of PBS or PBS containing nonlabelled DNA. Samples were taken at the times indicated. Filtration was carried out as described in Material and methods. Dissociation in the presence of ○——○, PBS; ●——● PBS with 500 μg of nonlabelled DNA

pH dependence of the membrane-DNA complex formation

Fig. 5 shows that maximal binding occurred in the lower pH region tested, and fell off rapidly below pH 5. This holds for both specific and nonspecific binding.

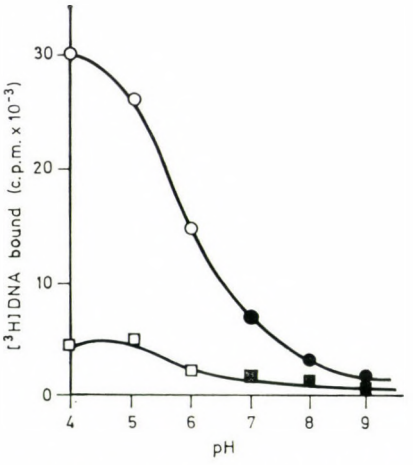

Fig. 5. pH dependence of (^{3}H) DNA binding to lymphocyte membranes. 20 μg of a membrane preparation in 0.05 ml of 0.01 M Tris-HCl, pH 7.4, was added to a mixture containing 0.1 ml buffer and 0.05 ml of 4×10^{-9} (^{3}H) DNA and incubated for 10 min at 37 °C. The incubation was performed at the pH indicated in either 0.1 M ○——○ acetate or ●——●, 0.1 M Tris-HCl. The circles and the squares represent specific and the nonspecific binding, respectively

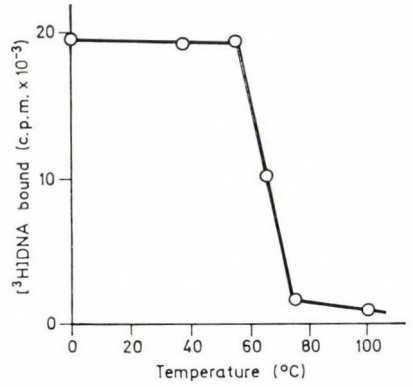

Fig. 6. DNA binding activities of membrane preparations pretreated at temperatures between 0 °C and 100 °C. 22 μg of a membrane preparation, in 0.05 ml of 0.01 M Tris-HCl buffer, pH 4.5, were incubated in 0.1 ml of PBS for 10 min at the temperatures indicated and chilled. DNA binding capacity with 2×10^{-9} M labelled DNA was measured as described in Materials and methods

Heat sensitivity of membrane-DNA complex formation

The effect of temperature on the capacity membrane preparations to bind foreign DNA was measured in PBS. Fig. 6 shows that the DNA binding remained unaltered with membranes pretreated up to 55 °C for 10 minutes but higher temperatures destroyed the membrane binding capacity.

DNA binding and DNase activities

The binding capacity of the lymphocytes and the membrane preparations was estimated both at acidic pH values under conditions when the acid DNase can work optimally, and in the presence of 10^{-2} M Na_2SO_4 which inhibits the hydrolytic activity of this enzyme (Figs. 7a and 7b). It can be seen that the amount of radioactive DNA bound in the presence of Na_2SO_4 was higher, suggesting an

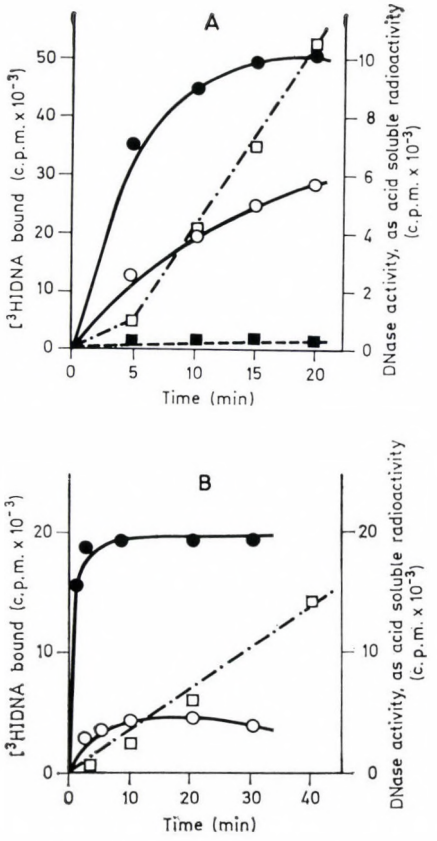

Fig. 7. Influence of pH and Na_2SO_4 on the binding of DNA by lymphocytes and lymphocyte membrane preparations and/or DNase activities. (*A*) Incubation at 37 °C was performed with 10^7 cells suspended in 0.5 ml of isotonic acetate buffer, pH 4.5, containing 1×10^{-9} M labelled DNA. Binding and nucleolytic activities were measured as described in Materials and methods. ●——●, binding in the presence of Na_2SO_4; ○——○, binding in the absence of Na_2SO_4; □——□, DNase activity in the total mixture; ■——■, DNase activity in the supernatant. (*B*) 20 mg of a membrane preparation in 0.05 ml of 0.01 M Tris-HCl, pH 7.4, were incubated in 0.1 ml of 0.1 M acetate buffer, pH 4.5, containing 1×10^{-9} M labelled DNA. Binding and nucleolytic activities were measured as described in Materials and methods. •——•, binding in presence of Na_2SO_4; ○——○ binding in the absence of Na_2SO_4 □——□, nucleolytic activity

interference of the acid DNase in the binding process. The figures also show that acid DNase activity increased during the observation period, both in the cells and in the membrane preparations. Although DNase activity was present in the incubation medium of the cells, the values obtained after 20 min incubation of lymphocytes with labeled DNA did not exceed 5% of the total activity. Consequently, about 95% ot the detected nuclease activity resides in the intact cells. In membrane preparations, both in the heavy and the light fractions, the same specific hydrolytic activity was found. This experiment indicates both the presence of acid DNase and a decreased DNA binding capacity at pH 4.5, the pH optimum of this enzyme.

Discussion

In the experiments reported in the present study both specific and nonspecific types of binding of foreign DNA to lymphocytes from human tonsils were estimated according to the criteria introduced in the analysis of hormone receptors.

There is reason to suppose that, during the observation period, a significant amount of the DNA trapped by the cells remains at the cellular surface. This is supported by the finding that more than 75% of the bound DNA can be removed by DNase treatment in accordance with the data reported by Farber et al. (1975). Dissociation data also confirm the above observation. This is in good agreement with the finding of Ehrlich et al. (1976), that after 30 min 95% of the cell associated bacterial DNA was bound to the cell surface.

Since DNA-cell association follows a regular hyperbolic saturation curve, this process can be characterized by its dissociation constant which has been calculated from a double reciprocal plot of the bound DNA and the concentration of DNA. An apparent K of 4×10^{-10} M was obtained. Despite the fact that the DNA molecules involved in the binding process have a molecular weight of 10^7 daltons, the value of the apparent constant obtained was in accordance with that reported for hormone receptors (Cuatrecasas, 1974). The most common method of analyzing the data of protein-ligand reaction is to plot the bound/free ratio of the labeled ligand as a function of the concentration of ligand bound to the receptors (Scatchard plot). In the experiment described in Fig. 1b the Scatchard plot analysis shows a non-linear curve. The first slope revealed an apparent dissociation constant of about 4×10^{-10} M similar to that calculated for cell-DNA interaction. This suggests that the binding site is identical in both systems. Nonlinear Scatchard plot steady state binding of labeled DNA by lymphocyte membranes and the data of the dissociation experiment in the presence and absence of nonlabeled ligand might have several explanations such as a weaker binding to less specific secondary sites, self aggregation of the ligand and negative cooperativity (De Meyts et al., 1976). Negative cooperativity may provide a defense mechanism of the cell against massive invasion in which binding to receptors is favored at low concentrations of DNA but becomes more difficult when the

concentration of the nucleic acid increases. It is quite clear that the nature of the membrane-DNA complex is influenced by subtle conformational changes of the nucleic acid (Printz, Hippel, 1968) as indicated by the effect of pH and salt concentration (Figs 3 and 4). Nevertheless, further studies are necessary to assert the role of the changes of lymphocyte membranes induced by changes in pH and salt concentration.

In the present experiments acid DNase was detected in the intact cells and the medium and also to the same extent in the heavy and the light fractions of the membrane preparations.

It is not suprising to find DNase at the mammalian cell surface since acid DNase is a well characterized protein and widely distributed in subcellular particles. In our experiments acid DNase could not be eliminated from the membrane preparations even by repeated washings with isotonic solutions. There is ample evidence to show that biologically active proteins, such as enzymes, after being synthesized on the polysomes of the rough endoplasmic reticulum, may be transported to the exterior via the smooth endoplasmic reticulum, usually through the Golgi apparatus and finally through vesicles that fuse with the plasma membranes (Schramm, 1967). A release of enzymatic activites, such as acid DNase, RNase and alkaline phosphatase, from cells in to the culture fluid has been demonstrated (Kiyoshi et al., 1974). One possible role of this protein, present on the cell surface, may consist in degrading the foreing DNA bound to it. Only DNA molecules, having the chance to avoid these molecules, can exert biological activity. Consequently, the function of DNase bound to the cell surface is to protect the cell against invasion by foreign DNA. This idea may explain the low frequency of transformation and low infectivity of DNA of viral origin. Another interpretation may be that DNA-membrane complex formation is part of a DNA uptake system in which the DNase combines with the extracellular DNA molecules at physiological conditions where it has little degradative activity. Some secondary step is then needed for the penetration or the rejection of these macromolecules.

We are most grateful to Dr Maria Staub for DNA polymerase determinations and helpful discussions, to Dr G. Bánfalvi for his help in preparing bacterial nucleic acids and to Mrs Borbála Fülöp for excellent technical assistance.

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Contraction of Muscle Fibril under the Effect of Salt Solutions without ATP

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It is possible to elicite contraction on isolated muscle fibrils of honey-bee in isotonic sugar-solution by adding certain salt solutions without the addition of ATP. From this phenomenon we can conclude that the muscle contraction can appear under the effect of electricity.

Introduction

In our institute as early as in the twenties of our century the view was established that to inorganic materials, especially ions and water play an important role in the activity of muscle, particularly in the formation of contraction process (Ernst 1928, 1963). According to Szent-Györgyi (1942) surviving muscles contract under the effect of certain salt solutions. Jacobson gives account of his finding that molecules of dezoxyribonucleic acid contract if a small quantity of electrolyte solution is added to the preparation (Jacobson, 1953; Jacobson et al., 1954). Ernst and Metzger-Török (1962) found, that fibrils dissected from different proteins contract if certain salt solutions were added without ATP. In investigations performed on the basis of the above mentioned facts and others it was found that a change in the ion-surroundings of muscle fibrils brings about a contraction in the muscle fibrils when ATP is not added to the system.

Methods[1]

Single isolated fibrils of indirect wing muscle of honey-bee *(Apis mellifica)* were prepared in isotonic glucose (55 g/l) solution. From the sample made in this way a hanging-drop preparation was made on a cover slide, and it was placed on the objective table of a micromanipulator furnished with phase-contrast microscope (Zeiss Gleitmikromanipulator). Besides the two needles of the manipulator a micropipette was also lead to the hanging-drop, containing the salt solution the effect of which was being examined. After squirting the salt solution to the preparation the setting in of shortening was shown by the change of length and form of the fibril, and this was recorded on microphotographs. The micro-

[1] G. Metzger-Török collaborated in the experiments.

photographs were evaluated with a KIPP (Delft-Holland) microphotometer in order to get quantitative values also of the degree of contraction. Two series of experiments were performed, the applied salt solutions were: 1. mixture of 50 mM KCl + 1 mM $MgCl_2$ (pH 7.0); 2. 1 mM Na_2HPO_4 (pH 7.2).

Results

In each experiment of the series a contraction was observed after salt-solution was added to the preparation. Figs 1a and 1b show a muscle fibril before and after the addition of the mixture of KCl and $MgCl_2$ solutions. Figs 2a and 2b show the state before and after the addition of Na_2HPO_4 to the preparation. The photographs made after the addition of salt solution show well the

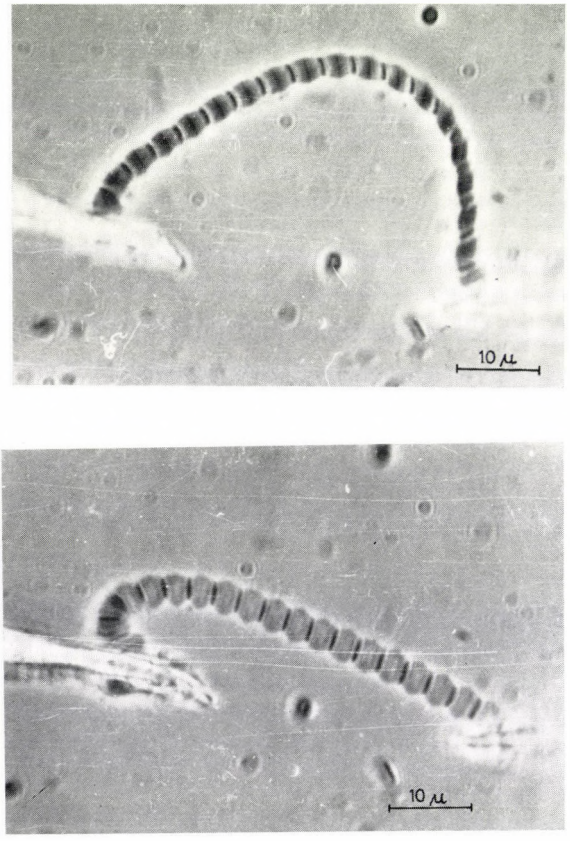

Fig. 1a, b. Phase-contrast microscopic image of a fibril of the indirect flight-muscle of honey-bee *(Apis mellifica)*: a, in isotonic sugar solution; b, after the addition of a mixture of 50 mM KCl + 1 mM $MgCl_2$ salt solution. ×1560

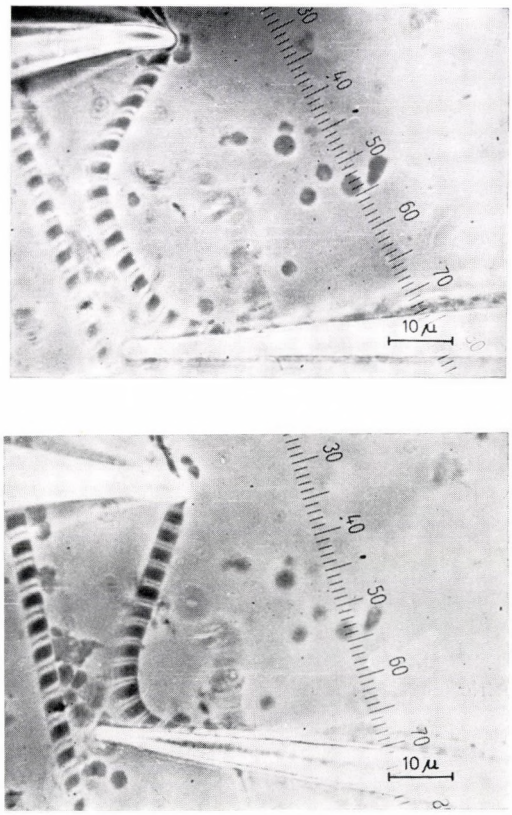

Fig. 2a, b. Phase-contrast microscopic image of a fibril of the indirect flight muscle of honeybee *(Apis mellifica)*: a, in isotonic sugar solution; b, after the addition of 1 mM Na_2HPO_4 solution. ×1300

shortened state of the fibril. Fig. 3 is the microdensitometric picture of the experiment shown in Fig. 2: Fig. 3a correspond to the fibril shown in Fig. 2a, and Fig. 3b to the fibril shown in Fig. 2b. With the aid of length-measurements we could demonstrate, that sarcomere shortened to two thirds of its length in this experiment.

In control experiments, when isotonic sugar solution was squirted to the preparation no contraction was found. No contraction appeared after the repeated addition of salt solution to the preparation for the second and third times either. These control experiments show, that mechanical stimulation cannot be the factor which elicits contraction, because the mechanical effect caused by squirting when sugar solution was added did not bring about contraction. A slight change in the concentration of salt solution did not influence the state of muscle either, because further salt addition following the first did not cause further contraction.

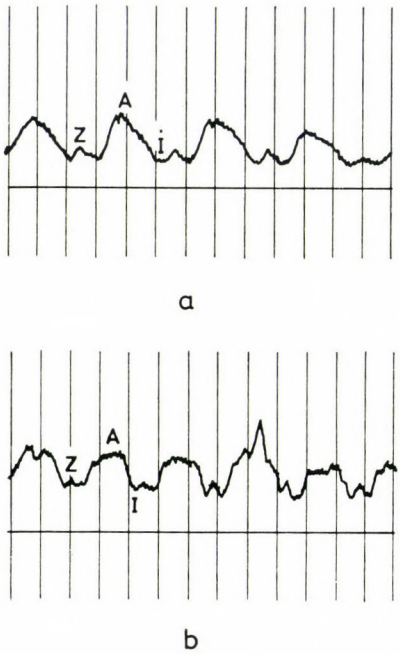

Fig. 3a, b. Densitometric picture of a section of fibrils shown in Figs 2a and b: a, fibril at resting length; b, shortened fibril

Discussion

Our results seem to prove that salt solution alone elicits the process of shortening. A significant difference between the two series of experiments is, that in the first series the change of state set in through ions, natively contained by the muscle when KCl and $MgCl_2$ were added, while in the second series, when Na_2HPO_4 solution which is not contained either by the muscle fibril or its surroundings in native state was added to the muscle fibril surrounded by the isotonic sugar solution. These facts corroborate the assumption, that salt solution (electrolyte) takes part in the development of the process of shortening through its electric effect.[2]

We do not deal with the question whether contraction or contracture appears in our experiments, we only ascertain that we demonstrated a shortening similar to that other research-workers produced with ATP (connecting it with energy release going together with the breakdown of the latter).

[2] This is supported by the opinion of Dempsey et al. (1946): "The breakdown of adenosine triphosphate to adenylic acid, to cite a single example, liberates additional charges into the environment." (See Szent-Györgyi, 1960.)

In a group of our control experiments the isotonic sugar solution contained 0.1 mM of EGTA in order to remove Ca and this way avoid the possibility of a contraction caused by an accidental Ca^{2+} contamination; at the addition of salt solution fibrils shortened also in this series of experiments. In connection with this it seems right to suggest that a certain degree of interaction between the behaviour and the activity of Ca and K appears in the muscle (and several other organs) (Jullien et al., 1960; Carvalho, Leo, 1967; Meech, 1974; Taylor et al., 1975); but this question, which is very important is beyond the intended scope of this paper.

In our experiments ATP was not added to the sugar solution surrounding the fibril, the contraction occurred without the addition of ATP. In the future the author intends to continue his investigation in this direction with muscle fibrils prepared from fibres treated with glycerine.

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The Study of Nitroxide Radical Active Esters as Spin Labels on Muscle Protein Actin

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Nitroxide radical active esters were used to label the muscle protein actin and to study the orientation dependence of the EPR spectra of the labelled protein. It is concluded that the labels are located at least at two different sites of the protein with strong polar environment and different mobilities. The EPR spectrum of the strongly immobilized labels exhibits orientation dependence, the N—O bond axis of the spin labels is nearly perpendicular to the long axis of the F-actin threads, and the labels undergo a rapid rotational motion about an axis directed perpendicular to the filament axis.

The application of the nitroxide radical active esters may be useful in the study of ordered systems as muscle or muscle protein systems.

Introduction

Nitroxide radical spin labels have been widely used to study structure and motion in biological systems. Using this technique several papers have been published on the contractile proteins of muscle as well (Seidel, 1972; Seidel, Gergely, 1973; Stone, 1973; Thomas et al., 1975a, 1975b; Belágyi et al., 1978). In earlier works Burley et al. (1971) and Sleigh and Burley (1973) have shown that the electron paramagnetic resonance (EPR) spectrum of an aligned F-actin sample spin-labelled with maleimide spin labels exhibits a strong orientation dependence. It was concluded that the $2p\pi$ orbital of the unpaired electron possesses an orientation which tends to be parallel rather than perpendicular to the long axis of the F-actin threads. The present paper is concerned with the study of the nitroxide radical active esters as spin labels to probe their application to muscle actin and the orientation dependence of EPR spectra in aligned F-actin threads.

Materials and methods

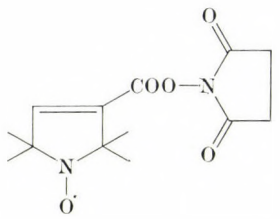
The following active esters were used in the experiments (Fig. 1):

HO 77 HO 88 HO 105 HO 164

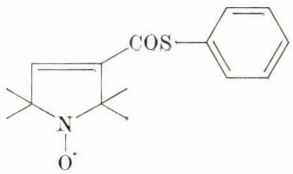
The active esters were prepared by K. Hideg.**

Actin was extracted in the monomeric form from an acetone dried muscle powder of rabbit skeletal muscle prepared according to Straub (1943) at 0 °C

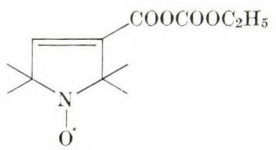
** The spin labels can be purchased from REANAL, Hungary (Catalogue of nitroxide spin labels, REANAL 1978).



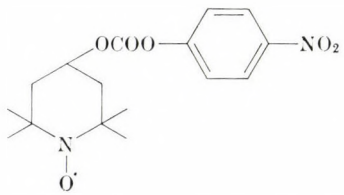
HO 77



HO 88



HO 105



HO 164

Fig. 1. The chemical structures of the spin labels. HO 77 1-oxyl-2,2,5,5-tetramethylpyroline-3-carboxylic acid N-hydroxysuccinimide ester; HO 88 3-(phenylthio) carbonyl-1-oxyl-2,2,2,5,5-tetramethylpyrroline; HO 105 [carbonic acid-1-oxyl-2,2,5,5-tetramethylpyrroline-3-carboxylic acid] anhydride ethyl ester; HO 164 p-nitrophenyl 1-oxido-2,2,6,6-tetramethyl-piperidinyl carbonate

with 5 mM Tris HCl buffer at pH 8.5 containing 0.2 mM ATP, 0.2 mM $CaCl_2$ and 0.1 mM dithiothreitol. The whole extraction procedure took 15 minutes. The actin was purified by repeated polymerization-depolymerization cycles, and labelled in F-form in 100 mM KCl and 0.6 mM $MgCl_2$. One mole of nitroxide

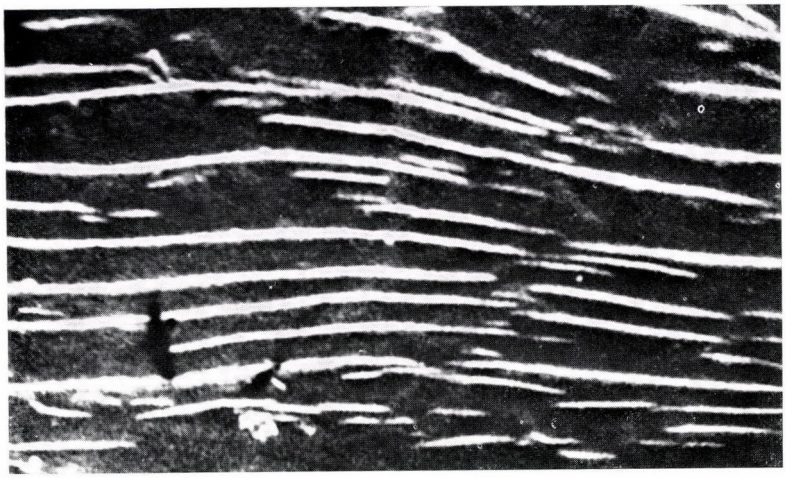


Fig. 2. Scanning electron micrograph of the oriented F-actin gel. ×3000

radical active ester reacted with 1 mole of actin at 0 °C for 180 min under continuous stirring. The unreacted labels were removed at 4 °C by overnight dialysis against a solution containing 0.1 mM ATP, 0.2 mM $CaCl_2$ in phosphate buffer, pH 7.6. The spin-labelled actin was polymerized again and centrifuged for 1 h at 100.000 × g. The F-actin pellet was aligned on the surface of a flat EPR cell. For EPR measurement a Zeiss ER 9 spectrometer was used at 9.5 GHz at room temperature. The morphological appearance of the aligned F-actin preparation was analyzed electron microscopically (Fig. 2).

Results and Discussion

Fig. 3 shows the EPR spectra of an oriented F-actin sample spin-labelled with HO 77 in two different orientation with respect to the applied magnetic field. Solid line shows the spectrum, where the filament axis was oriented parallel to the static magnetic field, and dotted line shows the spectrum of the same sample, where the filament axis was perpendicular to the applied field. All spectra reflect that the nitroxide radical active esters reacted at least at two different sites of the protein which can be distinguished by the restriction of the rotational freedom of the labels. The spectral component denoted by B arises from radicals which are loosely attached to the actin sample, whereas the spectral part denoted by A is typical for strong immobilization of the labels.

The isotropic splitting constant (a_{iso}) in spectrum B is 15.8 G which is the same as the value of the isotropic hyperfine splitting constant of the labels (except HO 164, which has the six-membered ring) in water, and therefore the labels are located in a strong polar environment on the protein. The rotational correlation time (τ_2) for labels in spectrum B was calculated according to the formula proposed by Kuznetsov et al. (1971), to be 0.5 ns.

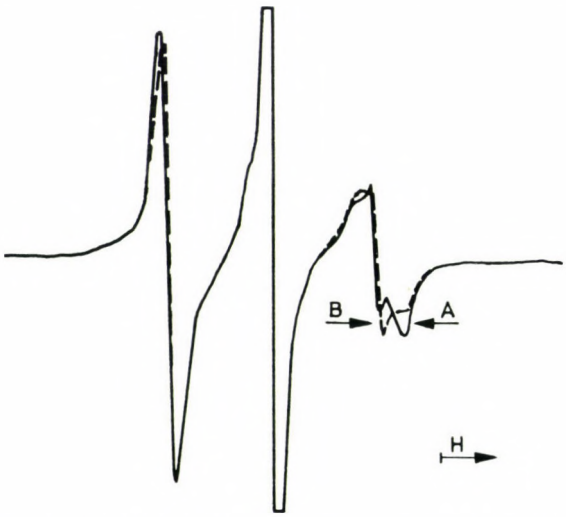


Fig. 3. EPR spectra of spin labelled oriented F-actin sample. The spin label used in the experiment was the HO 77 label. Solid line shows the EPR spectrum, when the alignment of F-actin was oriented parallel to the applied magnetic fields. Dotted line shows the EPR spectrum of the same sample in perpendicular orientation

As can be seen from Fig. 3 only the spectrum part A reflects the orientation dependence of the strongly immobilized labels. The spectra can be characterized by the hyperfine splitting constant measured between the two outermost hyperfine extrema. It was estimated to be $2A'_{||} = 38$ G. For the hyperfine splitting constant of the spin label HO 77 in a randomly oriented F-actin sample $A'_{zz} = 31.5$ G was measured. Therefore, from the EPR spectra of the oriented samples it is concluded that the labels are rigidly attached to the F-actin filaments, but undergo rapid rotation about the axis directed perpendicular to the long axis of the F-actin filaments. The axis of rotation is parallel to the N–O bond axis of the spin labels.

Fig. 4 shows the computer simulation of the EPR spectra of spin labels in oriented F-actin filaments. The EPR line positions were calculated using the following values for the hyperfine tensor components $A_{xx} = A_{yy} = 6.5$ G and $A_{zz} = 35.0$ G and g values: $g_{||} = 2.0057$, $g_{\perp} = 2.0061$. The individual line width was 3.0 G and line shape was chosen to be Lorentzian. In our model the axis of rotation executes a restricted random walk (wobble motion), the rotational correlation time of wobble motion was assumed to be 2.5 ns (Israelachvili et al., 1974; Gróf et al., 1977). In view of this assumption the motion-averaged components $A'^{1}_{||} = 19.0$ and $A'_{\perp} = 9.7$ G were obtained. The deviations of $A_{||} = \frac{1}{2}(A_{yy} + A_{zz})$ and $A_{\perp} = A_{xx}$ from $A'_{||}$ and $A'_{\perp}$ indicate that wobble motion occurs about the axis of rotation.

Using the value $A'_{||} = 19.0$ G measured on samples and $A'_{||} = 19.0$ G and $A'_{\perp} = 9.7$ G, which were obtained from the computer simulation of the EPR spectra, the isotropic hyperfine splitting (a_{iso}) can be calculated in the usual manner

$$a_{iso} = \frac{1}{3}(2A'_{||} + A'_{\perp}) = 15.9 \text{ G},$$

which is in a good agreement with the experimentally measured isotropic hyperfine splitting constant in water, and the same value as the splitting constant for weakly immobilized labels between the limits of error. This finding suggests that the strongly immobilized labels are also located in a polar environment of the protein moiety.

Stone et al. (1973) reported in an EPR study of actin polymerization that the mobility of the attached labels was markedly reduced after polymerization. The label reagents (maleimide label, iodoacetamide label) react specifically with the cystein-373 (Burley et al., 1971). The EPR spectra of the strongly immobilized labels exhibited no change after G → F transformation, therefore, it can be concluded that the nitroxide radical active ester labels are located at sites which

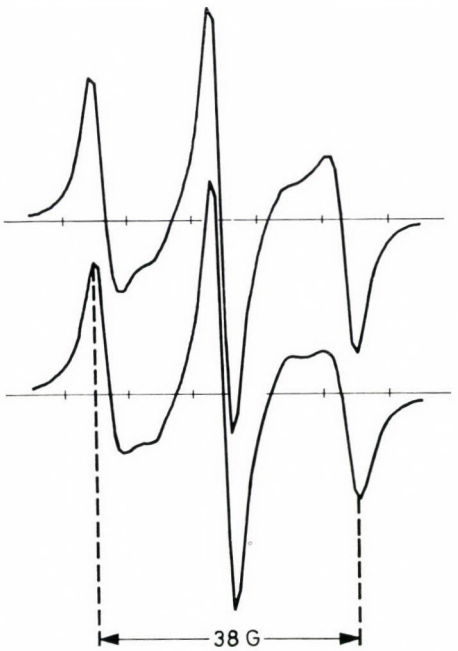


Fig. 4. Simulated EPR spectra of the HO 77 labelled F-actin sample. The filament axis of F-actin was taken parallel (top) and perpendicular (bottom) to the applied magnetic field. The EPR spectra of weakly immobilized labels are omitted. The motion-averaged parameters are $A'_{||} = 19.0$ G, $A'_{\perp} = 9.7$ G. The individual line width was 3.0 G and the line shape was chosen to be Lorentzian

are far from the polymerization sites. The fluorescence changes accompanying the G → F transformation support that the fluorescence probe (dansyl cystein, Cheung et al., 1971; dansyl aziridine, Lin, 1978) is located in a hydrophobic environment, and the polarity of the labelled site is sensitive to polymerization. Our results concerning the polarity of the environments of the spin labels indicate that the labels are located on the surface of the protein, and the polymerization does not perturb the environment of the labelled sites.

Loscalzo et al. (1975) pointed out the decrease in amplitude of EPR signal of maleimide spin-labelled actin on manganous ion – containing actin relative to that of Mn^{2+}-free F-actin. The decrease in amplitude is due to the spin-spin interaction (Leigh, 1970). The nitroxide radical active esters attached to actin have a higher mobility, therefore, they might be sensitive indicators to account for the spin-spin interaction.

On the other hand, the striated muscle is a highly ordered system, therefore, the application of the nitroxide radical active esters an spin labels on ordered contractile protein system may be a useful method to see changes in the degree of order of spin labels, when the actin interacts with myosin or the changes of the flexibility of actin filament under different circumstances (Belágyi et al., 1977).

The authors are indebted to Dr K. Hideg (Central Laboratory, Medical University, Pécs) who synthethized and kindly donated the nitroxide radical active esters.

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Ion Content of Synaptic Vesicles

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Proton induced X-ray emission analysis measurements were performed to determine the P, S, K, Ca, Fe, Ni, Cu and Zn ion content of presynaptic vesicles prepared from guinea-pig brain cortex. The number of different ions per single vesicle is calculated using the results of the additional protein content determinations. The ion contents of cholinergic and adrenergic vesicles are compared.

Introduction

The spatial distribution of different elements in cells could possibly reflect the function of the particular place where they are located. The method used in carrying out such studies is the electron microscopy with facilities to determine elemental composition. In such cases a transmission or scanning picture of the sample can first be seen, then the electron beam is focused on the loci in which the composition is to be determined. The energy of the emitted X-rays is characteristic of the element under bombardment; the X-ray intensity is proportional to the quantities of the material. The X-rays are usually measured by a Si(Li) solid state detector built into the electron microscope. A very high sensitivity ($\sim 10^{-17}$ gram) and good localization characterize this method. The quantitative evaluation of the data, however, is difficult and it is impossible to measure small concentrations (below $\sim$100 ppm) because of the high background of bremsstrahlung.

In this paper an alternative method is suggested for localization of ions in cell organelles.

The quantity of matter in these fractions is usually sufficient for an elemental analysis by observing the X-ray spectra emitted under high energy (2–4 MeV) proton bombardment (PIXE) (Johansson et al., 1976).

For the purpose of this study synaptic vesicles isolated from guinea-pig brain cortex (Nagy et al., 1977) were chosen.

Abbreviations: AE, adrenergic; CE, cholinergic; AEG, adrenergic vesicles after gradient separation; CEG, cholinergic vesicles after gradient separation; AEB, CEB vesicles with glass bead chromatography; PIXE, particle induced X-ray emission.

Materials and methods

Vesicles containing catecholamines (AE) and acetylcholine (CE) were extracted in separate fractions from guinea-pig brain cortex (Nagy et al., 1977) on a continuous D_2O-H_2O (1 : 1) sucrose density gradient and purified by chromatography on columns of glass beads of controlled pore size.

For the determination of the membrane-bound ion content it was necessary to remove the sucrose from the media and concentrate the fractions by centrifuging in a Beckmann SW 56 rotor at 300 000 g for one hour. The pellets were taken up in "ion free" three-times distilled water of conductancy less than 0.1 $(M\Omega cm)^{-1}$ and pipetted onto a square piece of 13 μm polypropylene foil using Eppendorf micropipettes.

The applied protein contents were $\sim$0.3 mg for the gradient and $\sim$0.05 mg for the chromatographically purified fractions. The volume of the samples varied between 100 μl and 500 μl. The mean diameter of the targets was about 5 mm.

The samples kept on a special metal frame were completely dehydrated at 40 °C in a vacuum chamber. After weighing, their surface densities were calculated. They were kept dry and isolated until the ion content determination to avoid any possible contamination.

The samples were investigated for protein content by the $^{14}N(d, p)^{15}N$ nuclear reaction (Varga, 1977). The weight of N multiplied by 6.5 gives the weight of protein in biological samples as a very good approximation (FAO, Nutritional Studies 1970).

The deuteron beam was accelerated in the 5 MV Van de Graaff accelerator of the Central Research Institute for Physics. The target arrangement can be seen in Fig. 1. The samples can be exchanged through a vacuum lock system within 20–30 seconds and moved $\sim$10 mm perpendicularly to the beam. The protons emitted in the (d, p) reaction are measured by a CsI(Tl) scintillation counter. An Al absorber of 60 mg/cm^2 thick cut off the backscattered deuterons and low energy protons. The Q-value of the $^{14}N(d, p)^{15}N$ reaction is 8.4 MeV, therefore the protons emitted at 160° with respect to the deuteron beam direction may have an energy of $\sim$10 MeV in the case of 1.5 MeV deuterons. These are easily separated from protons emitted in (d, p) reactions by any other usual elements in biological material. The homogeneity of the samples was studied by mapping the protein content with the above reaction across a radius of the

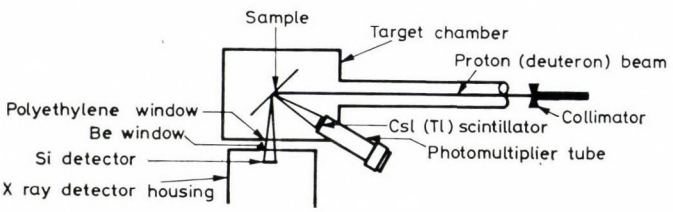

Fig. 1. Target arrangement

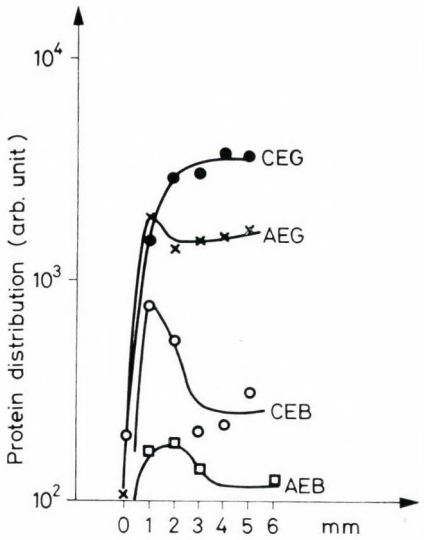

Fig. 2. Protein distribution across the target sample

samples. The beam diameter was 0.5 mm, and steps were 1 mm each. Some results are shown in Fig. 2. The middle parts are fairly homogeneous.

The N measurement was calibrated using an NH_4NO_3 target. As the vesicle samples were thick for the 1.5 MeV bombarding deuterons this calibration could be used. In Table 1 the protein content of the samples determined by the Lowry method (Lowry et al., 1951) and by the nuclear reactios on the middle homogeneous part are compared. The agreement of the data is very good in the case of gradient separation. Larger deviations appear, however, after additional purifica-

Table 1

Protein content determined by different methods
The protein content is given in mg/cm²

Sample	Lowry method	Nuclear reactions
Cholinergic vesicles (gradient separation)	0.40	0.33
Adrenergic vesicles (gradient separation)	0.15	0.14
Cholinergic vesicles (glass bead purification)	0.06	0.03
Adrenergic vesicles (glass bead purification)	0.005	0.008

tion by glass bead chromatography. As the Lowry method gives the average protein content and the nuclear reaction method gives the local one, this deviation is very probably caused by the inhomogeneous distribution of protein on the target surface in these cases (Fig. 2).

For the PIXE measurements the proton beam was also generated in the 5 MV Van de Graaff accelerator and its position was defined by a slit of 2 mm diameter. The beam energy used in these experiments was 2 MeV and beam currents were a few nA to reject dead-time difficulties. Targets were mounted in the same chamber used in the nitrogen determination measurements at 45° to the proton beam. X-rays passed through the 4 μm Mylar window of the chamber and 11 mm air before entering the 30 mm^2 Canberra Si(Li) X-ray detector positioned at 90° to the incident beam. The entrance window of the detector was 25 μm Be foil and an additional 25 μm polypropylene absorber was always used to prevent the detector from the scattered protons. The pulses were processed by the special Canberra X-ray amplifier system and spectra were stored in a multichannel analyser. The energy resolution of the system was 175 eV for the 5.89 keV Mn K_α line. In cases of thick targets (the thickness was compared to the range of the 2 MeV protons in the sample) the additional background due to target charging effects was suppressed by electron spraying of a hot tungsten filament (Ahlberg et al., 1976). The quantity of the evaporated tungsten on the target was below the detection limit. The peak areas of the spectra were determined by using an off-line TPA-i small computer. The conversion of the number of X-rays detected to the quantity of the element of interest was carried out by the computer taking into account the slowing down of the protons and the X-ray absorption in the thick target.

Results

X-ray spectra of cholinergic and adrenergic vesicles after sucrose gradient separation (CEG, AEG) and after purification with glass bead chromatography (CEB, AEB) were measured. Figure 3 shows typical CEG and AEG spectra. Spectra were also taken of the samples prepared from the starting material before density gradient separation (W_s), and from the supernatants of the final centrifugation (separating the sucrose from the vesicles). The number of P, S, K. Ca, Fe, Ni, Cu and Zn atoms/g of sample material was determined, and related to the protein content of the sample. The W_s fraction contained the same elements as CEG and AEG; in the suparnatants practically no elements were found.

The data are collected in Table 2 expressed as number of atoms per single vesicle. To get these data the following assumptions were made:

a) the size of a vesicle is 3×10^{-17} cm^3 and its weight is 3×10^{-17} g, i.e. its density is unity,

b) the weight of a vesicle is composed of 1/3 protein, 1/3 lipid, and 1/3 transmitter, water, etc. So 10^{17} vesicles contain 1 g protein.

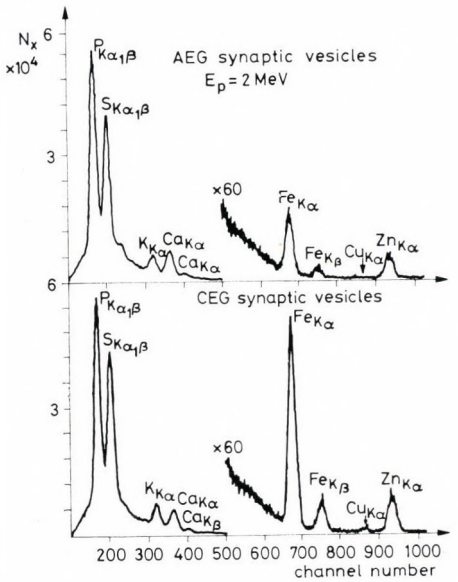

Fig. 3. X-ray spectra measured in AEG and CEG vesicles

Table 2

Number of atoms/vesicle

Vesicle	P	S	K	Ca	Fe	Ni	Cu	Zn
CEG	3.5×10^3	960	130	60	22	0.4	0.5	10
AEG	6.3×10^3	1050	150	90	12	0.7	0.8	14
CEB	2.0×10^3	980	110	80	25	0.7	0.7	10
AEB	3.0×10^3	1400	160	100	30	1.0	1.0	14

Discussion

Phosphorus

The data show that going over from gradient separated vesicle to vesicles purified by glass bead chromatography a factor of two loss of P occurred. The reason for this is not yet understood. The P content of AEG, and of AEB is 1.5 times greater than in CEG and CEB.

The P ions are mainly in the phospholipid molecules of the vesicles membranes. An estimation of the number of lipid molecules gives a value of ~9000 lipid molecules in one vesicle. (This number came out assuming an average molecular weight of 700 dalton for lipids or assuming a surface of lipid molecules

Table 3

Molar ratio of phospholipids to lipids

CEG	0.4
AEG	0.7
CEB	0.2
AEB	0.3

of ~ 50 Å^2 and close packing.) Assuming that one lipid molecule contains one P ion the molar ratio of phospholipids to lipids can be calculated. The data are given in Table 3.

Sulphur

S is mainly contained in the proteins. Two amino acids (cysteine and methionine) have SH groups. If the average molecular weight of the amino acids is ~ 100 then one vesicle contains $\sim 6 \times 10^4$ amino acids which means that the proteins in vesicles are rather poor in CYS and MET, their number is 1 out of 60 amino acids.

Potassium

The K ions play an important role in establishing membrane potential. Their concentration is greater inside (100 – 400 mM) than outside (4 – 22 mM) of excitable membranes. They are supposed to be dissolved in intra- and extracellular solutions. In the present case only intravesicular K ions may be supposed. If so, their concentration would be 16 mM which is very small. The freely moving K ions, however, were probably lost in the separation processes therefore the remaining ions ought to be bound to the membrane.

The average number of lipid molecules per vesicle has been determined above. Using this result we can assume that ~ 70 lipids in the case of CE and ~ 95 in the case of AE are present for each K ions. This means that a potassium ion binds on a lipid surface on every 7th or 8th molecule in a row.

Calcium

The Ca ions are thought to move in cells and in intracellular media, bind to lipids and proteins and in this way trigger some important function. The reasoning given for K is valid here too: the found Ca ions must be bound to membranes. There is one Ca ion for 125 and 90 lipid molecules in CE and AE vesicles, respectively, or on a surface every 10th – 11th lipid molecule binds a Ca ion in a row.

Iron

The data show a marked difference between the Fe content of CEG and AEG vesicles. The Fe content of AEB vesicles seems to be irregular; this can however be caused by some small Fe contamination because in the AEB samples the vesicle content is much less than in the other cases (see Fig. 2) and the unknown contamination has a comparatively greater effect.

The Fe ions should be mainly in proteins. Assuming that the vesicle proteins have a molecular weight of ~50 000 dalton there are about 120 proteins in one vesicle and in the case of CEG about 20 per cent, in the case of AEG about 10 per cent of them contain one Fe ion.

Nickel, copper

The Ni and Cu data are subject to rather large error. It seems that there is no more than one Ni or Cu ion per vesicle.

Zinc

The Zn ions may be bound to proteins too. In CE vesicles 8 per cent, in AE vesicles 11 per cent of the proteins contain Zn ions according to the estimation made in the case of Fe ions.

Summing up, one can state that the percentage of ion-containing proteins is ~28 per cent in cholinergic vesicles and 21 per cent in adrenergic vesicles, respectively. Further work is needed to separate and characterize these metalloproteins.

As could be seen in the above discussion, some rough conclusions regarding the biochemistry of vesicles were made based on the measurements of their ion content. The elucidation of the meaning of the data needs further work which will demonstrate the value of PIXE-type investigations in similar studies.

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Simultaneous Determination of Protein and Metal Ion Content of Metal-Containing Proteins

(Short Communication)

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Methods exist for determining the protein content of biological samples by measuring their nitrogen content. As a rule of thumb the weight of protein in a sample equals 6.5 multiplied by the weight of nitrogen (FAO, 1970).

A method of N measurement was worked out previously (Varga, 1977) where the sample is bombarded by deuterons of 1.5 MeV energy, and protons of 10 MeV energy from the $N^{14}(d, p)^{15}N$ reaction are measured by scintillation or solid state detector. The ion concentration in biological material can be determined by PIXE analysis (Johansson et al., 1976). By combining the two methods the ion content of biological samples related to the protein content can be determined. The method was utilized for a purified protein: superoxide dismutase (SOD) extracted from the algae Anacystis nidulans (Cséke et al. 1979). One particular problem concerned the identification of the type of metal ion in this peculiar superoxide dismutase molecule since Mn, Fe, Cu, Zn ions had previously been found in SOD molecules of different origin.

200 μl solution of protein (0.5 mg/ml) in 0.01 M TRIS-HCl buffer and 0.35 M NaCl was dropped on to polypropylene foil of ~ 1 mg/cm^2 thickness and dried. The CsI(Tl) scintillation counter measured the number of high energy protons under deuteron bombardment, the Si(Li) X-ray detector of 30 mm^2 had a resolution of 175 eV for 5.9 keV X-rays.

The number of N atoms/cm^2 in the sample was calibrated by an NH_4NO_3 target. This calibration and the multiplication factor for protein were checked with vesicle preparations by comparing the data obtained by this method and by the Lowry method (Lowry et al., 1951). Table 1 shows that the results obtained by the two methods are in reasonable agreement.

Although after drying the sample appeared to be very inhomogeneous, a scan with a deuteron beam of 0.5 mm diameter (Fig. 1) showed that the middle part of the sample was, in fact, rather homogeneous. The protein and ion content measurements were performed in this region.

Abbreviation: PIXE, particle induced X-ray emission.

Table 1

Protein content (mg protein)/(mg sample of vesicles) determined by two different methods

Type	Lowry	$N^{14}(d, p)^{15}N$
Cholinergic	0.4	0.33
Adrenergic	0.15	0.14

From the X-ray measurement it was possible to decide that the SOD from Anacystis nidulans contains Fe ions. The quantitative analysis of the data (after corrections to the N in TRIS, to X-ray absorptions, etc.) made the calculation of the molecular weight of the protein per one Fe ion possible. The result was

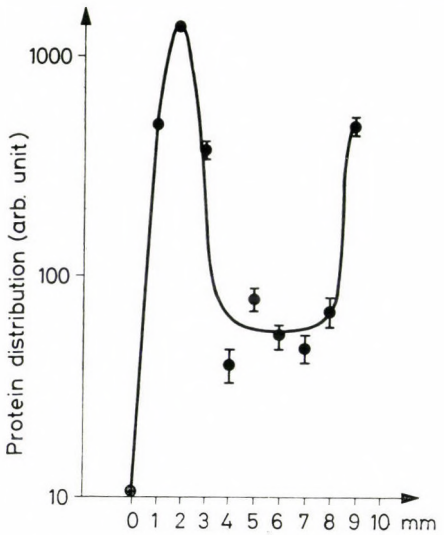

Fig. 1. Distribution of protein on the target sample

38 000 ± 8 000 dalton. Similar measurements by SDS electrophoresis and electron spin resonance gave values for molecular weight of 42 000 and 30 000 dalton with similar error.

The sensitivity of this method is very high: the bombarded material was 0.4 picomole protein, it contained 2.2×10^{-11} g Fe. This type of measurement could be performed with even 100 times less material.

We are indebted to Dr G. L. Farkas and Cs. Cséke for preparing the SOD proteins and to Dr Ágnes Nagy for measuring the protein content of vesicle preparation by the Lowry method.

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On the Effectiveness of Spin-labelled Procaine

(Short Communication)

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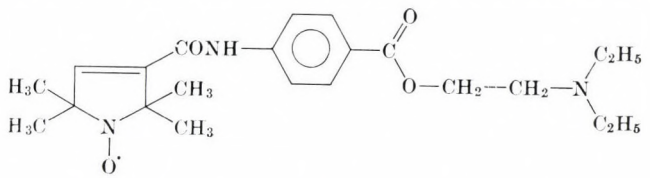
Excitable tissues such as the central nervous system, the myoneural junction, muscle, nerve, and the myocardium are all depressed and finally blocked by local anesthetics (Takman, Camoguis, 1970; Zipf, Dittman, 1971).

Procaine has a few clinical applications, like infiltration anesthesia, block and conduction anesthesia, besides it is used as smooth muscle relaxant and intravenous analgetics. The anesthesia is achieved by blocking impulse conduction in peripheral nerves or in the spinal-nerve roots of the cord. A spin-labelled local anesthetic, 2-[N-methyl-N-(2,2,6,6-tetramethylpiperidino-oxyl)] ethyl 4-alkoxybenzoates has recently been investigated. The modified basic side chain of parethoxy-



caine was labelled and the compound retained its activity (Gargiulo et al., 1973). In our experiments we wanted to examine whether the spin-labelling of procaine (Tigyi et al., 1978) diminished its local anesthetic effect or not. The free radical was bound to the aromatic group of procaine. The spin-labelling of procaine may be useful in more exact exploration of the mechanism of action of local anesthetics by ESR spectroscopy.

In our experiments frog sciatic nerve and isolated ileum of guinea pig were used. To perform our experiments we modified *Aström*'s method (Aström, 1965). We used two pairs of recording electrodes instead of one, and we applied the blocking solution between them. In this way we could observe if the nerve was in action apart of the blocked area. At first we tested the effect of both non-labelled and spin-labelled procaine on the impulse conduction of frog nerve. The frog sciatic nerve was excised and the perineurinum was removed. Then we placed the nerve into a moist chamber onto platinum wire electrodes. A small vessel of 0.5 ml volume was placed between the two pairs of recording electrodes. This vessel contained normal Ringer solution or one containing procaine in a concentration of 10 mM/l. Then we stimulated the nerve with a supramaximal stimulus and the nerve activity was indicated by a double beam cathode-ray

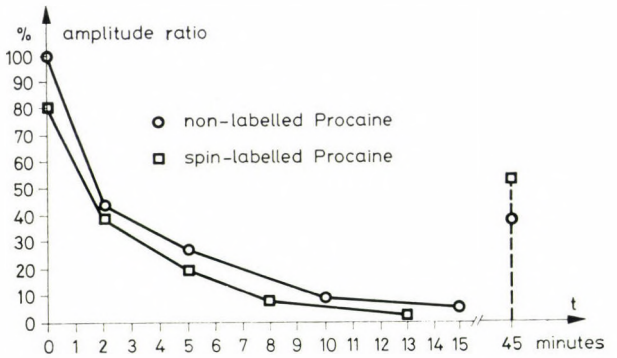


Fig. 1. The ratio of amplitudes taken from the first and second recording electrodes plotted against time. The first point represents the amplitude ratio before the application of procaine. The last point represents the amplitude ratio after the procaine was washed out with normal Ringer solution

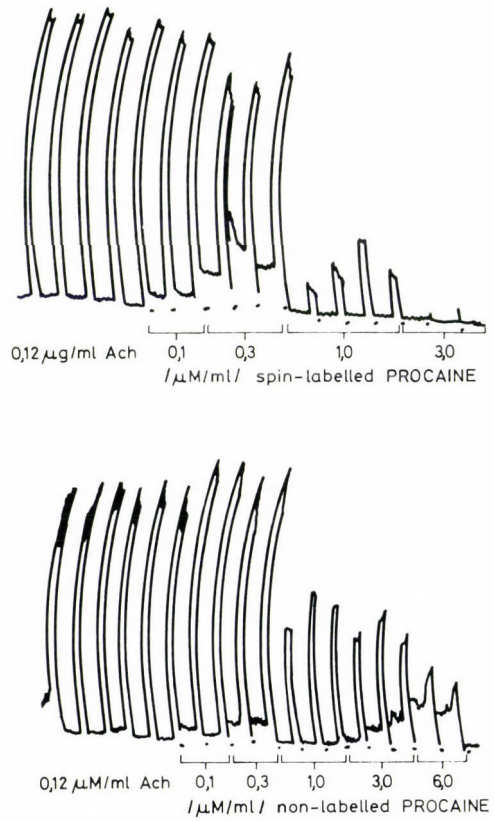


Fig. 2. The contracture of isolated ileum of guinea pig induced by Ach (0.12 µg/ml) is partly, and later totally prevented by treatment with spin-labelled or non-labelled procaine

oscilloscope. When either non-labelled or spin-labelled procain was put in normal Ringer solution in a concentration mentioned above, the action potential from the second pair of recording electrodes gradually decreased and finally totally disappeared. This indicated that the conduction of nerve impulse was blocked. The effect was reversible. When the procaine was washed out of the nerve with normal Ringer solution the action potential appeared again. The time course of the change of action potential is shown in Fig. 1. Both the non-labelled and spin-labelled procaine dissolved in normal Ringer solution were kept in a refrigerator for ten days and the experiment was succesfully repeated with this material. This showed that the spin-labelled procaine molecule was as stable as the non-labelled one.

The relaxant effect of both non-labelled and spin-labelled procaine was also tested on the smooth muscle of isolated ileum of guinea pig. The contracture of isolated ileum of guinea pig induced by acetylcholine (0.12 μg/ml) could be prevented by treating the preparation with either non-labelled or spin-labelled procaine if the concentration was at least 6 μM/l and 3 μM/l, respectively. When both compounds were applied in lower concentrations than that mentioned above, the contracture was only partly inhibited (Fig. 2). The phenomenon was reproducible, same results were obtained in repeated experiments. So it is demonstrated that the spin-labelling of procaine does not diminish the activity of this drug. Further experiments are in progress to investigate some other details of the problem.

We are grateful to Dr K. Hideg and his colleagues (Central Laboratory of Chemistry at our University) for the labelled compounds and the helpful discussions.

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Book Review

Aufbau und Eigenschaften der Materie im Mikrokosmos by Friedrich Klages. Sammlung Göschen (Walter de Gruyter Berlin, New York, 1979)

The material science became a hard attractive and relevant science from a collection of recipes in a few decades. It incorporated conclusions obtained from atomic physics, classical chemistry, empirical geology, solid state technology, but now it is more coherent and far more powerful. This is due to the foundation offered by quantum mechanics. Questions like the peculiar structure of water, the amazing difference between coal and diamond, the revolutionizing structure of DNA, all can be understood from a common root: from an *anschaulich* wave mechanics. This rapid change of views influences now the way of thinking far beyond physics: in chemistry, biology, earth science and material technology. It offers a new challenge for school science teaching as well. School chemistry does no longer necessarily seem to be a specific collection of rules, valid for substances occurring mainly in the chemical laboratory, but to be an efficient orientation in the real world. This new possibility attracts the attention of teachers and professors and tempts them to write books.

This small nice booklet is a realization of this new possibility. The book starts with simple properties of particles and atoms. It does not aim at building up the sophisticated dialectics of quantum mechanics, it gives only a few necessary pieces of information, in order to go over to the interaction of atoms as quickly as possible. It is somewhat surprising that the intermolecular forces (a tertiary phenomenon) comes earlier than chemical bonding (a secondary phenomenon, derivable from the direct Coulomb force). But the author is not a physicist, he is a chemist, and his goal is to show how one can get oriented in the universe of materials based on the knowledge of interatomic and intermolecular forces. The application is a strong side of the book rather than the introduction.

After having given the modern (restricted) concept of molecules, the book starts to explain the physical properties of material based upon their chemical structure. Beside covalent, ionic and metallic crystals an equal consideration is given to two- and one-dimensional patterns, to amorphous, colloidal and elastic structures. The author (professor of organic chemistry at the University of Munich) shows us around in the inorganic and organic world in the same elegant way. This persistent organic-chemical approach to the inorganic world is especially successful, it gives the characteristic flavor of the book. It is an enjoyable study not only for a wide range of experts, but for school teachers and secondary school students as well. Let us hope, books like this are forerunners of the schoolbooks of the near future.

G. MARX

Biochemistry of Wounded Plant Tissues by G. Kahl (ed.) p. 680, Walter de Gruyter, Berlin, New York, 1978.

The present volume contains 19 articles written by 29 authors on the biochemical aspects of wounding and wound healing in higher plants. The problem is very up-to-date. It has its intrinsic interest but, in addi-

tion, almost all aspects of plant pathology are associated with wounding effects and, unless one is working with intact plants, wounding and its consequences are unavoidable in most biochemical and physiological experiments with higher plants. Therefore, the literature on the problem of wounding is vast. Only part of it is covered in this volume. The system selected for discussion is a widely studied one: the biochemistry of sliced storage organs. This system has its special features but, at the same time, it allows extrapolations to other areas. In fact, such a detailed survey on the storage tissues will certainly stimulate research in other fields. The selection of specialists to write the various chapters is well balanced: in addition to well established authorities in the field, the younger generation is also well represented. The topics cover a wide range of subjects including the ultrastructural changes after wounding, the biochemistry of suberin formation, synthesis of cell wall glycoproteins, alteration of membrane systems, lipid metabolism including lipolytic enzymes and lipoxygenases, synthesis of terpenoids, formation of phenolics, control of glycolysis, induction and degradation of enzymes, biogenesis of cell organelles, development and control of respiratory pathways, changes in electron-transport system, control of ion-transport, ribosome metabolism, ethylene formation, and role of phytohormones in the regulation of the metabolism of tissues exhibiting wound-response.

Some articles are primarily review-papers, but others contain a high proportion of unpublished material. The extensive literature cited (well over 100 citations in most cases) is extremely useful for the newcomer in the field. The editor and the Publishers did not strive for unnecessary uniformization of style and format. This made it possible that the book is unusually up-to-date and makes an excellent source of information both on the "classical" and recent literature in this rapidly expanding field. G. L. FARKAS

Thermodynamics of Biological Processes by I. Lamprecht, A. I. Zotin (eds), W. de Gruyter, Berlin, New York, 1978.

For a description of the processes in living systems a thermodynamic theory encompassing regulation and control processes as well is called for. However, at the present stage of scientific development a commonly accepted theory of non-linear irreversible processes is missing. Certainly, new thermodynamics are required to describe a variety of phenomena proceeding in ordered, living systems far from equilibrium.

This book shows that the thermodynamics of irreversible processes can be the basis of the theory of evolution, of development of organisms, and of responses of living systems to external effects. This book reveals basic problems and summarizes the first results of thermodynamics applied to biology.

This monograph resulted from a cooperative effort between two laboratories: the Biophysical Laboratory of the Central Institute for Biochemistry and Biophysics at the Free University, West Berlin and the Laboratory of Developmental Biology of the Academy of Sciences of the USSR. The main theories discussed in the book are based on a global approach to non-linear irreversible processes, however, the fields of biology in which these theories are applied are confined to problems that are studied in the above two laboratories.

The main problems discussed are the following:

I. General problems of biological thermodynamics.

II. Qualitative phenomenological theory of the development of organisms.

III. Quantitative phenomenological theory of the development of organisms.

IV. Heat production of living systems.

V. Some problems of energetics of developmental processes.

VI. Dissipative structures.

VII. Probability state and orderliness of biological systems.

A very comprehensive list of the cited literature (about 700 references) enormously increases the value of this book. In contrast, the subject index is very poor and does not facilitate the use of this monograph.

This book will be useful to biophysicists and to those physiologists, biochemists and embryologists who are specially interested in modern thermodynamics or to those physicists who are specially interested in modern molecular and evolutionary biology.

T. KELETI

Advances in General and Cellular Pharmacology by T. Narahashi and C. P. Bianchi (eds), Volume I, Plenum Press, New York and London, 1976, pp. 252

The object of this series is to integrate the concepts and techniques of the many disciplines that contribute to our understanding of drug action — physics, chemistry, physiology, biochemistry and biophysics. The first volume contains five chapters, one for each of the following topics: cardiac automaticity, opiates and their antagonists, drug actions and developing cardiac tissues, dose-response relation and ganglionic transmission. Each chapter has been compiled by well-known investigators of the respective fields.

1. Cardiac-Cellular Pharmacology: Automaticity in Cardiac Muscle — Its Alteration by Physical and Chemical Influences, by Francis M. Weld and J. Thomas Bigger, Jr., gives a comprehensive and up-to-date review on the ionic determinants of diastolic depolarization and normal automaticity of pacemaker cells. Effects of ion concentration changes and neurohormons, physical factors and various drugs (Na- and Ca-conductance blockers, antiarrhythmic agents, digitalis) on the voltage-dependent and kinetic properties of the ionic conductances, on cellular metabolism and on transmembrane ionic gradients are analyzed.

2. Action of Opiates and their Antagonists on Cholinergic Transmission in the Guinea Pig Ileum, by Seymour Ehrenpreis, discusses in detail the actions of various opiates and their antagonists on ilea from normal and addicted guinea pigs. New concepts for the molecular mechanism of action of opiates, the development of tolerance, and production of withdrawal symptoms are suggested, with special regard to the interrelations between opiate effects and the prostaglandin system.

3. Pharmacology of Heart Cells During Ontogenesis, by Achilles J. Pappano, describes the techniques for studying heart cells in culture, morphological and functional development of cardiac tissues and drug induced modifications of the electrical and mechanical activity of embryo hearts.

Effects of cholinergic and adrenergic drugs, nicotine, tyramine, tetrodotoxin and digitalis glycosides are dealt with in detail, demonstrating the correlation between drug effects and the development of functional innervation.

4. Analysis of Dose-Response Relationships, by Douglas R. Waud, classifies the approaches to dose-response curves as empirical examination, kinetic analysis or mechanistic analysis. "While many of the ideas relevant to looking at dose-response relationship are simple one you have seen them, — writes the author — they are not trivial." This is why his review, first of all on kinetic analysis, dealt with in more detail, meets a long-felt want.

5. Cellular Pharmacology of Ganglionic Transmission, by Syogoro Nishi, gives an overall review of the field, summarizing the present knowledge of pre- and postsynaptic receptor sites, transmitter liberation and ionic and drug effects upon the postsynaptic neuron membrane.

The volume is of invaluable help to all those working in pharmacology and related fields.

K. KELEMEN

Intercellular Communication by W. C. De Mello, Plenum Press, New York, 1977

An increasing number of recent evidence suggests that many basic problems in cell biology, e.g. mechanism of intercellular material transfer, cell recognition during development, cell cooperation in immune response and failure to control cell multiplication after cancerous transformation, are closely connected with intercellular communication. It appears that a new branch of biology has recently emerged as a result of increasing interest in this field of research. This necessarily presumes a common approach to quite divergent topics and many lead to a common interpretation of different biological phenomena in general biochemical and biophysical terms.

The present volume has great merit in presenting up-to-date works with the clear tendency of revealing a biophysical and biochemical background for cell communication phenomena. Two of the nine chapters in the book, Chapter 2 and 5, present a pure

biophysical approach to the problems of cell-to-cell contact and electrical coupling. In this way the experimental material discussed in the preceding chapters, Chapters 1, 3 and 4, is provided with a firm theoretical background. Chapters 6—9 recent findings are collected and discussed that clearly demonstrate the relevance of intercellular communication to important topics in biology and medicine such as cell communication and cancer, cell interactions by neuronal and target cells, cell cooperations in the immune response, intercellular communications in the early embryo etc. These chapters, too, are characterized by an intention of throwing light on the biochemical changes in membrane functions that may determine both the biology and pathology of cell-cell interactions. Plenty of high quality microphotographs give information on the morphological basis of cell coupling, i.e. on the structure of gap functions between cells of different origin under various conditions. In the discussions medical aspects are given due consideration.

Therefore, the book is of great importance to researchers working in a broad range of biological and medical sciences. It may be particularly recommended to biochemists working with cultured cells.

The authors of the individual chapters are prominent experts of the presented subject and do work in outstanding research institutions.

The references are up-to-date and exhaustive.

L. LATZKOVITS

Interpretation of Biochemical Multitest Profiles. An analysis of 100 important conditions by Wolf, P. L. Masson Publishing, USA, Onc. New York, Paris, Barcelona, Milan, 1977, 296 pages, 102 charts and subject index.

It is one of the most fascinating handbooks that I came across in the past few years. According to the author a well known professor of pathology, the aim of this work is to help first of all the practicing physicians interpret biochemical multitest profiles.

Since the advent of the wide use of automatized analytical procedures in clinical chemistry, it has become customary to use one blood sample for multiple analyses. Depending on the capacity of the analyzer 6 or 12 different parameters can be estimated. This is, of course, cheaper and more rapid than carrying out the individual determinations one by one. The computerized analyzer draws a chart on which the abnormal patterns can be clearly distinguished. This is supposed to be of great help to the physician in evaluating the data.

The introductory part of this compendium contains a detailed but incomplete list of different conditions that will cause high or low values of various parameters.

The introduction is followed by 100 different charts obtained from 100 patients suffering from various pathological conditions. Each chart is completed by a brief clinical history and a number of references to help the reader in orientation.

Since the title of each chart contains only the main clinical symptoms and the most striking abnormal biochemical parameters, the final diagnosis of each case can be found in a numbered list which closes the whole compendium.

The author writes in the preface of the book, that the use of automated multitest screening is somewhat controversial, and its most important benefit is to obtain diagnostic information that is not apparent from the history, physical, hematology data and urinanalysis. In general I agree with this statement, but I think it needs a more circumstantial explanation. First about the contradiction. The undiscriminate use of computerization in practical medicine helps the physician neither in the hospital, nor in the general practice. The laboratory data per se do not resolve the diagnostic problems, especially if delivered in superfluous quantity, since careful observation of the patient is invariably the most important task of the physician. For example: panels 2, 3 and 4 are dealing with the biochemical parameters of a child suffering from diarrhea. It is obvious that severe loss of body-fluids leads to hyperelectrolytemia and hyperosmolarity and there is no surprise observing this phenomenon in the blood sample. But it is most surprising that with the most up-to-date laboratory facilities at hand, the subsequent panel shows the results of an obviously

inadequate treatment resulting in a markedly elevated blood glucose level. Nobody will think that in the course of events this patient has had diabetes mellitus. Now, the next panel shows the results of further 12 analyses and now we see the entire harmful effect of inadequate therapy. It is indeed very useful and perhaps practical to have a rapid computerized analytical procedure at hand, but in my oppinion adequate therapy could have been introduced without preliminary laboratory examinations and its result could have been correctly evaluated by the improvement of the patient's condition. Therefore it is only a virtual advantage to know 10—12 unimportant biochemical data, when any careful observation would guide the treatment in the proper direction.

The titles of the panels are also misleading. One example: Elevated serum calcium in a black male with pulmonary infiltrate. The calcium level in an adult does not depend on sex differences, has nothing to do with the colour of the skin or with the observed pulmonary lesion. A lot of unspecific but clearly abnormal findings were obtained in this case, but these did not help establish the correct diagnosis and consequently did not lead to the correct therapy.

The examples just mentioned are not rare in the compendium, and the reader, if not well acquainted with the whole clinical picture, may be misled by the pure laboratory data.

And here lies the most important controversy. The laboratory data alone do not mean too much. They mean only help — a very important one for that matter — in the exact clinical diagnosis. The clinician who must have the ability to synthetize all findings concerning the patient, has to distinguish clearly between the important and the unreliable facts. So far, these 100 panels collected in one volume, together with the case histories and references (of which two-third have already been updated) do not mean too much help in every day practice. Therefore it seems to me that this book is a well organized collection of very different, but not very interesting clinical histories rather than a guidance for the use of multiple biochemical analytical procedures.

P. KISS

Protides of the Biological Fluids, Proceedings of the 25th Colloquium, Bruges, 1977. Vol. 25, by H. Peeters (ed.), Pergamon Press, 1978

The Colloquium on "Protides of the Biological Fluids" was organized for the twenty fifth time in 1977. This annual meeting has become a tradition for a quarter of a century. This tradition is highly appreciated among workers in this field: those who took part in one or more of the meetings enjoyed the pleasant atmosphere and the vivid discussions as well as the charms of the town Brugge and its surrounding, whereas for those who did not participate in these meeting a carefully edited set of papers on mostly relevant topics by prominent authors has been made available. On the occasion of the silver jubilee, the organizer of the whole series of colloquia Dr. H. Peeters gave the Tiselius Memorial Lecture summarizing the 25 years of history of protein research as reflected in the meetings. I think he was right in stating that a textbook of protein biochemistry could be written up using the material of the proceedings of the colloquia only, together with a second volume on clinical implications pertaining to abnormal protein patterns in different biological fluids.

Turning to the scientific contents of the 25th volume we notice that the largest part is devoted to lipoproteins with various aspects of structure, metabolism, genetics, clinical diagnosis and therapy of disorders and methodology. As an introduction to this topic A. M. Gotto and R. L. Jackson summarized the present status of our knowledge on plasma lipoprotein structure and metabolism. P. Laggner's paper contained highly relevant new data collected by small angle X-ray scattering studies. Based on X-ray scattering data Laggner proposed a "lipid core" model for LDL assuming an amphilic outer shell and a core composed mainly of cholecterol ester molecules, while Essex et al. interpreted their data as being consistent with an insulating shell-conducting core model. Several studies were presented on the protein composition of lipoproteins and on the problems of protein-lipid interactions within the lipoprotein molecules. G. M. Kostner outlined the distribution of apolipoproteins in human serum lipoprotein subfractions. This highly informative review

was followed by a number of contributions on important details. A further series of papers discussed problems of lipoprotein biosynthesis and metabolism followed by thirteen reports of enzymatic processes associated with lipoproteins. Lipoprotein lipases and the lectincholecterol acyltransferase were the subject of most of these latter studies. In the section devoted to the genetics of lipoproteins there were reviews on recent studies of polymorphic systems of lipoproteins, the Ag and Lp system (K. Berg). The latter property is confined to a distinct, minor class of lipoproteins. Particular importance was attributed to the LpA lipoprotein because the risk of coronary heart disease is significantly higher in LpA+ than in LpA− individuals. Uterman et al. recognized allelic variations of apoprotein E by isoelectric focusing and showed that there was an association of type III hyperlipaemia (Broad-disease) with one of the autosomal alleles. Further topics related to lipoproteins included clinical data, animal models, drug effects and new methods in lipoprotein research.

The second main topic of the Colloquium was "Cell lines in the study of Lymphocyte Antigens and Receptors". Needless to say, this topic is highly relevant not only to modern immunobiology but also to genetics and cell membrane biology. *In vitro* cell lines can now be established by fusion of normal lymphocytes with mouse myeloma cells. The first paper of this section was by C. Köhler, H. Hengartner and C. Milstein who had done pioneering work in developing this new experimental approach. In this paper the authors reported clonal variants derived from hydrids of antibody-secreting cells with myeloma cells. Secretion of the polypeptide chains corresponding to the parental cells have been studied and loss of heavy chain expression was found to occur more frequently. Studies of hybridoma have been presented also by Hämmerling et al. They studied monoclonal antibody production by hybrid cells and also reported on hybridization of a T-lymphoma with normal sensitized lymphocytes. All the hybrid cell lines expressed Thy-1 antigens. Among the further papers there was a report on the establishment of an IgE secreting rat myeloma cell line (Beunich et al.) and an other on the *in vitro* induction of cytotoxic lymphocytes against Ly alloantigens (Röllinghof et al.) as well as several studies on the characterization of leukaemic cells and lymphoid cell lines. The reader can easily realize the vigorous progress in this very important field promising an insight into both malignancy and normal differentiation in the haemopoietic system. Further series of papers covered dynamics of surface receptors and antigens as well as studies of isolated cell surface, antigenic and receptor structures. The latter permit an insight into the chemistry of the cell surface structures while the former ones contain relevant informations on cell membrane mobility. The last part of the volume contains the proceedings of a symposium on IgA held in memoriam of J. F. Heremans. Professor Heremans was one of the founders of modern immunoglobulin research. A great part of this work was devoted to structural and functional studies of IgA. Papers presented in the symposium demonstrate that IgA research is continously producing new concepts and important results.

G. A. MEDGYESI

Quantitative Aspects of Allosteric Mechanisms by A. Levitzki (Springer Verlag, Berlin, Heidelberg, New York 1978), Published as the 28th volume of the Series Entitled "Molecular Biology, Biochemistry and Biophysics"

It has been a long time since I read such a good *and* such a bad book at once. This is neither a misprint nor a slip of the pen. The book is excellent because the topics discussed in it are very important for both the theoretician and the practicing enzymologist and have long been neglected; and the book is bad as it has no uniform sequence of ideas, it contains contradictions and it is not a mature work.

The book discusses the concept of allosteric regulation, the structure of oligomeric proteins, then the basic ideas about the cooperative interactions of the latter. The comparison of the Hill and Adair equations and the discussion of statistical corrections are very interesting. However, the treatment of the thermodynamics of interactions is too

brief and shallow. The exposition of the molecular models of the allosteric interaction and of cooperativity, their comparison with each other and with the thermodynamic model are useful and extensive.

As regards the unadvised writing, let us take just one example out of the many to be found. On page 26, in connection with the mixed negative-positive cooperativity it is stated that "In such a case the quantity v/V_{max} is not identical with the saturation function $\bar{Y}_x$ as determined from direct bonding studies". On page 27, however, it stands that "It is worth pointing out that in general it is found that the ratio v/V_{max} obtained from kinetic measurements represents the ligand saturation function $\bar{Y}_{\bar{x}}$ and therefore represents the ligand saturation function expected from binding measurements". And this is not true. It has not been generally *found* to be so; it has been generally, but incorrectly assumed to be so. As a matter of fact, v/V_{max} equals $\bar{Y}_x$ only (i) if the conformational change preceding the rate-limiting step is fast and reversible; (ii) if, in the case of a symmetry model, the system in question is a pure K-system; and (iii) if, in the case of a sequential model, the various complexes (ES_1, ES_2, . . . ES_n) dissociate to free enzyme and product with an identical rate constant. The validity of the above conditions must be proved by independent experiments before using the v/V_{max} ratio for the determination of $\bar{Y}_x$. Be it said only incidentally that what has been said above applies only to enzymes of "simple kinetics" and the identity of v/V_{max} with $\bar{Y}_x$ is still less true of enzymes that are e.g. inhibited or activated by excess substrate. However, this is not the end of this series of inconsistencies. On page 82, it is written that ". . . many authors have assumed that the extent of ligand saturation, $\bar{Y}_x$ can be measured by the ratio v/V_{max} . . . Hence as a working hypothesis, kinetic experiments are useful as a diagnostic tool but they must always be followed by binding experiments to be sure of the cooperative pattern." Well, that is exactly what should have been said on page 27 already!

Of the 61 works listed in the References, 20 are by Levitzki or Koshland, which would be just all right if some basic works such as Endrényi, L., Fajszi, Cs., Kwong, F. H. F. (1975): Evaluation of Hill slopes and Hill coefficients when the saturation binding or velocity is not known (Eur. J. Biochem. *51* 317) or Kurganov, B. I., Kagan, Z. S., Dorozhko, A. I., Yakovlev, V. A. (1974): Kinetic manifestations of allosteric interactions in models of regulatory enzymes with "indirect" cooperativity (J. Theor. Biol. *47* 1) had not been omitted, since it is just these theoretical problems that are mostly discussed in this book. No less conspicuous is the absence of the results of a series of publications by Kurganov et al. [The theoretical analysis of the kinetic behaviour of "hysteritic" enzymes I—IV, J. Theor. Biol. *60* 247 (1976), *60* 271 (1976), *60* 287 (1976), *68* 521 (1977)].

It would not be fair to blame the author of a book published in 1978 for having not cited the basic work of W. G. Bardsley and R. D. Waight (1978): The determination of positive and negative cooperativity with allosteric enzymes and the interpretation of sigmoid curves and non-linear double-reciprocal plots for the MWC and KNF models (J. Theor. Biol. *70* 135), had Bardsley not published part of the problem of graphic analysis previously [J. Theor. Biol. *65* 281 (1977), *67* 121 (1977), *67* 407 (1977)]. Since, according to the Preface, the book was put to press in January, 1978, the reader is justified for disapproving the absence in this book of closely related works published in 1974—1977. Except for one article published in 1975, all of the most recent citations (apart from those by Levitzki and Koshland) date from 1973 (6 altogether) and the rest from even earlier.

In spite of all these faults this book is one that should be at hand in the library of the enzymologist as it contains many excellent and often needed formulations that are rarely found elsewhere. The explicit expression of the Hill coefficient for all the models discussed (including the thermodynamic model, too) both for dimers and tetramers should be specially emphasized as it indeed meets a long-felt want and may help put an end to many previous misinterpretations.

The book was issued in the usually neat presentation of Springer Verlag and, what is more important, in a very short time.

T. KELETI

Biophysikalische Aspekte der elementaren Zellfunktionen (Jacob Segal), VEB Georg Thieme, Leipzig, 1978

This book is the 4th part of a series of "Aspekte". Professor Jacob Segal, emerited Professor of the Humboldt University, Berlin, is not biophysicist, but in this book he summerizes up his biophysical views. He did this in a very fortunate form. This book practically covers all the fields of biophysics, though nobody knows the exact borders of this discipline of science. Biochemists and physiologists regard themselves biophysicists and this is the case in Segal's one, as well, who is worker in general biology, and perhaps therefore he needed a book, in fact dealing with the main fields of biophysics.

The book is up-to-date and good, in spite of the dumping of informations of the last years. He presents a lot of literature, though not in the most fortunate form. It is trough that it may be advantageous to cite the most recent work of a given author — from which his earlier works may be detected — but this aim unevitably leads to the result that classical works of this field will be neglected.

Just this is the case in the topic of the membrane function. The author is very objective. He is discussing the membrane theory, as well as the phase theory, but without mentioning the bound Potassium concept of the Hungarian biophysical school.

At any rate, the book deals with practically all fields of biophysics, but knowing the author's views, his dearest topic is motility.

The topic of motility extends from the amoeboid movement to the cell mitosis, but the most important part of it is muscle movement. It is just this part — though carefully written — in which I do not agree in some aspects. Professor Segal discusses the muscle movement on the basis of the sliding filament theory, without taking into consideration its paradoxons. At resting length the length of the overlap is not 50 per cent but — except a short zone, called pseudo H-zone by H. F. Huxley and L-zone by Sjöstrand, not containing myosin heads, — is practically total. This may be demonstrated by LASER-diffraction technics in a very convincing manner. This means furthermore, that in the resting sarcomere there is no H-zone, it appears merely, when the muscle will be streched. On the other hand, when the muscle shortens, starting from the resting length, it seems that — after passing the M-line — it forms a zone of double overlap of thin filaments successively broadening with the degree of shortening. The sliding model did not deal with this important range of contraction. I agree with professor Segal that M-lines are no membranes but many of us demonstrated that — apart from additional M-material — it consists of a system of stable cross-bridges (not to be confused with myosin-actin cross links). This is, why I do not agree with the author to call the M-line H-line, since this term is already occupied for the H-zone.

I am not convinced that the Z-line would represent a real membrane in the proper meaning of the word, neither am I convinced that it would belong to the connective tissue.

A proposed principle of the architecture of the myosin filament seems in fact very ingenious.

The description of the mechanisms of non-muscular movement is very extensive. It is now a frequently returning question and it is a great merit of the book that it summarized up this highly important problem.

Perhaps the most immature but most daring chapter is cancerogenesis. This does not mean that it were unnecessary.

Summarizing up, Professor Segal's book is a useful one. It deserves to be published in a 2nd Edition, together with its three forerunners. The time of publication seems somewhat slow, from the side of the Publisher.

Finally I express that I deeply agree with the following statement of Professor Segal: "We do not believe that a long-living theory were necessarily a good theory. Not exactly formulated theories offering no points for criticism are very often sterile ones. Our aim was in contrary, to stimulate to critical disputes, from which valuable thoughts have to originate . . ."

N. GARAMVÖLGYI

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THE NEW YORK ACADEMY OF SCIENCES

Title: Calmodulin and cell functions

Sponsor: The New York Academy of Sciences

Dates: May 21—23, 1980

Place: The Roosevelt Hotel, New York City

Theme: Calmodulin is a calcium binding protein that has no known enzymatic activity but will apparently regulate several enzyme activities, affect microtubule assembly *in vitro* and bind antipsychotic drugs. This conference will bring together investigators from a variety of fields to discuss the biochemistry, physiology, enzymology, cell biology and pharmacology of calmodulins and the possible role of calmodulins in regulating cell functions. The conference will feature invited lectures and a limited number of poster presentations. Preference in selection of poster sessions will be given to young investigators and to novel and rigorous studies. The deadline for submission of abstracts is March 1, 1980. The abstracts should include the name of the author(s), institution and 100—200 word summary of the work to be presented in the poster. Send abstracts for poster sessions and inquiries about details of the program and registration to:

Conference Director
The New York Academy of Sciences
2 East 63rd Street
New York, NY 10021.

Chairs: Dr. FRANK F. VINCENZI, Department of Pharmacology, School of Medicine, University of Washington, Seattle, Washington 98195; and Dr. MARTIN WATTERSON, Department of Cell Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10021.

For further information, contact: Conference Department, The New York Academy of Sciences, 2 East 63rd Street, New York, New York 10021. (212) 838—0230.

SECOND INTERNATIONAL CONGRESS ON CELL BIOLOGY

August 31—September 5, 1980 Berlin (West) sponsored by the International Federation for Cell Biology (IFCB), organized by the European Cell Biology Organization (ECBO)

SCIENTIFIC PROGRAM

The entire scientific program is being arranged in such a way that all events will take place at the International Congress Center. The Organizing Committee, assisted by the Scientific Advisory Board, is planning a program that will include topics of broad and current interest in cell biology. There will be about 25 major symposia and a series of special workshops. Symposia will include contributions by invited speakers, platform papers and posters. Special Workshops, probably on Wednesday morning and Thursday night, will be dedicated to topics of special interest and critical discussions of important and novel methods used in cell biology. In addition, there are plans for workshops dedicated to the discussion of special problems of experimenting with cultured cells. Poster contributions will, if possible, be organized in association with major symposia and workshops, and will be located in the immediate vicinity of the respective lecture halls.

A tentative list of symposia and workshops includes the following:

Gene Arrangements and Re-Arrangements in the Nucleus
Arrangements and Activities of Mitochondrial and Plastidal Genes
Supranucleosomal Levels of Organization in Chromatin and Chromosomes
Organization of Transcription and Posttranscriptional Events
Non-Chromatinous Structures of the Nucleus
Meiosis and Control of Meiotic Stages
Cultured Plant Cells, Protoplast Fusion and Plant Cytogenetics
Membrane Architecture and Experimental Manipulation of Membrane Composition and Properties
Signal and Energy Transduction in Biological Membranes
Translocation, Fusion and Recycling of Membranes
Liposomes as Models and Tools in Membrane Research
Mechanisms of Membrane Synthesis and Assembly
Ribosomes and Ribosome-Membrane Interactions
Modes of Formation of Extracellular Skeletal Components (e.g. Chitin, Cellulose, Silica, Collagen, Elastin)
Endocytosis and Lysosomes
Cell-Cell Recognition and Intercellular Communication
Microfilaments, Actin, Myosin and Associated Proteins
Microtubules and Associated Components
Intermediate-Sized Filaments
Disorders of Arrays of Contractile and Cytoskeletal Structures and their Pathological Significance
Teratocarcinoma Cells in Research on Differentiation
Formation and Maintenance of Differentiated and Transformed States
Early Embryonic Development
Cellular Immunology

Cell Polarity
Cellular Clocks and Rhythms
Biomineralization
Identification and Functions of Epithelial Cells in Culture
Intracellular Distribution of Water and Ions
Monoclonal Antibodies and their Potential in Cell Biology Research
Special Cell Culture Methods, incl. Mass Cultures
Cell Biology of Tropical Parasites
Disorders in the Extracellular Matrix
Cell Biological Studies on Invasive Growth and Metastasis
Host-Fungus Interaction
In addition, it is planned to have, throughout the whole week, a common methodological poster exhibition in which few methods in cell biological research will be displayed and discussed

HISTORIC EXHIBITIONS

There will be two historic exhibitions on methodical developments central to progress in cell biology:
The Development of the Light Microscope — Chairman: D. Olins, Oak Ridge, USA
The Development of the Electron Microscope — Chairman: E. Ruska, Berlin (West)
Organization and coordination of both are in the hands of P. Giesbrecht, Berlin (West) and K. Lemmerich, Berlin (West)

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SECOND INTERNATIONAL CONGRESS ON TOXICOLOGY

BELGIUM-BRUSSELS, JULY 7-11, 1980

The congress will be dedicated to "Mechanisms in Toxicity and Hazard Evaluation".

The main topics to be treated are:

- recent developments in mechanisms of neurotoxicity
- short-term tests for predicting long-term effects
- early changes in chemical carcinogenesis
- long-term exposure to occupational intoxicants
- clinical toxicology
- legislative scientific and socio-economic consideration underlying toxicological texting of new chemicals

We would appreciate it if you could announce this congress in either the "Calendar of Events" or "Congresses" in your journal or mention this congress in a write-up. The first announcement is enclosed for your information.

Information about this congress can be obtained at:
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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 14 (4), pp. 213—216 (1979)

Effect of Specific Antibodies on Membrane Microviscosity of Human Lymphocytes

(Short Communication)

Margit BALÁZS, J. SZÖLLŐSI, B. SOMOGYI, S. DAMJANOVICH

Department of Biophysics, Medical University of Debrecen, Debrecen, Hungary

(Received July 19, 1979)

Emission anisotropy of the fluorescence dye DPH is extensively used to monitor intramembrane microviscosity of surface cell membranes (Shinitzky et al., 1976; Haggerty et al., 1978). The lipid soluble dye dissolved in the lipid moiety of he fluid mosaic membrane gives information about the overall fluidity of the cell surface membrane, which is in close relation to cellular regulation. That is why differences in parameters controlling cellular metabolism can result in different values of membrane viscosity by the use of DPH (van Hoeven et al., 1979; Cossins et al., 1977; van Blitterswijk et al., 1977).

The presence of bound or adsorbed proteins on the cell surface can also influence membrane fluidity (Hilgers et al., 1978; Inbar et al., 1973). In spite of the many investigations carried out with the DPH-technique, little attention has been paid to this latter point (Rédai et al., 1980).

This paper deals with the effect of specific antibodies on membrane fluidity of lymphocytes as determined by DPH. The experiments show remarkable effects of antibodies on membrane fluidity. It is also shown that, by changing the sequence of adding the DPH and the antibody, different effects can be obtained suggesting that either the DPH produces a remarkable structural change in the cellular membranes interfering with the action of antibodies or that the antibodies alter some characteristics of the DPH binding sites.

Human lymphocytes were prepared according to Boyum (1968). Labeling of cells with DPH (Sigma) was carried out as follows: 0.01 ml of 6×10^{-3} M DPH in tetrahydrofuran was injected into 20 ml of vigorously stirred PBS, pH 7.2. Stirring was continued for 120 min at room temperature. The clear dispersion of 3×10^{-6} M DPH has practically no fluorescence. One volume of DPH dispersion was mixed one volume of cell suspension and incubated for 30 min at 37 °C. The labeled cells were washed twice and resuspended in PBS. One volume of DPH-treated or control cells (1.5×10^6 cell/ml) was incubated with one volume of antibody solution for 60 min at 37 °C. The antibody solution was anti human lymphocyte globulin gained from horse (Behringwerke AG, Marburg-Lahn) and containing 50 mg

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; PBS, phosphate buffered saline

protein/ml. It consisted of 330 mg% IgG, 42 mg% IgA and 17 mg% IgM as determined by immunoelectrophoresis. The solution did not contain active complement components. Then the cells were washed twice and resuspended in PBS. Control cells were labeled with DPH after the antibody treatment.

Steady state fluorescence polarization was measured in a Hitachi MPF-4 fluorescence spectrophotometer equipped with polarizers. Thermostated (37 °C) 300 μl cells were used and all samples were measured immediately after completig the different treatments. To reduce experimental error due to scattered light in fluorescence polarization measurements (Shinitzky et al., 1973; Inbar et al., 1973), cell concentration was kept below 5×10^5 cell/ml during the measurements (Inbar et al., 1974).

Fluorescence anisotropy (r) was calculated as:

$$r = \frac{I_{VV} - G\, I_{VH}}{I_{VV} + 2G\, I_{VH}}$$

where I_{VV} and I_{VH} are the fluorescence intensities measured with vertical and horizontal analyzers, respectively, keeping the polariser in vertical position, and G is the correction factor ($G = \frac{I_{HV}}{I_{HH}}$). Microviscosities were calculated according to the Perrin equation (Perrin, 1926):

$$\frac{r_0}{r} = 1 + C(r)\frac{T\tau}{\eta}$$

where $r_0 = 0.362$ is the limiting fluorescence anisotropy (Shinitzky et al., 1971; Shinitzky et al., 1974), r is the measured fluorescence anisotropy, $\tau = 9.7$ nsec is the excited state lifetime of DPH at 37 °C, as estimated indirectly from the temperature profile of the fluorescence intensity, assuming the limiting value $\tau_0 = 11.4$ nsec at 0 °C as determined by Shinitzky and Barenholz (Shinitzky et al., 1974), η is the microviscosity of the medium in which the DPH molecules are embedded, and $C(r) = (8.5 \pm 0.4) \times 10^5$ poise $deg^{-1}\, s^{-1}$ is the parameter which relates to the molecular shape of the fluorophore and has a specific value for each r (Shinitzky et al., 1974).

The membrane viscosities of human lymphocytes labeled only with DPH were practically independent of the applied concentrations of DPH and lymphocytes and of the emission wavelength within the range of 425–450 nm.

Antibody-labeled human lymphocytes showed marked differences in membrane fluidity as determined by the DPH fluorescence polarization probes.

When antibody was added to DPH-treated lymphocytes, the emission anisotropy of DPH indicated an increase in membrane viscosity compared to that of the control. This increase in membrane viscosity depended on the antibody concentration (Table 1).

However, upon changing the sequence of treatments, i.e. adding first the antibody and then the DPH, there was practically no observable change in mem-

Table 1

Effect of antihuman lymphocyte globulin on the outer membrane of human lymphocytes

Antibody dilution	Cell + DPH + antibody		Cell + antibody + DPH	
	r	η (poise)	r	η (poise)
1 : 100	0.241	5.65	0.186	2.74
1 : 200	0.202	3.27	0.181	2.57
1 : 300	0.192	2.92	0.170	2.29
Control	0.171	2.32	0.175	2.42

brane viscosity. This indicates that DPH, thought to perturb the membrane structure only very slightly, can produce a remarkable change of the membrane at least in relation to the structure mediating the action of antibodies. This is supported by the results of fluorescence microscope studies, in which FITC conjugated antiglobulin was used for antibody treatment. This experiment clearly showed that there was no observable capping when the cells were treated first with DPH. However, capping always developed when DPH treatment followed the antibody treatment.

Thus, DPH treatment produces certain changes in cellular membranes which prevent the capping. A possible interpretation of this phenomenon is the following: the binding of antibody to its receptors results in a stiffening of membrane structure in the near vicinity of the complex. This effect can be monitored by the DPH molecules located near the complexes. When capping has developed, only a few DPH molecules are close enough to the complexes to indicate the change, while the remaining majority show an unchanged viscosity.

It seems to be clear from the above that the binding of specific antibodies results in a structural change of the outer membrane that can be monitored by the emission polarization of DPH. This structural change should account for the fact that the fluorescence intensity of DPH incorporated in membranes is three times lower if cells are incubated first with the antibody. It has been proved that specific bound antibodies can modify the membrane structure, at least in the vicinity of their receptors. This could result in artefacts when cells are characterized by their viscosity or other membrane parameters, without checking for antibody content.

There are experimental facts to prove that emission anisotropy of DPH cannot be directly related to membrane viscosity (Sene et al., 1978). On the basis of the above results the use of DPH as a tool to measure membrane viscosity is questionable from a different viewpoint as well. Since DPH itself causes substantial changes in membrane structure great care and thorough analysis is required before any conclusions are drawn concerning fluidity changes deduced from alterations in the fluorescence anisotropy of the DPH dissolved in the lipid regions of the membrane.

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Acta Biochim. et Biophys. Acad. Sci. Hung. Vol. 14 (4), pp. 217—222 (1979)

Localization of Deoxyribonucleic Acid-Stimulated Adenosine Triphosphatases in Human Lymphocytes

Adel OHLBAUM, S. CSUZI, G. BÁNFALVI, F. ANTONI

Institute of Biochemistry I. Semmelweis University Medical School, Budapest, Hungary

(Received July 12, 1979)

Two enzymes hydrolyzing ATP (ATPase A and ATPase B) were purified from freshly isolated lymphocytes of human tonsils. Both enzymes are stimulated by single-stranded DNA and seem to be localized in the chromatin. ATPase A and ATPase B appear to be distinct enzymes as judged from their elution profiles obtained after DEAE-cellulose and ATP-Sepharose column chromatography, from their behavoir towards actinomycin D, a DNA intercalating agent, and from their sensitivity to monovalent salt concentration.

Abbreviations: NET, buffer (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0); RSB, reticulocyte standard buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.0); SSC, buffer (15 ml NaCl, 1.5 mm M sodium citrate pH 7.5), Tris, buffer (10 mM Tris-HCl, pH 8.0, 0.2 mM 2-mercaptoetanol); buffer A, (0.2 mM EDTA, 5 mM 2-mercaptoethanol, 50 mM Tris-HCl, pH 8.0), buffer B, (buffer A plus 30 v/v % glycerol); incubation buffer (50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 μg bovine serum albumin).

Introduction

Although the individual steps of DNA replication are better understood in prokaryotes than in enkaryotes, many components of the multienzyme complex participating in replication and repair of DNA in eukaryotic cells have been identified in the last years (Sheinin et al., 1978). DNA replication is dependent on ATP (Kornberg, 1974) and a search for eukaryotic enzymes that utilize ATP has been succesful (Otto, 1977; Hachmann, Lezius, 1976). Since DNA synthesis occurs in the cell nucleus, it seems reasonable to look for the localization of enzymes engaged in DNA replication in this particular cell organelle. The present paper deals with the localization, purification and characterization of two ATP phosphohydrolases. These ATPases were stimulated by single-stranded DNA and were isolated from chromatin of human tonsillar lymphocytes.

Materials and methods

Preparation and fractionation of lymphocytes. Lymphocytes were extracted from 20 human tonsils as described previously (Ohlbaum et al., 1977). All following operations were performed at 0°C. Cells were fractionated essentially according to Bekhor and Feldman (1976). The cells were washed with NET buffer, suspended in

100 ml of RSB buffer and stirred for 5 min. The swollen cells were homogenized with about 20 strokes in a teflon-glass homogenizer. Nuclei were harvested by centrifugation at 1000 *g* for 5 min. The supernatant constituted the cytosol. The pelleted nuclei, after washing twice with RSB buffer, were suspended in 50 ml of Tris buffer, stirred for 5 min and homogenized with a total of 40 strokes in a teflon-glass homogenizer. The chromatin was pelleted by centrifugation at 20 000 g for 10 min and the supernatant was saved as nucleosol. The chromatin was resuspended in 30 ml of buffer A. Samples from each step were kept at −20 °C.

Enzyme extraction and purification. The enzymes were extracted mainly by using the technique described by Hachmann and Lezius (1976). Chromatin preparations were slowly thawed at 4 °C. Five ml portions were disrupted with 2 × 30 sec pulses from a Sonic 300 dismembrator or by stirring overnight in buffer A and centrifuged at 20 000 g for 30 min. The chromatin, the cytosol and the nucleosol fractions were dialyzed overnight against 100 vol of buffer B and subjected separately to the following purification steps: adsorption to DEAE-cellulose, chromatography on DEAE-cellulose and chromatography on ATP-Sepharose.

Enzyme assays. Adenosine triphosphate hydrolase activity was determined by measuring the orthophosphate liberated from γ-^{32}P-ATP. The standard reaction mixture contained 50 μl of dialyzed enzyme in buffer B, 100 μl incubation buffer 80 ng heat-denatured DNA from chicken erythrocytes and 0.5 mM labelled ATP (10 – 50 cpm per mol). After 20 min incubation the reaction mixture was stopped by addition of 300 μl 4% charcoal suspension in 0.25 M HCl and 200 μl of ethanol. After vigorous shaking the charcoal was removed by centrifugation. 300 μl supernatant was added to 5 ml distilled water and radioactivity was measured by means of the Cerenkov effect. Control assay without enzyme was always made in parallel.

Deoxyribonuclease activity was measured with native and denatured DNA as previously described (Ohlbaum et al., 1977) using the same conditions as in the ATPase assay.

γ-^{32}P-ATP was prepared by the method of Post and Sen (1967). ATP was coupled to AH-Sepharose 4B as described by Lamed et al. (1973). Protein was determined by the method of Lowry et al. (1955). Heat denaturation of chicken erythrocyte DNA (80 μg per ml) was performed by heating it at 100 °C for 10 min in SSC buffer followed by quenching the sample in ice.

Results

Purification. Fig. 1 shows the distribution of single-stranded DNA-stimulated ATPase in a chromatin preparation after DEAE-cellulose chromatography. DNase and DNA-independent ATPase activities are located close to the DNA-dependent ATPase activity. Fractions 8 to 16 were pooled and dialyzed against buffer B and applied to an ATP-Sepharose column. DNase was not absorbed by this column. Fig. 2 shows that ATPase activity in the absence of single-stranded DNA (ssDNA) was reduced to about one third of the original value. ssDNA-stimulated ATPase

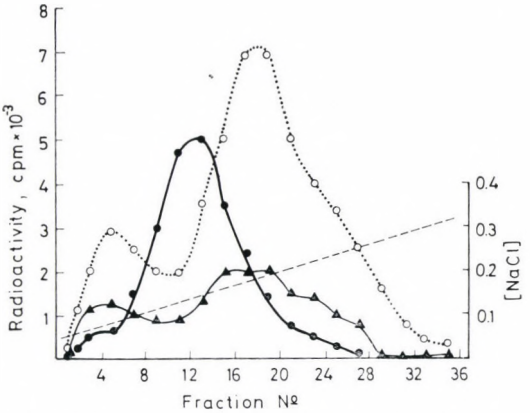

Fig. 1. DEAE-cellulose chromatography of a chromatin preparation. A DEAE-cellulose column (2.5 $cm^2 \times 8$ cm) was equilibrated with buffer B and loaded with 2 mg protein. Proteins were eluted with a linear gradient of 0 — 0.4 M NaCl in buffer B. 4 ml fractions were collected and 50 μl was used for enzyme assay as described in "Materials and methods". ATPase activity in the presence of ssDNA (○ . . . ○) and in the absence of DNA (▲ — ▲). DNase activity (● — ●), NaCl concentration gradient (- - -)

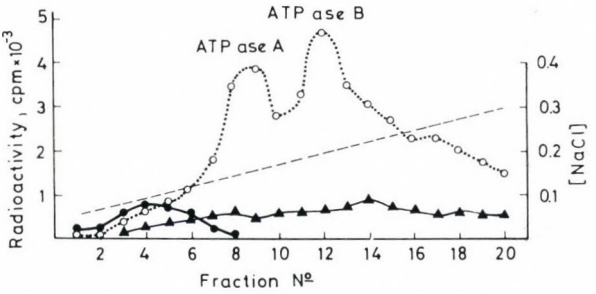

Fig. 2. ATP-Sepharose chromatography of the pooled fractions obtained by DEAE-cellulose chromatography. Elution from a 2.5 $cm^2 \times 1$ cm column was performed by a linear gradient of ○ — ○. 4 M NaCl in buffer B. 4 ml fractions were collected and 50 μl was used for enzyme assay as described in "Materials and methods". ATPase activity in the presence of ssDNA (○ . . . ○) and in the absence of DNA (▲ — ▲). DNase activity (● — ●), NaCl concentration (- - -)

activities appear in two peaks: one at 0.15 M and the other at 0.20 M NaCl concentration. These will be referred to as ATPase A and ATPase B, respectively. Fractions of each peak were pooled separately, dialyzed against buffer B and stored at −20 °C.

Localization. Different cell fractions were separated from each other and purified as described in "Methods". The ATPase activities of the cytosol, nucleosol and chromatin fractions were determined and expressed in nmol inorganic phosphate per mg protein. As shown in Table 1 isolated chromatin exhibited the highest specific activity amongst the cell components tested.

Table 1

Specific activity of ATPase after an ATP Sepharose purification step
Specific activity of ATPase means the sum of the specific activity of ATPase A and ATPase B obtained after ATP-Sepharose purification

	ATPase activity nmol Pi/mg prot
Cytosol	90
Nucleosol	91
Chromatin	345

Table 2

Effect of bivalent cations on the activities of DNA stimulated ATPases
Purified ATPase A and ATPase B were tested as described in "Methods" with bivalent cations present at 2 mM concentrations. ATPase activities are given in nmoles of hydrolyzed inorganic phosphate (Pi) and also expressed as per cent relative activities

Divalent cation 2 mM	ATPase A		ATPase B	
	nmol of Pi	%	nmol of Pi	%
Mg^{2+}	120	100	110	100
Mn^{2+}	118	100	110	100
Zn^{2+}	43	36	62	56
Ca^{2+}	43	36	40	34
Cu^{2+}	0	0	0	0
$Mg^{2+} + Mn^{2+}$	120	100	112	100
$Mg^{2+} + Zn^{2+}$	48	34	61	55
$Mg^{2+} + Ca^{2+}$	42	35	31	28
$Mg^{2+} + Cu^{2+}$	0	0	0	0

Table 3

Effect of NaCl concentration on ATPase A and ATPase B
Purified enzymes were tested as described in "Materials and methods" in the presence of indicated concentrations of NaCl

NaCl mM	ATPase A %	ATPase B %
0	100	100
10	84	109
50	64	91
100	48	80
200	24	43
500	7	17

ATP optimum and effect of ATP analogues. ATPase A and ATPase B follow the same saturation curve, and the apparent K_M values calculated were 0.28 mM for ATP in both cases. Experiments with two ATP analogues – adenosine 5'-(β,γ-methylene)-diphosphate and adenosine 5'-(β,γ-imino)-diphosphate used in non radioactive form in combination with labelled ATP – showed that only the imino compound was recognized by both enzymes.

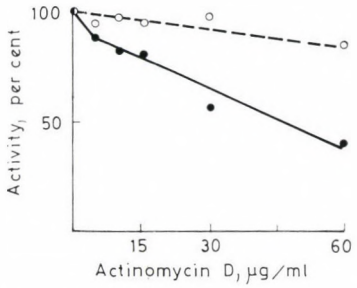

Fig. 3. Effect of actinomycin D on the activity of ATPase A and ATPase B. Purified enzymes were tested as described in "Materials and methods" with the concentrations of actinomycin D as indicated. Activity of ATPase A (● – ●) and that of ATPase B (○ – ○)

Specificity for nucleic acid cofactors. Both enzymes required ssDNA as cofactor. The optimal concentration for the A and B enzymes was reached at 0.5 μg per ml ssDNA. ssDNA was a six times more effective cofactor than double-stranded DNA.

pH optimum. The ssDNA-stimulated ATPase activity of both enzymes was relatively unaffected by pH changes. The enzymes showed a broad pH optimum ranging from 4 to 9.5.

Requirement for bivalent cations. The activities of enzymes A and B were strongly dependent on bivalent cations. There was no difference in Mg^{2+} requirement between enzymes A and B, maximal activity was reached in the presence of 1–2 mM $MgCl_2$. The effect of other bivalent cations, either alone or in the presence of Mg^{2+} was also investigated. The data presented in Table 2 indicate that Mn^{2+} can substitute Mg^{2+} and that Cu^{2+} completely inhibits the activity of both enzymes. Zn^{2+} and Ca^{2+} are less effective cofactors than Mg^{2+}.

Effect of monovalent cations. The influence of NaCl in the incubation medium was assayed for both enzymes, and data presented in Table 3 show that ATPase A is more sensitive to monovalent cations than ATPase B.

Effect of actinomycin D. The effect of the DNA intercalating agent, actinomycin D is presented in Fig. 3. The drug preferentially inhibits ATPase A.

Deoxiribonuclease activity. In the first steps of purification the enzymes copurified with DNase, however, after purification through an ATP-Sepharose column, ATPase A and ATPase B failed to show nuclease activity either with ssDNA or with the duplex as substrate.

Discussion

The biochemical properties of the ATPases purified from human tonsillar lymphocytes are similar to those obtained with corresponding enzymes from myeloma cells (Hachmann, Lezius, 1976), from stimulated lymphocytes (Otto, 1977) or from Vaccinia virus core (Paoletti et al., 1974). Both ATPases, as far as tested, were free of nucleolytic activities after ATP-Sepharose chromatography. It has been demonstrated that Concanavalin A-stimulated lymphocytes, in contrast to resting cells, contain at least three DNA-stimulated ATPases (Otto, 1977). However, the localization of these ATPases has not been reported yet. The presence of more than one DNA-stimulated ATPase activity in tonsillar lymphocyte populations suggests that freshly prepared cells from tonsils are in a stimulated state, confirming the observation made by another research group of our Institute (Antoni and Staub, 1978).

Our experiments presented in this report show that ssDNA-stimulated ATPases occur mainly in the chromatin of eukaryotic cells. If the A and B enzymes are proteins involved in the replication chain, it is not surprising to find them in the cell compartment where these events actually take place.

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Changes in Membrane Fluidity of Mast Cells, Induced by Antigen-Anaphylactic Antibody Reaction

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Binding of IgE to rat mast cell receptors caused a distinct decrease of membrane fluidity. The antigen-IgE antibody reaction was followed by rapid changes in membrane fluidity of sensitized mast cells. In the earliest phase of the antigen-antibody reaction membrane fluidity decreased, however, this decrease was followed by a quick increase 10 min. later.

It is known that, in anaphylactic reactions, binding of antigens to sensitized mast cells results in a release of histamine and other biologically active substances, however, the mechanism of the trigger is not clear. The present paper provides data showing that sensitization of mast cells as well as the antigen-anaphylactic antibody reaction is followed by membrane fluidity changes.

Materials and methods

Peritoneal exudate cells from unstimulated Wistar rats were collected by peritoneal washing with 15 ml Hanks' BSS. The peritoneal cells were sedimented by centrifugation at 800 *g* for 5 min and washed twice. The mast cells were purified on 35% Ficoll according to the method of Conrad (Conrad et al., 1975). The recover of mast cells was approximately 60% in the final preparation, which had a purity of 75–90%. The contaminating cells were mostly small lymphocytes.

Immunization of rats and titration of IgE and IgG_{2a}: Wistar rats (200–250 g) were immunized by intraperitoneal injection of 30 mg egg albumin and Bordatella pertussis vaccine containing 10^{10} organisms (Human, Budapest). Then 30 mg egg albumin was injected i.m. every second day. The animals were bled two weeks after the last treatment and the IgE and IgG_{2a} antibody contents of sera were estimated by passive cutaneous anaphylaxis (PCA) reaction according to Jarret (Jarret, Stewart, 1974).

As shown in Fig. 1, there was a marked positive PCA reaction at a 1 : 8 dilution of the sera. A negativ PCA reaction was observed after 24 and 48 hours

Abbreviations: BSS, Hanks' buffered salt solution DPH, 1,6-diphenyl-1,3,5-hexatriene PBS, phosphate buffered saline PCA, passive cutaneous anaphylaxis.

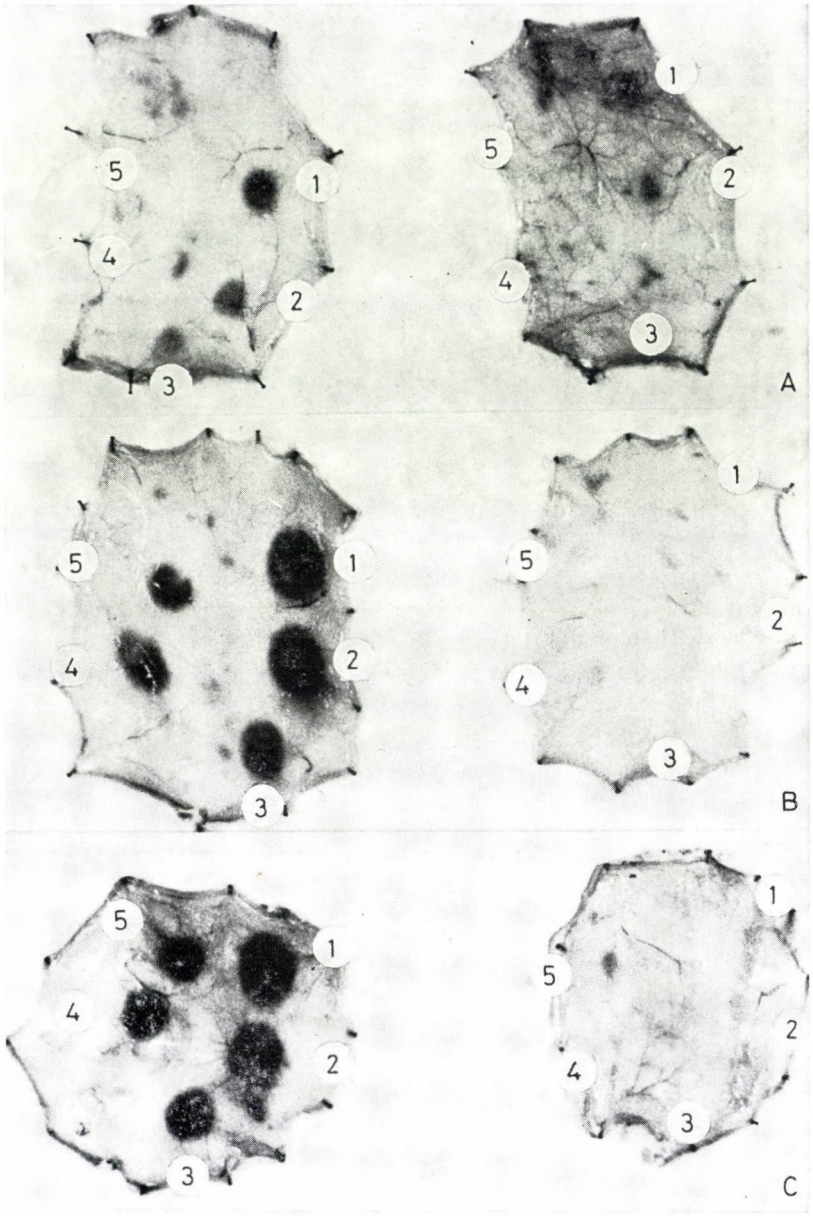

Fig. 1. PCA reactions induced by intact IgE-rich rat sera and sera inactivated at 56 C°. Challenges were made 3 h (A), 24 h (B) and 48 h (C) after i.c. injection of sera, the numbers represent the serum dilution from 1 : 4 to 1 : 64

with sera treated at 56 °C for 1 hour, at the same time a strong positive PCA reaction was obtained with nontreated sera at a dilution of 1 : 64. Thus the serum was classified as an IgE-rich serum.

Mast cell activation: 50 μl of the cell suspension (10^6 cell/ml) were added to 50 μl of undiluted immunoserum. Passive sensitization of mast cells was achieved by incubation at 37 °C for 30 min. The cells were washed twice with PBS and labelled with 2×10^{-6} M 1,6-diphenyl-1,3,5-hexatriene (DPH) at 37 °C for 30 min according to Shinitzky (Shinitzky and Inbar, 1974).

Measurement of fluorescence polarization: A practical method for monitoring microviscosities of membrane lipids is fluorescence polarization utilizing DPH as fluorophore. Fluorescence polarization (P) was determined by simultaneous measurements of I_W and I_{VH}. I_W and I_{VH} are the fluorescence intensities of the emitted fluorescent light vertical and horizontal to the plane of polarization of the excited beam, respectively.

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \qquad G = \frac{I_{HV}}{I_{HH}}$$

G is the correction factor of the polarizers. The P value and the lipid fluidity have reciprocal relationship i.e. the higher the polarization of the cell the lower the lipid fluidity. The accuracy of the measurement of P is about ± 0.005 (Shinitzky, Inbar, 1974; Shinitzky et al., 1971). The polarization of DPH-labelled mast cells was determined at 37 °C in a Hitachi MPF-4 fluorescence spectrophotometer equipped with polarizers. Microviscosities were calculated according to the Perrin's equation (Perrin, 1934):

$$\frac{r_0}{r} = 1 + C(r)\frac{T\tau}{\bar{\eta}}$$

where r_0 = the limiting fluorescence anisotropy having the value of 0.362 (Shinitzky and Barenholz, 1974).

r = the fluorescence anisotropy, i.e. $r = \dfrac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$

τ = the excited state lifetime of DPH

$\bar{\eta}$ = the microviscosity of the medium where the DPH molecules are embedded

$C(r)$ = the parameter which is related to the molecular shape of the fluorophore and has a specific value for each r (2). Its value is 8.6×10^5 poise deg. $^{-1}s^{-1}$.

The value of for DPH at 37 °C was estimated indirectly to be 9.7 nsec from the temperature profile of the fluorescence intensity determined by Shinitzky and Barenholz (Shinitzky and Barenholz, 1974).

Results and discussion

The changes in the membrane fluidity of the mast cells after treatment with IgE or egg albumin are given in Table 1. The microviscosity parameters of mast cells treated with IgE-rich sera or egg albumin exhibit similar features. In both cases microviscosity increased.

Table 1

Modification of surface membrane fluidity of mast cells by IgE and egg albumin

Protein	P	$\bar{\eta}$ (Poise)
None	0.186	1.63
IgE-rich serum	0.234	2.48
Egg albumin (1 mg/ml)	0.243	2.69

The antigen-IgE antibody reaction was followed by rapid changes in membrane microviscosity of the mast cells as shown in Fig. 2.

As can be seen, in the earliest phase of the antigen-antibody reaction the membrane fluidity distinctly decreased, but 10 min later it quickly increased.

To test whether the changes observed were the consequence of the antigen--antibody binding, unsensitized mast cells were incubated in normal rat sera and the membrane fluidity was determined. Then the cells were treated with egg albumin and the membrane fluidity was measured immediately and ten minutes later. The changes in membrane fluidity of mast cells treated with normal rat sera and egg albumin are also presented in Fig. 2.

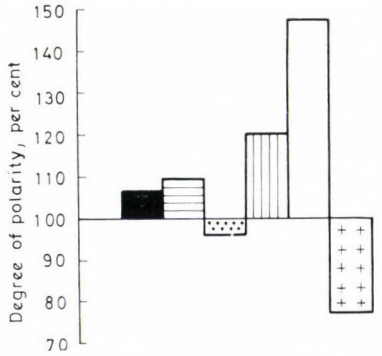

Fig. 2. Changes in membrane fluidity of rat mast cells after incubation in normal rat sera and IgE-rich sera, respectively, as well as after adding antigen. The changes are expressed as the percentage of control. ▩ = normal rat sera; ▤ = normal rat sera + egg albumin 1 min later; ⁘ = normal rat sera + egg albumin 10 min later; ▥ = IgE-rich sera; □ = IgE--rich sera + egg albumin 1 min later; + + + = IgE-rich sera + egg albumin 10 min later

As can be seen, the incubation of unsensitized mast cells in normal rat sera and a subsequent addition of egg albumin changed the membrane fluidity in a small degree.

Our results can be interpreted by assuming that the non-specific proteins attach to the lipid region of the cellular membrane. Consequently the outer part of the lipid gets into direct contact with the protein, at the same time the infiltrating proteins compress the lipid region.

Mast cells and basophils bind monomeric immunoglobulin E to their surface via the F_c portion of the antibody molecule. The binding of IgE takes place on well-defined parts of the membrane, the IgE receptors. The binding results in a change of the configuration of the IgE-receptors. When such cells are exposed to antigens capable of reacting with the F_{ab} portion of the bound IgE, the cells are stimulated to release histamine. serotonin, and other active components enclosed in the basophilic granules.

It has been suggested (Segal et al., 1977) that bridging of two molecules of IgE is sufficient to generate a unit signal; the summation of such signals leads to degranulation of mast cells. Our results suggest that the bridging of two molecules of IgE with antigen is followed by an increase in the membrane microviscosity of the mast cells. On the basis of our findings we propose, that this change in membrane microviscosity is the first signal after the IgE antigen binding.

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Yellow Mutations Alter Chloroplast Ribosomal Proteins in *Chlamidomonas reinhardii*

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Ribosomes and ribosomal proteins from wild-type and three *yellow* mutants of *Chlamydomonas reinhardii* were analyzed and compared by two-dimensional gel-electrophoresis.

Mixotrophycally grown mutants differ from wild-type cells in lowered chlorophyll content.

Analytical ultracentrifugation analysis of cell extracts showed a reduced amount of 70*S* ribosomes and an increased level of 50*S* subunits in mutants *y*-27 and *y*-28.

Two-dimensional gel electrophoresis indicated considerable alterations in the protein composition of 70*S* ribosomes of the mutants.

Two proteins of 70*S* ribosomes were altered in the *y*-27 and *y*-28 mutants. Two proteins were absent from the electrophoretograms of the yellow-76 mutant and seven proteins were present in lowered concentrations.

The mutations are inherited in a non-Mendelian manner. The protein alterations in 70*S* ribosome are most probably localized in the chloroplast DNA.

Introductions

Recent studies have shown that chloroplast ribosome biogenesis in *Chlamydomonas reinhardii* requires the participation of both nuclear and chloroplast genes (Surzycki et al., 1970). Mutations which directly affect chloroplast ribosome structure or function are known in both genomes and can be distinguished by their Mendelian and uniparental patterns of inheritance (Boynton et al., 1972; Mets, Bogorad, 1972; Harris et al., 1972; Schlanger, Sager, 1974).

Chromosomal and extrachromosomal mutants with altered chloroplast ribosomes are useful tools for the identification and localization of the structural gene or genes of a given chloroplast ribosomal component. One type of such mutants is characterized by a reduced amount or total absence of chloroplast ribosomes (Goodenough, Levine, 1970; Bourque et al., 1971; Katterman, Endrizzi, 1973; Börner et al., 1973; Harris et al., 1974). Another type of mutants contains 70*S* ribosomes at nearly normal levels, but with altered physical or functional proper-

Abbreviations: MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; 25/25/25 TKM buffer, 25 mM TRIS-HCl (pH 7.8) + 25 mM KCl + 25 mM $Mg(CH_3COO)_2$; 25/25/5 TKM buffer, 25 mM TRIS-HCl (pH 7.8) + 25 mM KCl + 5 mM $Mg(CH_3COO)_2$.

ties (Schlanger et al., 1972; Boschetti, Bogdanov, 1973). A number of single-step mutations in Chlamydomonas confer resistance to antibiotics such as spectinomycin, erythromycin, streptomycin and neamin. These mutations belong to the type of ribosomal mutants with alterations in one or more ribosomal proteins (Burton, 1972; Schlanger, Sager, 1974; Davidson et al., 1974; Ohta et al., 1975; Boynton et al., 1975a, b; Brügger, Boschetti, 1975).

Cells blocked in chloroplast ribosome formation show a characteristic syndrome of defects in photosynthesis, including inability to make the CO_2-fixing enzyme ribulose diphosphate carboxylase in normal amounts. They will grow when acetate is supplied as a carbon source.

The mutants with defective chloroplast ribosomal RNA also exhibit this syndrome. Bourque et al. (1971) and Goodenough and Levine (1971) have established that the reduction in chloroplast ribosomes is accompanied by a reduction in both 16*S* and 23*S* rRNAs. Another type of mutants can form structurally normal ribosomes but shows the syndrome of deficiency in chloroplast protein synthesis if the auxiliary components of protein synthesis (such as transfer RNAs, initiation factors, aminoacyl-tRNA) are absent (Harris et al., 1976).

In the present paper we report the results of ribosomal analysis of *yellow* mutants of Chlamydomonas reinhardii. Ultracentrifugal analysis of ribosomes and the pattern of two-dimensional gel electrophoresis of 70*S* ribosomal proteins show distinct differences between wild-type and mutant strains.

Material and methods

Organisms and culture conditions

The wild-type strain 494 (originated from strain 137c) and three yellow mutants derived from it, were used in the experiments. The mutations had been induced with MNNG and the mutants were selected from a dark culture. The cells were grown at 25 °C in liquid cultures to late logarithmic phase (10^6 cells per ml) on the high salt acetate medium of Sager and Granick (1953). The cultures were synchronized by exposing the cells to a cycle of alternating 12-hour periods of light and dark and bubbled with 3% CO_2 in air. The light intensity was 600 lux in case of determination of chlorophyll content and photosynthetic activity and 3 500 lux in experiments with ribosome.

Determination of chlorophylls

The cells were homogenized with acetone and diethyl ether (1 : 1) in a Potter homogenizer with teflon pestle. After adding water to the solution the pigments were transferred to diethyl ether. The chlorophyll content of the solution was determined by the two-wavelength method (644 and 663 nm). The pigment concentration (nmol $ml^{-1}cm^{-1}$) was calculated according to French (1960) by using the formulas: $chl_a = 11.44\ D_{663} - 0.96\ D_{644}$ and $chl_b = 18.11\ D_{644} - 2.90\ D_{663}$.

Activity of photosynthesis

Synchronized *Chlamydomonas* cells were pelleted and resuspended in 10 ml of high salt medium containing 0.1 ml of $NaH^{14}CO_3$ (specific activity 1 μCi/ml) and shaken in the light (1000 lux) for 1 hour. The reaction was stopped by turning off the light and the cells were washed three times in cold, inactive medium. Radioactivity was determined according to Bush and Hansen (1965) in a Nuclear Chicago 724 liquid scintillation spectrometer.

Estimation of DNA and RNA in the cells

Total DNA and RNA per 10^7 cells were determined according to Smillie and Krotkov (1960). The RNA and DNA contents were measured in a UNICAM SP 500 spectrophotometer and the concentrations calculated according to Sunderland and McLeish (1961) by taking 1 absorbance unit at 1 cm light path as 32.5 μg RNA per ml and 35.0 μg DNA per ml respectively.

Analytical ultracentrifugation

The alga cells were harvested by low-speed centrifugation (300 x *g*) at 0 °C. About 5 g of pelleted cells were washed twice in cold 25/25/25/ TKM buffer containing 6 mM 2-mercaptoethanol and resuspended in the same buffer to get a concentration of 5×10^8 cell per ml. This suspension was homogenized in a French-press at 100 kPa/cm^2 and -60 °C. The homogenate was centrifuged at 10 000 x *g* for 5 min and the supernatant was used for ultracentrifugation. Analysis of ribosomes was carried out at 20 °C in a Beckman Model E analytical ultracentrifuge using Schlieren optics.

Isolation of total ribosomes

For the isolation of total ribosomes 50 g of harvested cells were washed twice in cold 25/25/25 TKM buffer and homogenized as described above under "Analytical ultracentrifugation". To the 10 000 x *g* supernatant, a 25% solution of Triton X-100 in 25/25/25 TKM buffer was added to a final concentration of 1%. This solution was layered on a cushion of 7 ml of 1 M sucrose in 25/25/25 TKM buffer and centrifuged in a SKB-65 type ultracentrifuge at 48 000 rev/min for 3 hours. The slightly yellow pellets contained the ribosomes.

Separation of 70S and 80S ribosomes

For the separation of the ribosomes on a small zonal rotor RZ-47T (volume 650 ml) a hyperbolic or linear gradient was used (Sypherd, Wiremann, 1974).

For making linear gradient the rotor was filled from the periphery by pumping 200 ml of cold 50% (w/v) sucrose (in buffer 25/25/5 TKM and 6 mM 2-mercapto-

ethanol) through a mixing chamber, containing 210 ml 7.5% (w/v) sucrose into the rotor. Finally 50% (w/v) sucrose solution was pumped as a cushion from the same direction. 15 ml of the sample, containing 300 – 500 mg of ribosomes and 3% (w/v) sucrose was layered from the center. The sample was overlayered with 180 ml of 25/25/5 TKM buffer, containing 6 mM 2-mercaptoethanol.

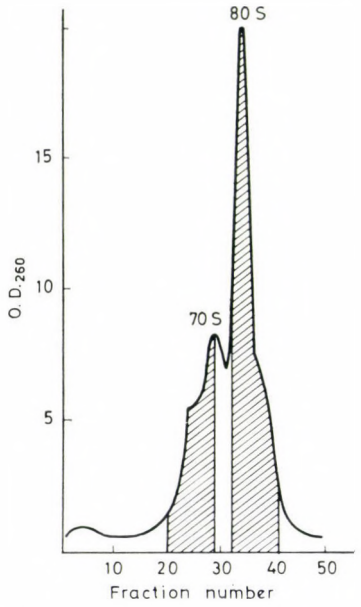


Fig. 1. Ultraviolet profile of 70*S* and 80*S* ribosomes after separation in a small zonal rotor RZ-47T. Separation was accomplished in a linear 7.5 – 50% (w/v) sucrose gradient. Only the fractions within the hatched areas were collected

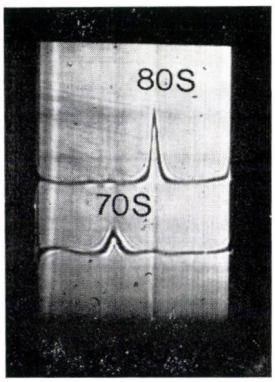


Fig. 2. Sedimentation profiles of separated 70*S* and 80*S* ribosomes

After a 2.5-hour centrifugation at 45 000 rev/min and 2 °C the rotor content was displaced through the center by pumping 50% of sucrose from the periphery and the UV profile was recorded (Fig. 1). Ten-milliliter fractions were collected. The sucrose concentration of the ribosome-containing solution was reduced by dialysis againts 25/25/25 TKM buffer overnight and the ribosomes were sedimented by centrifugation at 48 000 rev/min for 3 hour. The purity of the isolated ribosomes was tested by analytical ultracentrifugation (Fig. 2). There was no cross-contamination between 70*S* and 80*S* ribosomes.

Isolation of ribosomal proteins

The ribosomal proteins were extracted from sedimented ribosomes by the LiCl-urea method (Spitnik-Elson, 1965). After 1 day in the cold the precipitated RNA was removed by centrifugation, and the supernatant solution was dialyzed against 6 M urea – containing 0.005 M acetate buffer, pH 4.5.

Two-dimensional poliacrylamide gel electrophoresis of ribosomal proteins

The apparatus and modified method for two-dimensional gel electrophoresis described by Kaltschmidt and Wittmann (1970) were used.

In the first dimension the gel contained 8% acrylamide and 0.25% bisacrylamide, pH 8.7. The electrophoresis time was 17 hours at 5 mA per gel and 4 °C. The acrylamide concentration and the pH were 18% and 4.5, respectively in the second dimension. The electrophoresis time was 24 hours at 90 mA per slab and 4 °C.

Results

As shown in Table 1 the mutant cells contain about half or less then half as much chlorofyll as the wild-type. The chlorophyll-a and chlorophyll-b ratios are nearly the same in all strains (Table 1).

Table 1

The chlorophyll content of wild-type and yellow mutants of Chlamydomonas re inhardii

Strains	Chlorophyll content		chl-a / chl-b
	nmol chl – a + b / 10^7 cells	%	
494 (wild)	21.4	100.0	2.7
y-27	10.4	48.6	2.6
y-28	7.4	34.6	2.6
y-76	3.5	16.7	2.6

Table 2

Photosynthetic characteristics of wild-type and yellow mutants of Chlamydomonas reinhardii

Strains	Photosynthetic activity			RuDPCase activity %
	n mol $H^{14}CO_3^-$ / 10^7 cells	%	nmol $H^{14}CO_3^-$ / nmol chlorophyll %	
494 (wild)	16.0	100.0	100.0	100.0
y-27	7.3	45.6	93.9	100.0
y-28	5.4	33.5	97.7	88.5
y-76	1.9	11.7	72.7	70.0

Table 3

Nucleic acid content of the wild-type and yellow mutants of Chlamydomonas reinhardii

Strains	DNA		RNA		RNA / DNA
	μg/10^7 cells	%	μg/10^7 cells	%	
494 (wild)	6.10	100.0	59.1	100.0	9.7
y-27	5.58	91.6	29.8	50.4	5.4
y-28	5.59	91.7	32.1	54.3	5.3
y-76	5.95	97.5	47.1	79.5	7.8

The photosynthetic activities are presented in Table 2. It is well noticeable that the light-stimulated incorporation of $H^{14}CO_3^-$ per 10^7 cells is lower in the mutants, but the activity of photosynthesis as a function of chlorophyll content is nearly the same as that found in the wild-type. On the percentage basis, photosynthetic activity and RuDPCase activity are identical in the *yellow*-76 mutant (Table 2).

The nucleic acid content of algal cells is summarised in Table 3. There is no significant difference in DNA content between the wild-type and the yellow mutants while there is considerably less RNA in the mutant cells especially in the *y-27* and *y-28* mutants (Table 3).

Since the majority of cellular RNA is ribosomal RNA, the lowered amount of RNA in cells of the mutants directed our attention to the analysis of their ribosomes.

Fig. 3 shows the sedimention patterns obtained with the extracts of algal cells. Each of them contains two types of ribosomes and the 50*S* large subunit of the chloroplast ribosomes.

Two-dimensional electrophoretograms of the proteins of 70*S* and 80*S* ribosomes are shown in Figs 4 and 5.

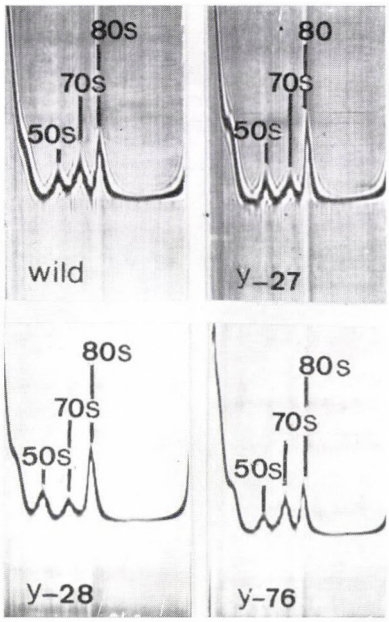


Fig. 3. Sedimentation profiles obtained with the extracts of algal cells. Sedimentations were carried out in the AnD rotor at 42 040 rpm and at a temperature of approximately 20 °C. The photograph was taken at a Schlieren bar angle of 55°, 12 min after reaching the set speed. Sedimentation medium: 25 mM TRIS-HCl, pH 7.8, 25 mM $Mg(CH_3COO)_2$; 25 mM KCl; 5 mM 2-mercaptoethanol

In the 80*S* ribosomes of the wild-type (Fig. 4a) 76 proteins can be detected of which 69 migrate in the first dimension to the cathode and 7 to the anode at pH 8,7. No alteration in the proteins of the 80S ribosomes (Fig. 4b, c, d) could be detected in cells of the mutant strains compared with the wild-type.

The electrophoretic patterns of 70*S* ribosomal proteins of the yellow mutants are different from the corresponding pattern of the wild-type (Fig. 5a, b, c, d). In the electrophoretograms of the 70*S* ribosomes of wild-type cells (Fig. 5a) 57 proteins were detected. 5 of them migrated to the anode at pH 8,6.

One intensive spot No 6 in the electrophoretogram of the wild-type is absent in the electrophoretogram of the 70*S* ribosomal protein of the *y*-27 mutant (Fig. 5a and 5b). There are two additional, so called extraproteins (marked with an arrow) in the electrophoretogram of the *y*-27 mutant.

There are two extraproteins (marked with an arrow) in the electrophoretogram of the *y*-28 mutant (Fig. 5c) which could not be found on the slab prepared from the 70*S* ribosomes of the wild-type.

The electrophoretic pattern of the 70*S* ribosomal proteins of the *yellow*-76 mutant (Fig. 5d) markedly differs from the corresponding pattern of the wild-type:

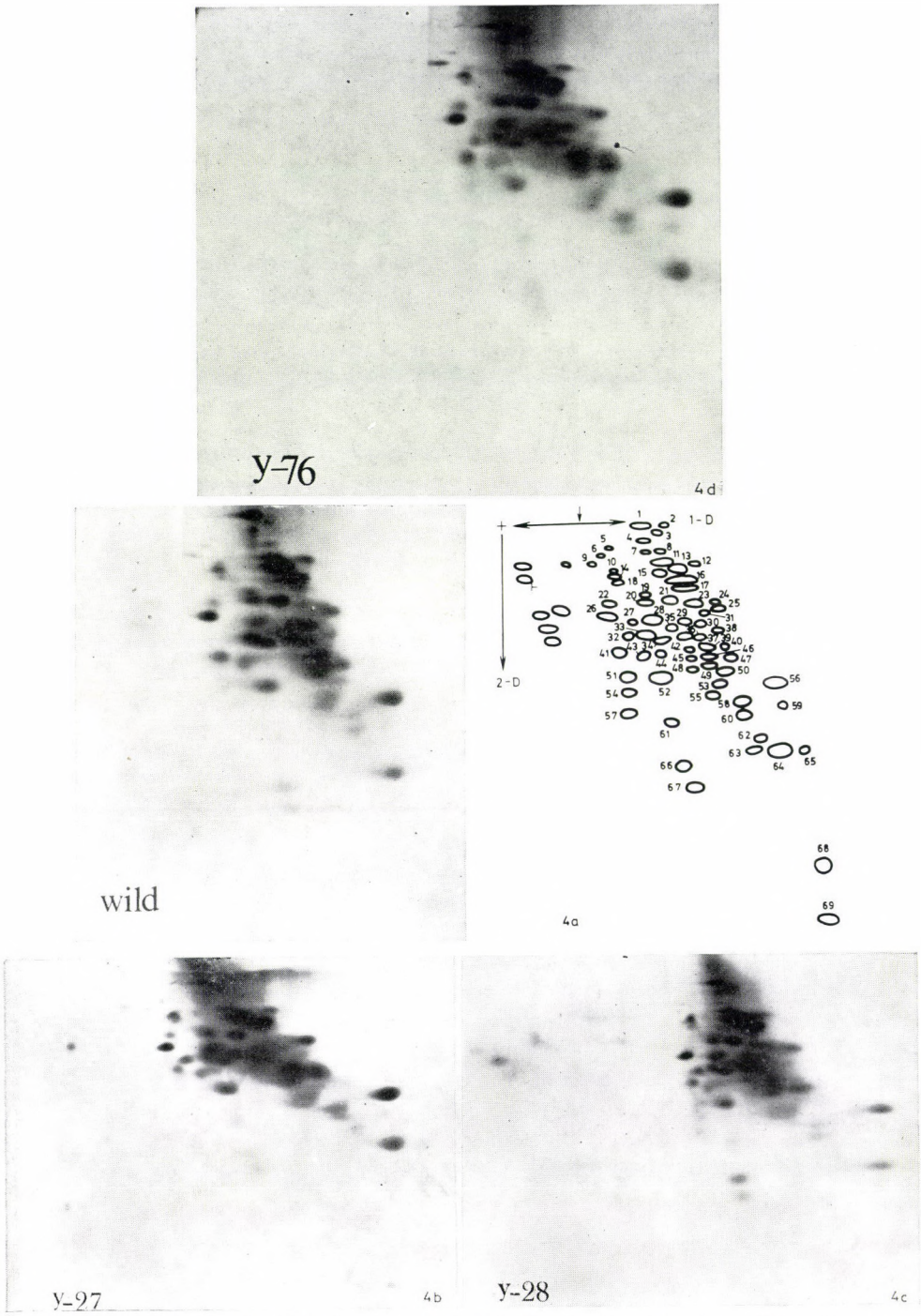
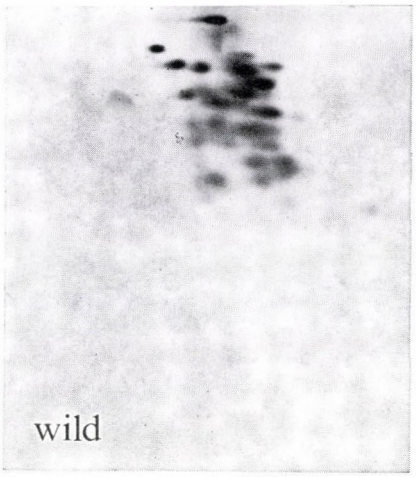
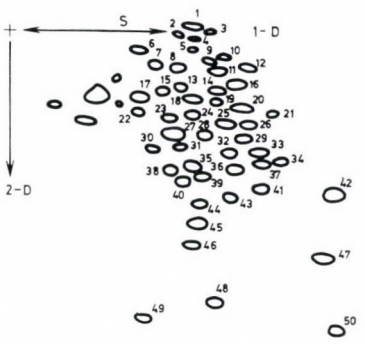
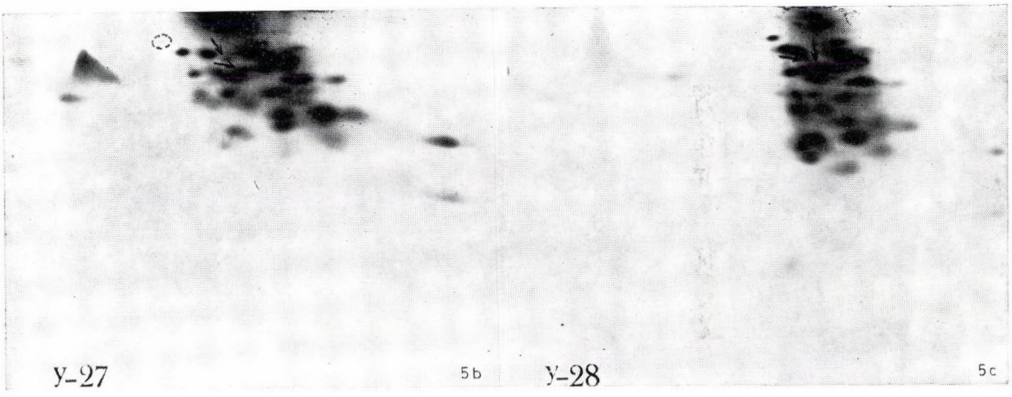


Fig. 4. Two dimensional gel electrophoresis of 80*S* ribosomal proteins from *Chlamydomonas reinhardii; a.* wild-type; *b.* yellow-27 mutant; *c.* yellow-28 mutant and *d.* yellow-76 mutant. The buffer used for both dimensions contained 6M urea, but the acrylamide concentration and the pH were 8% and 8.7 respectively in the first dimension and 18% and 4.5 respectively in the second. Approximately 1000 μg of ribosomal proteins were applied





5a



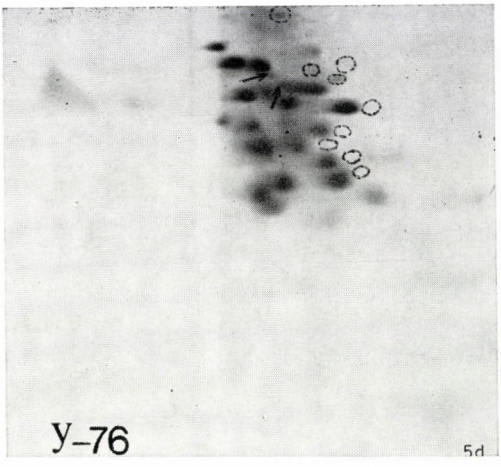


Fig. 5. Two dimensional gel electrophoresis of 70*S* ribosomal proteins from *Chlamydomonas reinhardii; a.* wild-type *b.* yellow-27 mutant; *c.* yellow-28 mutant and *d.* yellow-76 mutant. For more details see legends to Fig. 4

two proteins (spot 12 and 21) are absent and seven proteins (spots 1, 11, 15, 26, 29, 33 and 37) are present at lower concentrations. In addition, two slightly staining extraproteins (marked with an arrow) are present.

Discussion

Yellow mutants show nearly normal functions in photosynthetic CO_2 fixation and normal activity of the enzyme RuDPCase. The normal level of RuDPCase activity of the mutants indicates that there are enough 70*S* ribosomes to ensure proper functioning of the protein synthesizing system of the chloroplast.

In *ac-20* mutants of Chlamydomonas reinhardii the 70*S* ribosome content is correlated with RuDPCase and Hill activities, but not with chlorophyll content (Harris et al., 1974). This lends further support to the conclusion that functioning chloroplast ribosomes are specifically required for RuDPCase synthesis and for the formation of some components of photosystem II, but it is not required for chlorophyll accumulation.

The lowered level of free 70*S* ribosomes in the *y-27* and *y-28* mutants is either due to their increased dissotiation or, as in a certain ribosome-assembly mutant, to a relatively higher number of the large subunits of 70*S* ribosomes.

In the 70*S* ribosomes of the *y-27* mutants two protein alterations can be detected: One protein with high molecular weight is practically absent while in the wild-type cells this protein is present in large quantities (spot No 6).

From the lack of one protein and the occurrence of two new ones in the 70*S* ribosomes two conclusions can be draw. It is possible that protein No 6 is altered in molecular weight and/or in charge as a result of mutation. The second possibility is that two proteins had been altered. A primary structure analysis of these proteins would answer this question. The protein alteration of 70*S* ribosomes in *yellow-28* mutant cells raises the same problems.

The extensive protein alteration of 70*S* ribosomes in *yellow-76* mutant cells seems to be unusual. On the basis of the "one gene one polypeptide" theory this mutant could arise from a deletion or silencing of a certain chromosomal fragment or some chromosomal fragments which contain the genes of ribosomal proteins. It is known that Mendelian genes which directly affect the structure, assembly and function of the chloroplast ribosomes in *Chlamydomonas reinhardii* are not closely linked. No single nuclear operon contains information for all of the chloroplast ribosome determinants. In contrast, all the known chloroplast genes that affect chloroplast ribosomes appear to fall in a single linkage group (Harris et al., 1976).

Zygote clone analyses (Gyurján et al., 1979); show uniparental inheritance of the mutants. It may well be that the protein alterations in 70*S* ribosomes are localized in the chloroplast DNA.

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Kinetic Study of the Inhibition of Myosin ATPase Activity by ADP

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The kinetic parameters of the inhibition of monovalent cation activated myosin ATPase by ADP were investigated. The inhibitor constant (K_I) was 1.65×10^{-4} M and the maximal velocity (V) was 1.28 μmol P_i /mg myosin/min in the presence of 0.3M KCl at 20 °C. The dependence of $1/v_0$ on inhibitor concentration and the pH dependence of K_I and K_m (i.e. $pK_i \cong pK_m$) show that the inhibition has a pure competitive character. The results are supported by energetic parameters, too. The enthalpy of the formation of (EI) complex was calculated.

Similar results were obtained also in the presence of Rb^+ activated myosin ATPase and subfragment-I K^+ ATPase.

Introduction

Considerable progress has recently been made in the study of the mechanism of myosin ATPase reaction. Attempts have been made to develop further the basic scheme proposed by Lymn and Taylor (1970). One of them, accepted in general, is referred to as the Bagshaw-Trentham mechanism which assumes only one pathway of ATP hydrolysis (Bagshaw and Trentham, 1973; 1974). But the role of the cation in the ATPase reaction has not been completely elucidated yet.

In the presence of divalent cations ADP is not only a product but also an inhibitor of the myosin ATPase reaction. As to the extent of its inhibiting effect, contradictory results have been reported. Green and Mommaerts (1954) have found ADP to be a strong inhibitor of the ATPase activity of myosin.

Blum (1953, 1955) reported a relatively poor inhibition of not more than 30% even when the concentration of ADP was ten times higher than that of ATP. As to the effect of pH on the degree of inhibition, Baev (1958) found that at pH 6.4 it was smaller than at pH 9.

Nanninga (1962) found a competition between ADP and ATP in the case of Ca-ATPase. According to his kinetic analyses, the value of the inhibition constant was 33 times larger than that of the Michaelis constant.

Yoshida at al. (1975) determined the value of K_I by gelfiltration in the presence of 10 mM Mg^{2+} and found it to be 1.5×10^{-6}M, the value of K_m being 3.6×10^{-8}M.

All the above cited data refer to inhibition in the *presence* of divalent cations.

As to the mechanism of ATP splitting by myosin in the *absence* of divalent cations (Kielley, Bradley, 1956; Mühlrad et al., 1964; Seidel, 1969; Kelemen, Mühlrad, 1971; Mandelkow, Mandelkow, 1973; Kelemen, Magyar, 1975), it can be described by a simple two-step Michaelis mechanism (Lymn, Taylor, 1970).

$$M + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} M.ATP \xrightarrow{k_2} M + ADP + P_i$$

If $k_{-1} \ll k_2$, the Michaelis constant (K_m) can be expressed by the equation $K_m = k_2 / k_1$, where $k_2 = V / E_t$ (if V is calculated per mole enzyme, numerically $k_2 = V$).

In this case $k_1 = k_2 / K_m$, i.e. k_1 is the rate constant of the Michaelis complex formation and k_2 is that of decay.

In this study an attempt is made to indentify the conditions of the inhibitory effect of ADP on monovalent cation activated ATPase of myosin and to determine its kinetic parameters.

Materials and methods

Myosin was perpared by a modified version of Portzehl's (1950) method. Subfragment-I was isolated from myosin by papain digestion (Bálint at al., 1975). Myokinase was removed by several reprecipitations, and the solution purified by ultracentrifugation for 1^h at 105 000 *g*. Only fresh protein preparations, not older that 3 days, were used in the experiments. The protein concentration was determined by the biuret method of Gornall at al. (1949).

For the calculations presented in this paper, the molecular weight of myosin and subfragment-I were assumed to be 5×10^5 and 1.2×10^5 daltons, respectively.

The ATPase activity of myosin was measured by a modification of Bárány's method (1967). The test solution contained 6–20 μg/ml myosin, 9 mM EDTA, 40 mM Tris-HCl buffer, pH 8, and 0.3 M KCl or RbCl (if not stated otherwise). The substrate was [^{32}P] ATP, synthetized by the procedure of Glynn and Chappell (1964); its concentration varied between $10^{-6} - 10^{-4}$ M (around the value of K_m to be measured). The ADP concentration varied between $10^{-5} - 10^{-4}$ M so that it should be 1.5 – 10 times higher than that of ATP.

The measurements were carried out generally at 20° (in some cases at 0° or 10°) using 8 ml samples, and the reaction was stopped by adding sulfuric acid. After mixing with charcoal suspension, the reaction mixtures were filtered and the radioactivity of $^{32}P_i$ liberated in the filtrate was measured in a Nuclear Chicago J 24 liquid scintillation counter.

The specific activity of myosin is given in μmole P_i liberated per mg of protein per min.

For determining V and K_m the values of v_0 were measured at five different concentrations of ATP (around K_m) with three parallels each. The experiment was repeated three times using different preparations of myosin. The overall number of

experimental data obtained was 45 at each ionic strength and at each pH values. At least four different preparations of myosin were used for the determination of K_I. In every experimental series the mixture (with three parallels) contained the ADP at five different concentrations at each value of the three different ATP concentrations. Thus, e.g., the overall number of data obtained in experiments conducted at different ionic strengths is 216. The data were evaluated by the least square method. The correlation was 90 per cent or higher and the standard deviation for the values of V, K_m and K_I was 10 per cent or less.

Results

Systems with inhibitors are usually analysed by Dixon plots in which the values of $1/v_0$ are plotted against inhibitor concentrations at different concentrations of substrate (Fig. 1).

The profile of $1/v_0$ *vs* (I) lines (Fig. 1) shows a typical competitive inhibition. The value of the inhibitor constant (K_I) is 1.65×10^{-4} M with a maximal velocity (V) of 1.28 μmole P_i/mg myosin/min.

The K_m values from the above experiments can also be calculated by using a Lineweaver Burk plot. At different concentrations of ADP the intercept of the intersecting lines on the 1/S axis is given by $\frac{1}{K_m'} = \frac{1}{\left[1 + \frac{(I)}{K_I}\right] K_m}$.

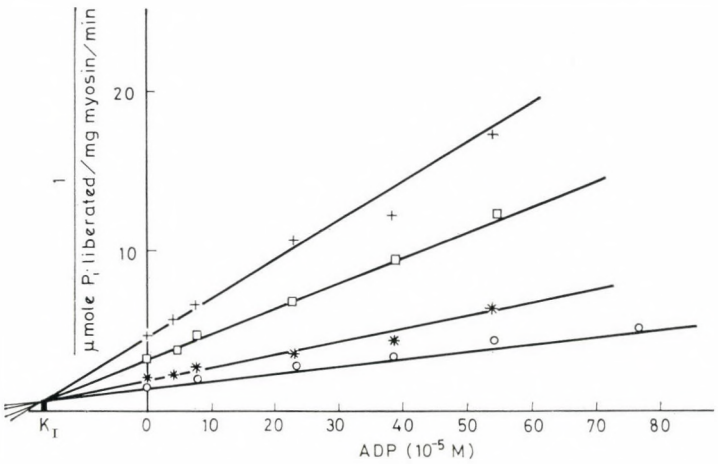

Fig. 1. Dependence of the reciprocal of the sepcific activity of K^+ ATPase on ADP concentration at different ATP concentrations. For details of ATPase measurements see Methods. ATP concentrations were: +, 2.72×10^{-5};, □, 5.16×10^{-5}; ✳, 8.16×10^{-5} and ○, 13.6×10^{-5} M

Table 1

Apparent Michaelis constants (K_m') and K_m (calculated from K_I) for myosin ATPase at different ADP concentrations

In 0.3 M KCl, 9 mM EDTA, 40 mM Tris-HCl buffer, pH 8, at 20 °C

ADP (10^{-5} M)	K_m' (10^{-4} M)	K_m (10^{-4} M)
3.83	2.0	1.62
7.67	2.3	1.63
23.0	3.57	1.49
38.35	5.0	1.5
53.69	6.25	1.48

From this, knowing K_I and K_m' at different values of (I), K_m can be calculated. The dependence of K_m' and K_m on ADP concentrations (at $K_I = 1.65 \times 10^{-4}$ M) is shown in Table 1.

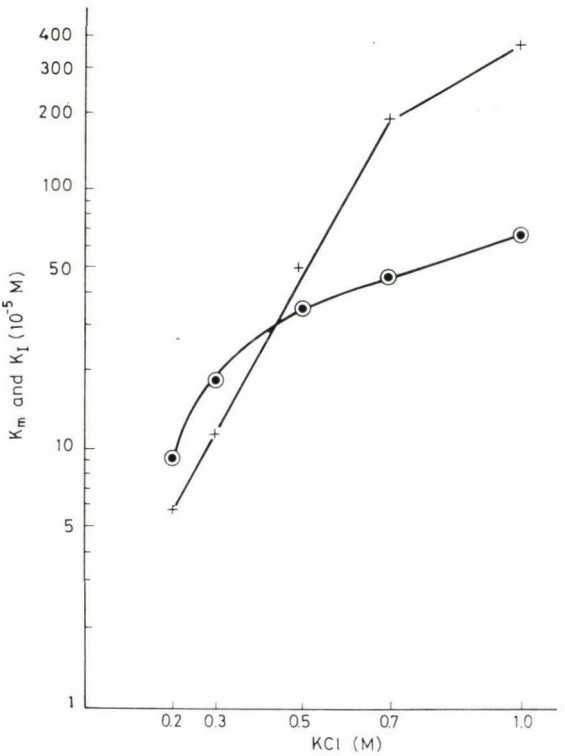

Fig. 2. Michaelis constants and inhibitor constants of K^+ activated myosin ATPase at different KCl concentrations. ATPase measurements see Methods. With K_I the concentration of ADP varied between $0 - 80 \times 10^{-5}$ M; +, K_m; ⊙, K_I

Table 1 shows that the value of K_m is practically independent of ADP concentration, and its average 1.54×10^{-4} M is in good agreement with the $K_m = 1.12 \times 10^{-4}$ M value of uninhibited myosin.

Since both V, i.e. k_2, (Fig. 1) and K_m (Table 1) are independent of the inhibitor concentration, the rate constant k_1 must also be independent of it. This means that the inhibition has a competitive character.

The inhibition is also competitive in the case of Rb^+ activated ATPase. In the presence of 0.3M RbCl at 20 °C, $K_I = 1.34 \times 10^{-4}$ M, V = 0.98 μmole P_i/mg myosin/min, and $K_m = 1.04 \times 10^{-4}$ M. The differences between the constants in the presence of Rb^+ and K^+ are due to the differences between their ionic radii (Kelemen, Magyar, 1975).

ADP is also a competitive inhibitor of ATP in the case of K^+ activated subfragment-I ATPase. The value of K_I is 1.24×10^{-4} M and the K_m and V values are also independent of the ADP concentrations.

As shown above, the values of uninhibited K_m and K_I hardly differ from each other, therefore it is of interest to compare their changing with ionic strength (Fig. 2).

It can be seen in Fig. 2 that up to 0.4 ionic strength K_m is smaller than K_I, At higher ionic strengths $K_m > K_I$.

The pure competive character of the inhibition can also be supported by the pH dependence of K_m and K_I. The pK values for the enzyme-inhibitor and enzyme--substrate complexes are given by the values on the abscissa belonging to the inflection point. Identity of the two pK values indicates a pure competitive inhibition,

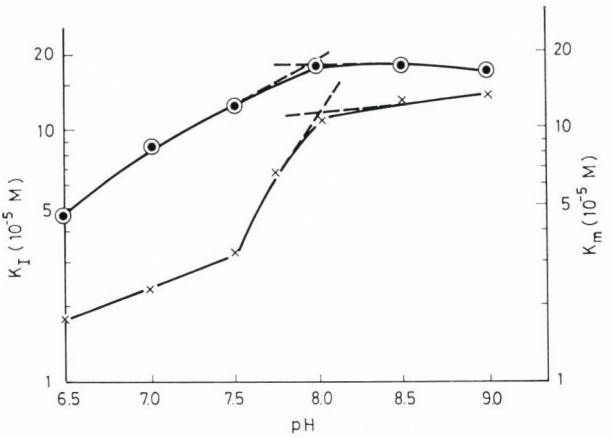

Fig. 3. pH dependence of the values of K_m and K_I for K^+ activated myosin ATPase in 9mM EDTA and 30 mM KCl, 40 mM Tris-maleate buffer was used between pH 6 and pH 7.75 (ATP concentration varied between 1.2×10^{-6} M and 13.0×10^{-6} M), and 40 mM Tris-HCl buffer was used between pH 8 and pH 9 (ATP concentration varied between 2.7×10^{-5} M and 15.0×10^{-5} M). The ADP concentration was $0 - 60 \times 10^{-5}$ M. +, K_m; ⊙, K_I

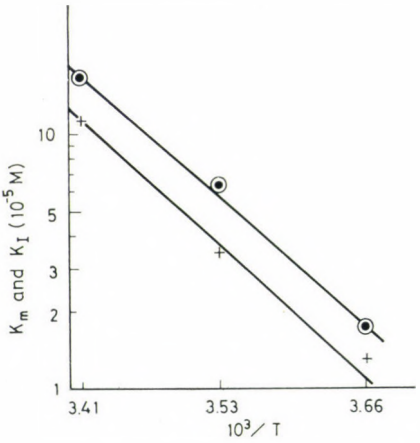

Fig. 4. Dependence of log K_m and log K_I on 1/T for K^+ activated ATPase. For details of the measurements see text and Fig. 2. +, K_m; ⊙, K_I

because both the inhibitor and the substrate react with the same group of the active site of the enzyme (Webb, 1963; Laidler, Bunting, 1973).

Changes of K_m and K_I as a function of pH are shown in Fig. 3. The values of pK obtained from pK_I and pK_m are 7.92 and 8.0, respectively, i.e., practically indentical.

The value of enthalpy (at 0.3 M KCl) are calculated on the basis of the temperature dependence of K_I (Fig. 4).

Table 2

Free enthalpies for the formation of (ES) complex by K^+ and Rb^+ activated ATPase of myosin
The experimental conditions are described in Table 1

		ΔG° kcal/mol	
		at 0.3 M KCl	at 0.3 M RbCl
System E + S ⇄ ES	uninhibited	− 5.29	− 5.17
	with ADP	− 5.01	− 5.33

In the case of the formation of the (EI) complex, $\Delta H_i^\circ = 17.90$ kcal/mol.

Table 2 shows that the ΔG° values (calculated from K_m, K_m' and K_I) in the presence of 0.3 M KCl and RbCl, respectively, do not differ significantly.

Discussion

The binding of the nucleotide to myosin S-I in the presence of Mg^{2+} is a two-step process involving the initial formation of an encounter complex followed by a proteinligand isomerisation, possibly representing a conformational change of the protein (Bagshaw et al., 1974). This assumption is supported by numerous experimental data obtained, among others, from the investigation of the binding of ADP to myosin. All these data, however, refer to the case in which the whole breakdown of ATP takes place in 7 steps.

For myosin ATPase activated by monovalent cations, another relation between myosin and ADP must be assumed, in view of a different mechanism of the splitting of ATP.

The rate (v_0) of the activity of myosin ATPase activated by K^+ and Rb^+, as well as that of subfragment-I decrease with increasing ADP concentration (as measured at $5-53 \times 10^{-5}$ M), while K_m and V are unchanged.

Our results also show that there is no significant difference between the values of ΔG° of unihibited and inhibited systems (Table 2).

These data refer to the fact that the type of the inhibition is competitive.

The pH dependence of K_m and K_I also supports the assumption of pure competition (Fig. 3).

The pK_m and pK_I values are at the same pH, suggesting that the nucleotide-binding amino acid residues are located near each other, or that they are identical in the active site.

The influence of the moderate salt concentration (0.3 – 0.4M) on the formation of (ES) and (EI) complexes is similar (Fig. 2).

The temperature dependence of K_I, or rather ΔH° and ΔG° (calculated from K_I shows that the binding of inhibitor to myosin is weak.

On the basis of these results it may be concluded that ADP is a competitive inhibitor in the myosin ATPase reaction. The changes in reaction rates (v_0) are due to the formation of an (EI) complex and, therefore, the quantity of myosin reacting with ATP will decrease. The energetic parameters and the structure of the (ES) complex are independent of the presence of inhibitor.

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Analysis of the Electron Spin Resonance Spectrum of Human Liver Tissue Embedded in Paraffin

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The ESR spectra of the liver samples embedded in paraffin of newborn, adult, cirrhotic and control individuals always display the $g \sim 2.05$ signal and occasionally also the $g = 4.3$ line and the signal of free radicals. In adults, the amplitude of the $g \sim 2.05$ signal is negatively correlated with that of the $g \sim 4.3$ signal; in infants aged from three months to one year this correlation is positive. The copper content of the liver is in linear correlation with the amplitude of the $g \sim 2.05$ signal. The hyperfine structure of the part of the $g \sim 2.05$ signal recorded at low magnetic field may be of three types: it may correspond to Cu^{2+} centers of axial symmetry of type I, II or I + II. The parameters of the signal are the following: type I center, $g_{||} = 2.35 \pm 0.05, g_{\perp} = 2.05 \pm 0.01$, $A_{||} < 100$ gauss; type II center, $g_{||} = 2.25 \pm 0.03$, $g_{\perp} = 2.05 \pm 0.01$, $A_{||} = 160 \pm 20$ gauss. On the basis of the amplitude of the $g \sim 2.05$ signal, the copper content of the liver tissue can be estimated from paraffin blocks with an error of 30%, without losing the tissue embedded for histological examination.

Chicken liver tissue displays paramagnetic absorption even after histological processing (Elek et al., 1977). The question arises whether any conclusions can be drawn from the ESR spectra of the embedded tissues. In order to decide this, the spectra of healthy and pathalogical liver samples were compared. In this work the data of liver samples from unselected premature infants and from adult, cirrhotic and control individuals are presented, according to which the intensity of the $g \sim 2.05$ line of the spectrum shows a direct correlation with the copper content of the samples.

Materials and methods

Our samples were taken from the 1975–1976 autopsy material of the I. Institute of Pathology. The number of the cases compared is shown in Table I.

As controls, we used blocks made of livers on the histological sections of which no pathological alteration was observed. The cirrhotic and prematurely born cases were studied in unselected blocks fixed in formalin and embedded in paraffin. According to their histological appearence the cirrhoses were without exception of the Laennec type, with variable amounts of fibrosis and fatty infiltration. The few liver cancer cases studied were primary hepatocellular carcinomas.

Table 1

Grouping and characteristics of the ESR spectra of liver samples embedded in paraffin

Chosen			Studied		Total No. of cases in which		$g \sim 2.05$ signal of the spectrum of the case				Copper content of liver samples (μg/g wet weight)	
	group	No. of cases	No. of paraffin blocks	No. of ESR spectra	$g = 4.3$ signal occurs	signal of free radicals occurs	type I	type II	type I + II	amplitude	data from the literature for native tissue	atomic absorption in paraffin blocks
Infants	prematurely born	10	11	12	4	0	7	1	2	10 ± 4.3	77.9 ± 5.1[c]	65.4 ± 24
	3 months —1 year	10	10	11	6	2	5	1	4	3.9 ± 2	5 ± 8[c]	18 ± 5.9
Adults	normal liver 9—65 years	6	20	24	5	2	2	1	3	5.0 ± 1.9	5.3[a] 6.97 ± 2.36[b] 8.87 ± 1.00[c]	
	cirrhotic liver 20—66 years	10	30	43	7	4	4	1	5	8.0 ± 4.2	9.4[a] 9.86[b] 20 ± 2.0[c]	
	liver cancer	4	1	14	2	1	1	1	2	6.6 ± 3.2		
Total		40	81	104	24	9	19	5	16			

[a] Gubler et al., 1957. Color reaction
[b] Smallwood et al., 1968. Neutron activation analysis
[c] Butt et al., 1958. Arc spectroscopy

From the paraffin blocks, rods measuring 1.8 cm in length and 4 mm in diameter were trimmed. One rod contained the liver tissue, while another one was made from the part of the block containing only paraffin. The rods containing liver tissue were weighted on an analytical balance; their average weight was 200 ± 10 mg. ESR spectra were registered in the presence of Mn^{2+} : MgO standard in a JES ME−3X spectrometer (for the details of ESR spectroscopy, see the volume containing the review by Vänngård, 1972). The spectra of the paraffin rods were recorded as controls, in order to exclude possible paramagnetic contaminations of the materials used for embedding. Amplitude measurement was carried out by taking the distance of the maximum and minimum of the $g \sim 2.05$ (and in some cases the $g \sim 4.3$) signal of the ESR spectrum, projecting it on the ordinate and measuring it (signal amplitude). This value was divided by the amplitude value of the 6th line of the Mn^{2+} : MgO standard. When the weight of the sample studied diverged considerably from the average weight of 200 ± 10 mg, the amplitude was corrected also for weight (normalization).

The liver samples were washed three times in 100 volumes of xylol and dried at 56 °C until constant weight was reached. The dry matter content was determined after the measurement of the wet weight of the autopsy samples, followed by drying at 110 °C and then at 140 °C (Butt et al., 1956) until constant weight was reached.

Copper content was determined after cremation by the flame atomic absorption technique (Perkin Elmer 403 type atomic absorption spectrophotometer). Correlation and regression were calculated according to Juvancz (1955) and Plohinsky (1967).

Results

The characteristic spectra of the liver preparations are shown in Figs 1 and 2. Three signals of different types can be observed. The central (2700−3300 gauss), asymmetric $g \sim 2.05$ signal is discernible on all of the spectra. This signal is accompanied by several peaks (hyperfine structure). These are brought about by the formation of several absorption waves depending upon the position of the paramagnetic molecule relative to the magnetic field; on the other hand, the magnetic momentum of the copper nucleus creates an inner magnetic field, the relation of which to the outer magnetic field modifies the condition of resonance. Around the molecule containing the copper(II) ion, a cylindrically symmetric electric field (so-called crystal field) is supposed to exist. The "orientation of the molecule" means the relation of the favoured axial orientation of the crystal field to the orientation of the outer magnetic field (Vänngård, 1972). The part of the signal of high magnetic field (strong field) corresponds to the vertically oriented position, with a g value of 2.05 ± 0.01. The value of "g" is calculated from its position between the 2nd and 3rd Mn^{2+} lines (the g values of the latter are 2.08 and 2.03, respectively).

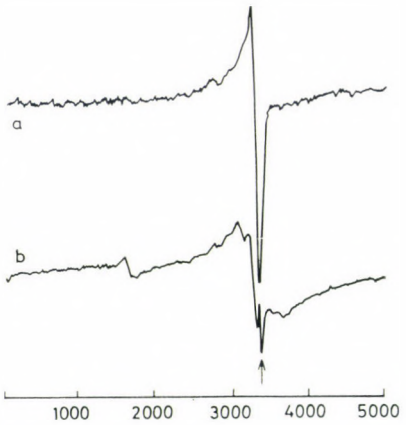

Fig. 1. X-band ESR spectrum of liver samples embedded in paraffin in the 0 – 5000 gauss region, at room temperature. *a.* sample containing 15 μg of copper. Only the $g \sim 2.05$ signal is seen on the spectrum. Amplification 200-fold. *b.* sample containing 6 μg of copper. The weight of the sample is identical with that of the previous one. Inside the 2.05 signal, the signal of free radicals can also be observed (arrow); at the beginning of the spectrum the $g \sim 4.3$ signal is seen. Amplification 200-fold. The recording time (t) in both cases was 5 min, the time constant (τ) 0.3 sec, the microwave power of the resonator cavity 10 mW. On the abscissa, field intensity is indicated in gauss units

On the stretch of low magnetic field (weak field) (2700 gauss, right before $g = 2.14$), the hyperfine structure is brought about by the position parallel with the field. This part of the spectrum may be different in the individual samples (Fig. 2). The parameters of the parallel position in the case of a type I copper center (ICu^{2+}) are : $g_{||} = 2.35 \pm 0.05$, and the coupling constant ($A_{||}$) is lower than 100 gauss. Therefore the lines of the hyperfine structure merge (Fig. 2a). The corresponding values of the type II copper centrum ($IICu^{2+}$) are $g_{||} = 2.25 \pm 0.03$ and $A_{||} = 160 \pm 20$ gauss (Fig. 2c). Finally, spectra in which both of the above types can be recognized may also occur (Fig. 2b).

The less intensive $g \sim 4.3$ signal was not observed on every spectrum. This line is found at the beginning of the spectrum, at about 1700 gauss (Fig. 1b). It was remarkably intensive in some cases in which the amplitude of the $g \sim 2.05$ signal was low. Therefore we looked for a correlation between the amplitudes of two signals ($g \sim 2.05$ and 4.3) of the same spectra. With liver samples taken from adults, a correlation coefficient of -0.4 was calculated, which means that a high g 2.05 signal tends to be accompanied by a low g 4.3 signal; this correlation, however, was significant only at the lowest level ($p = 0.05$). With infant livers, the correlation was positive (0.89) and significant at the level of 0.001. This means that in the latter case a high $g \sim 4.3$ line is accompanied by a high $g \sim 2.05$ signal.

The third ($g = 2$) signal was registered only in one-fourth of the cases. Its g value is ca. 2.004 and is found between the 3rd and 4th Mn^{2+} lines ($g = 2.03$

and 1.98). It is therefore observed on the lower peak of the $g \sim 2.05$ signal (Fig. 1b). In most of the cases, this signal which probably comes from free radicals was even lower than the one presented here and hardly recognizable.

The spectra of the groups listed in Table 1 (newborns, adults etc). were alike. The spectra of different blocks of the same liver were qualitatively similar, that is, if several lines of a certain spectral type were registered in one block, the same were also observed in another block.

The $g \sim 2.05$ signal was studied quantitatively. As shown in Fig. 2, the peak--to-peak amplitude values at the $g \sim 2.05$ region of the ESR spectrum, expressed in arbitrary (Mn^{2+} line) units show a significant positive correlation with the copper content measured by the atomic absorption technique (correlation coefficient 0.85; $p = 0.001$). The correlation is linear, the error of the directional tangent of the straight line is 6%. This result substantiates the conclusion, drawn from the "g" and A values, that the signal comes from Cu^{2+} ions.

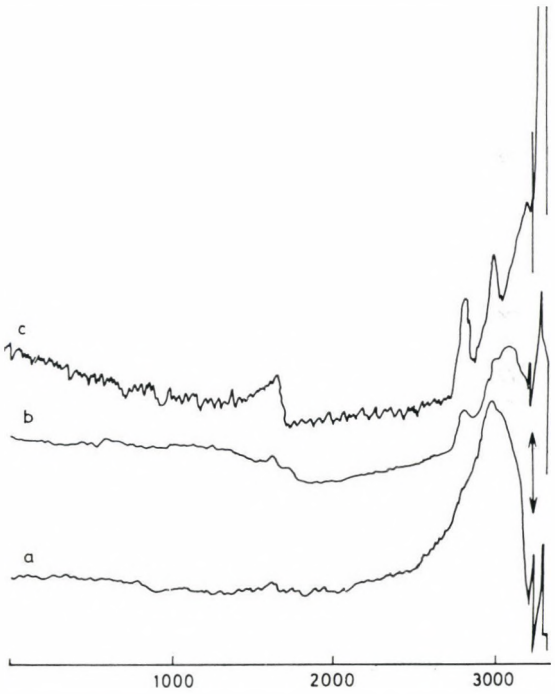

Fig. 2. The hyperfine structure of the part of the $g \sim 2.05$ signal recorded at weak (low) magnetic field in liver samples embedded in paraffin. *a.* type I Cu^{2+} complex, the hyperfine lines are merged, the parallel part of the hyperfine coupling constant ($A_{||}$) is below 100 gauss. Amplification 320-fold. *b.* spectrum containing type I and II Cu^{2+} complexes. Amplification 250-fold. *c.* type II Cu^{2+} complex, coupling constant ($A_{||}$) = 160 ± 20 gauss. Amplification 560-fold. The first line of the Mn : MgO standard is seen on every spectrum (arrow, $g = 2.14$), on spectra *a* and *b* the second line is also seen ($g = 2.08$). Recording time, time constant and microwave power are as in Fig. 1. On the abscissa, field intensity is indicated in gauss units

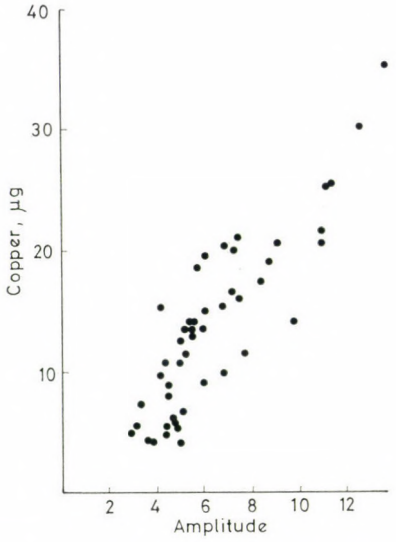

Fig. 3. Correlation of copper content with the amplitude of the $g \sim 2.05$ ESR signal in liver samples embedded in paraffin. Abscissa: signal amplitude expressed in units of the 6th line of Mn^{2+}. Ordinate: copper content in μg as determined by the flame atomic absorption technique. With copper content, determined by flame atomic absorption, taken as the independent variable (x), the regression equation of $y = 0.4x - 0.22$ is obtained for the size of the amplitude

In the control paraffin rods the copper content measured by atomic absorption was found to be lower by two orders of magnitude (0.1 μg) than in the embedded tissue, therefore our results did not have to be corrected for the copper contamination in paraffin. In the spectrum of the paraffin rods containing no tissue, at the $g \sim 2.05$ region only some unevenness, not comparable to the spectrum of copper indicated paramagnetic contamination. Part of this came from the wall of the Dewar flask.

The copper contents published in the literature refer to native liver tissue or to dry matter content and are expressed in various units. Therefore we had to determine the amount of native tissue in units of wet or dry weight to which the weighted tissue embedded in paraffin corresponded. From 20 different liver samples the amount necessary for histological studies (100 – 500 mg) was cut, weighed and after embedding in paraffin, the embedded tissue was again cut from the paraffin and weighed. The weight of the liver tissue embedded in paraffin was $64.3 \pm 10\%$ of that of the native sample. The dry matter content of the liver tissue was found to be $19.7 \pm 2\%$ of the native weight on the basis of 20 further measurements. These values were used for converting the data in the literature (Gubler et al., 1957; Smallwood et al., 1968; Butt et al., 1958) (Table I).

In the estimation of copper content by means of ESR, three possible sources of error must be taken into consideration: trimming, the measurement of the am-

plitude in the spectrum and the error of the determination of the weight of the sample. We tried to estimate the extent of these errors by comparing the data of tissue rods cut out parallelly from different blocks containing the same liver. In these samples of identical origin the standard error of the copper content was parallel with that of the amplitude. However, the standard error of the weight determination of the samples was not parallel with the standard errors of the copper content and the amplitudes. It might be supposed that copper is unevenly distributed in the rods, but this could be of importance only in the case of samples or histological sections of very small volumes (Sipponen et al., 1976). As the error of weight determination was higher than that of the determination of the amplitude (or of copper content), another possibility had to be considered, namely, that the parallel samples contained different amounts of paraffin besides the tissue. The uncomplete trimming of the embedded tissue may increase the weight of the sample, which renders the calculation of the wet weight of the tissue incorrect. This error can be prevented and the measurement made more precise if paraffin is removed from the sample by washing in xylene. The weight of the liver sample treated with xylol is roughly equal to its dry weight: the dry weight content is $19.7 \pm 3\%$ of the wet weight while the weight of the sample treated with xylol is $21 \pm 5\%$ of the wet weight. Washing in xylene does not influence the ESR signals. After re-embedding, the samples can still be used for sectioning.

The error of amplitude registration by the ESR spectrometer was 10%. Further error may be introduced by the instability of the shape of the signal. By comparing the peak areas of $g \sim 2.05$ signals of identical amplitudes but of different shapes by planimetry, a standard deviation of $\pm 5\%$ was determined. The error of the amplitude measurement is therefore about 15%.

Considering that the errors of independent manipulations add up, we also had to sum up the errors of trimming out, of the amplitude measurement and of the weight determination of the embedded sample. This means that in samples embedded in paraffin, the error of the estimation of copper content is $10 + 15 + 10 = 35\%$, while in samples extracted by xylol the same value amounts to $10 + 15 + 5 = 30\%$.

Discussion

The ESR spectra of liver samples embedded in paraffin are similar to those registered for frozen human serum (Foster et al., 1973; Bomba et al., 1977; Pocklington et al., 1977), inasmuch as it is the $g \sim 2.05$ and $g = 4.3$ paramagnetic signals that dominate also the spectrum of the serum. The $g \sim 2.05$ signal is unambiguously related to copper (Mailer et al., 1974), originating from coeruloplasmin (Bomba et al., 1977). It is, however, only about 50% of the copper content of coeruloplasmin that is paramagnetic (Broman et al., 1962; Vänngard, 1972). Also, the serum contains copper other than that in coeruloplasmin which amounts to some percentage of the total copper content of the serum and it is so far un-

known how much of this amount gives any ESR signal (Gubler et al., 1957). The $g \sim 2.05$ signal dominates the spectrum of serum, but it is absent from the ESR spectrum of adequately treated native liver tissue (see e.g. Fig. 9 in Swartz et al., 1973; Fig. 6 in Dodd et al., 1975). The $g \sim 2.05$ signal is detectable only in relatively oxidized tissue components, such as mitochondria (Sands, Beinert, 1960). In tissue samples embedded in paraffin, the oxidized state may be a consequence of fixation in formalin. After fixation in formaline and proton loss, methylene groups are attached to the molecules of the fixed material ($HCHO + H \rightarrow CH_2OH$, cf. Gabe, 1976).

The divalent copper-protein complexes described in the literature are divided into two groups. In type I (ICu^{2+}), the parallel part of the coupling constant of hyperfine structure $|A_{||}|$ is less than 100 gauss, and the copper ions are in a ligand field of tetrahedral symmetry. In type II ($IICu^{2+}$), $A_{||}$ is more than 100 gauss and the copper ion is surrounded by four planar nitrogen atoms. The embedded liver tissue contained mostly type I and a mixture of types I and II, and the number of purely type II samples was minimal. For the time being we do not know whether or not this observation has any practical significance. Although, for example, coeruloplasmin produced in the liver may occur in both types (Vänngård, 1972), it is possible that the above distribution is only the result of the processing and embedding of the sample. A picture similar to type I may be produced by clustered copper ions or even other metals.

The age-dependent change of the copper content can be also observed physiologically in the liver of infants. Before the age of three months, or in the case of prematurely born babies the copper content of the liver is higher than from the third month to the end of the first year. After the first year the copper level increases again (Butt et al., 1958). We attempted to make use of this phenomenon for finding a correlation between the amplitude of the $g \sim 2.05$ signal and the copper content of the liver. The correlation between these two characteristics indicates that the ESR spectrum could yield information for the determination of copper content. However, the correlation does not reach a maximal value. The reasons of this phenomenon may be the following:

1. It is only paramagnetic copper that gives a signal and the tissue may contain additional copper, not contributing to the ESR signal.
2. The signal of different paramagnetic metal complexes other than copper may blend into the $g \sim 2.05$ signal, modifying its shape and amplitude. In the case of liver samples with a low copper content, this would cause a virtual increase in the amount of copper traced.
3. The weight and the paraffin content of the rods cut out of the embedded tissue samples may be slightly different, and this may also bring about a fluctuation in the efficiency (quality) constant of the resonator of the ESR spectrometer.
4. For the determination of copper content we used the size of the amplitude of the $g \sim 2.05$ signal and not its second integral (its area). Because of variations in the shape of the signal, integration would have yielded a more precise result. In the literature, however, the measurement of the amplitude, which is much simpler

than integration, is equally accepted for the comparison of signals from tissue samples of identical type (Dodd, 1975; Foster et al., 1977).

The copper content of cirrhotic liver may be higher than that of normal liver. In the case of unselected cirrhotic cases, this increase falls within the limits of standard deviation (Gubler et al., 1957; Smallwood, 1968). The same was observed by us in our cirrhitoc and normal groups with respect to the $g \sim 2.05$ signal.

The second signal, observed in the $g = 4.3$ region of the ESR spectrum may theoretically come also from Cr^{2+}; in the case of serum and tissues, however, this absorption is unequivocally attributed to protein-bound trivalent iron occurring in surroundings of rhombic symmetry, like in transferrin. The $g = 4.3$ signal of serum transferrin and the $g \sim 2.05$ signal of coeruloplasmin often change simultaneously. This change may be not only parallel but also reverse, i.e. an increase in the coeruloplasmin line is accompanied by a decrease of the transferrin line (Dodd, 1975; Bomba et al., 1977). It is supposed that the iron in transferrin is oxidized to Fe^{III} by the Cu^{2+} ions of coeruloplasmin, and the higher coeruloplasmin level is a compensatory response to the increased Fe^{3+} ion requirement. The same was observed by us on the embedded liver samples.

The $g = 2$ signal, attributed to free radicals cannot be observed in mammalian sera. The often observed absence of this signal is in contrast with our earlier observations on embedded chicken liver, obtained by the application of a microwave power similar to that used in these experiments (Elek et al., 1977). This may be explained by the fact that chicken livers were fixed in formalin right after killing the animals, while the autopsy samples studied in our present experiments were subjected to fixation only after the autopsy, at least 24 hours after death.

The similarity of the spectrum of embedded liver tissue to the ESR signals of the serum is not surprising, since the serum proteins, i.e. transferrin and coeruloplasmin, detected by ESR, are produced in the liver (Grace et al., 1974). The investigation of the copper content of the serum has supplied some valuable data in connection with the effects of anticoncipients and hormones (Russ, Raymunt, 1956; Carruthers et al., 1966; Briggs et al., 1970). It may turn out later that only part of the copper content of liver is detectable by paramagnetic resonance. However, these data can still be useful, because at present no chemical technique is available that would discriminate among the different forms of copper in the liver.

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Valyl-tRNA Synthetase from Chick Embryo Brain. Properties of the Sulfhydryl Groups

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The number of thiol groups in valyl-tRNA synthetase [L-valine tRNA ligase (AMP) E.C.6.1.1.9] isolated from chick embryo brain was determined. Titration with 5.5'-dithiobis(2-nitrobenzoic) acid showed 11 free SH groups calculated on the basis of a molecular weight of 110 000 daltons. In 8M urea reaction with DTNB revealed 1 additional SH-group. Binding of substrates to the active site of the molecule decreased the number of titratable SH-groups. The modification of enzyme activity was studied by the use of thiol reagents: metal ions Ag^{+}, Cu^{2+}, Hg^{2+} and pCMB. The ATP-PP_i exchange activity of valyl-tRNA synthetase was significantly less inhibited by metal ions than the aminoacylation activity. SH-groups essential for tRNA acylation were not required for the activation of valine. After gamma irradiation of valyl-tRNA synthetase the number of SH-groups diminished parallel with the decrease in enzyme activity.

Introduction

The aminoacyl-tRNA synthetases are a group of enzymes responsible for the specificity of the genetic code (Novelli, 1967; Kisselev, Favorova, 1974). In spite of their particular importance, little is known about the role of specific amino acid residues in the catalytic activity (Mehler, Chakraburtty, 1971). The finding that all tRNA synthetases investigated so far had sulfur-containing amino acids in their structure has led to a study of the function and role of the SH--groups in enzyme activity (Cassio, 1968; Kuo and Deluca, 1969).

George and Meister (1967) titrated, with pCMB, 16 SH groups in valyl--tRNA synthetase prepared from E. coli. Yaniv and Gros (1969) found 4 SH groups in highly purified VRS isolated from E. coli K 12 and observed a decrease in the number of titratable thiol groups as a result of binding of substrates to the active site of the enzyme. Paradies (1975) reported two active forms of VRS from E. coli and the titration of SH-groups in 6M urea revealed 12 thiol groups in form II, and between 12 and 13 groups in form I. The larger fragment of form I contained 8.5 SH-groups, whereas the smaller polypeptide chain only 4.

Abbreviations: VRS, valyl-tRNA synthetase; tRNA, transfer ribonucleic acid; DTNB, 5,5'dithiobis-(2-nitro-benzoic)acid; pCMB, para-chloro-mercuribenzoate; 2-ME, 2-mercapto ethanol.

Previously we have described the isolation and the properties of VRS from chick embryo brain (Bölöni et al., 1978) and studied its radiosensitivity (Bölöni, Szabó, 1978).

The present paper deals with the properties of thiol groups in VRS with special reference to alterations induced by metal ions, thiol reagents and ^{60}Co--gamma irradiation as well as with their participation in enzyme activity.

Materials and methods

All reagents used for the isolation and assay of VRS were of analytical grade. DTNB, Norit A were purchased from Calbiochem, glycerol from SERVA, sodium borohydride and urea from REANAL. ^{14}C-L-valine (specific activity 130 mCi/mmol) and ^{32}P-sodium pyrophosphate, carrier-free, were obtained from UVVVR, Prague and Radiochemical Centre, Amersham, respectively.

VRS was prepared by a procedure described previously (Bölöni et al. 1978). The purity of the enzyme was about 90–95 per cent based on gel-electrophoretic analysis. The same process was used to prepare VRS from chicken embryo brain after irradiation *in vivo*. As a result of irradiation with doses less than 500 rad neither qualitative nor quantitative changes in the course of preparation were observed. tRNA free of protein was isolated by Kirby's method (1956), the OD_{260}/OD_{280} ratio was about 2.00.

Enzyme activity of VRS was assayed by two methods: amino acid activation was measured by the valine dependent ATP–PP_i exchange reaction according to Bergman (1962) and the formation of valyl-tRNA by the method of Yaniv and Gros (1969) using ^{14}C-valine.

Acylation assay

Enzyme activity was measured by charging tRNA with ^{14}C–L-valine. The reaction mixture contained in a total volume 250 μl : 2.5 μmoles of Mg^{2+}, 0.5 μmole of ATP-Na_2, 50 μmoles of TRIS-HCl buffer, pH 7.8, 3 μmoles of 2-ME, 2.0 μmoles of ^{14}C-valine, 80 μg tRNA and 20 μg of VRS preparation. After addition of 10 μg bovine serum albumin the mixture was incubated for 10 min at 37 °C and thereafter stopped with 3 ml of cold 10 per cent TCA. The precipitate was filtered onto Whatman GF/C glass filters which were then immersed into 4 ml of toluene, containing 200 mg POPOP and 5 g PPO per liter. Radioactivity was measured in a Packard scintillation counter.

One unit of VRS is defined as the amount of enzyme catalyzing the formation of 1 nmole of valyl-tRNA per 10 min at 37 °C. Specific activity is expressed as units/mg protein.

Amino acid activation assay

The first step of tRNA aminoacylation is the activation of amino acid. This reaction is measured by the aminoacid dependent ATP–PP_i exchange reaction, using ^{32}P labelled pyrophosphate.

The reaction mixture contained in 400 μl: 4 μmoles of Mg^{2+}, 0.8 μmole of L-valine, 0.8 μmole of ATP-Na_2, 40 μmoles of TRIS-HCl buffer, pH 8.0, 0.5 μCi of PP_i and 10–20 μg of enzyme. After incubation for 15 min at 37 °C the reaction was stopped by cooling and adding 0.2 ml of 7% PCA, 0.2 ml saturated $Na_4P_2O_7$ and 1.0 ml of 1% w/v washed Norit A suspension. After 30 min the ATP-P^{32} adsorbed to Norit A was collected on a Whatman I filter paper disc. The filter papers were dried and the radioactivity measured. One unit of VRS activity is defined as the amount of enzyme that catalyzes the incorporation of 1 nmole $^{32}PP_i$ into ATP during 15 min at 37 °C. Specific activity is expressed as units/mg protein.

Optimum conditions for both reactions have been described elsewhere (Bölöni, Holland, 1978).

Protein concentration was determined by the method of Lowry et al. (1951) using crystallized bovine serum albumin as a standard. The activity of the reaction product containing radioisotope was determined in a 688 MARK II Type Liquid Scintillation Counter (SEARLE Analytic Inc.). ^{32}P radiation was measured by the Cerenkov effect (Nascimento, 1977). The counting efficiency was 40 per cent.

Polyacrylamide gel disc electrophoresis was carried out according to Davies (1964).

Irradiation in vitro and in vivo

Irradiation of the enzyme preparations *in vitro* was carried out with a ^{60}Co Noratom-Norcontrol Irradiation Unit operating at a dose rate of 250 rad/sec. A special ^{60}Co γ-radiation source constructed in our institute was used for the irradiation of the embryos in eggs. The dose rate was 80 rad/min.

Assay of sulfhydryl groups

The titration of free thiol groups was performed with DTNB according to Ellman (1959). 2-ME used for the isolation procedure was removed by gel filtration before assaying. The titration was carried out as follows: 20 μl of 10 mM DTNB was added to 1.00 ml of a reaction mixture containing 0.4–0.5 mg VRS in 20 mM phosphate buffer. Change in optical density at 412 nm was determined spectrophotometrically (Unicam Sp Series 2). The reaction was completed in 4–5 hours. The buried SH-groups were freed by keeping the enzyme in 8M urea at room temperature for 30 minutes. After this treatment 20 μl of 10 mM DTNB was added to the reaction mixture and the optical density at 412 nm was measured. The maximum absorbancy was observed in 15–20 minutes. Assays were performed against control samples containing all components except the enzyme. In the calculations

the molecular weight of VRS was taken as 110 000 the molar extinction coefficient of reduced DTNB as 13.6×10^3 The DTNB dissolved in 50 mM TRIS-HCl buffer, pH 7.8. The titrations were made at room temperature. Assays were carried out also in the presence of the substrates of VRS using them together and separately. Substrates were added to the enzyme in quantities suitable for the aminoacylation reaction to occur. Incubation at 37 °C for 10 min caused no change in the number of titratable SH-groups.

Results

Thiol groups in VRS from chick embryo brain

Based on a molecular weight of 110 000 daltons 11 free and 1 buried SH--groups per mole were determined. When the native VRS was exposed to DTNB in the absence of denaturing agents only 4—4.5 SH-groups reacted rapidly (within 5 min), further 2 SH-groups reacted during 15 min and the others even slower. During 150 min 10 SH-groups and during 300 min 11 free SH-groups were titratable with DTNB (Fig. 1). The SH-groups of VRS can be divided into three types with different reactivities. The number of SH-groups per type and the pseudo-first-order rate constant of reaction with DTNB were calculated by a least square fitting program using a Hewlett—Packard calculator, type 9825 A, as well as from plots such as those in Fig. 2. The first type (I), comprising 3.8 SH-groups, was very reactive; its rate constant (k_I) 3.8×10^{-2} sec^{-1}. The rate constant (k_{II}) for the second type was 3.0×10^{-3} sec^{-1}, (2 SH-groups), and that (k_{III}) for the third type was $1.5 \times 10^{-4} sec^{-1}$ (5 SH-groups). The correlation were 0.97, 0.99 and 0.94 for the I, II and III linear plots, respectively.

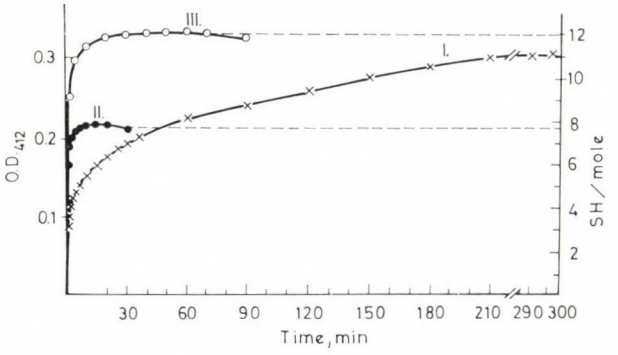

Fig. 1. *Titration of SH groups by DTNB according to Ellman.* VRS was freed from the protecting SH-reagent (2-ME) by filtration on a Sephadex G-25 column equilibrated with 20 mM phosphate buffer, pH 7.8. The reaction mixture (1.0 ml) contained 0.3—0.5 mg VRS in 20 mM phosphate buffer, pH 7.8. After addition of 20 μl of 10 mM DTNB the increase in absorption at 412 nm was measured. I, in the presence of buffer only; II, in the presence of 4×10^{-6} M tRNA, 2×10^{-3} M L-valine, 2×10^{-3} M ATP; III, in the presence of 8 M urea

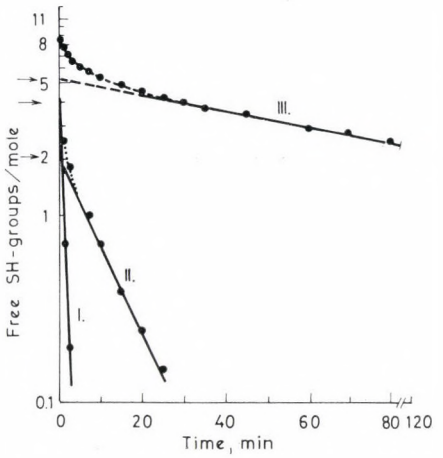

Fig. 2. *Semilogarithmic plot of the titration of VRS SH-groups by DTNB.* Total titration of native VRS, the data are from curve I in Fig. 1

In 8M urea (Fig. 1, curve III) 12 SH-groups reacted with DTNB within 30 min. After addition of the substrates to the enzyme the number of reactive SH--groups in VRS changed. The number of SH-groups immediately reacting amounted to 7 in 5 min and reached its maximum in 20–25 min, in contrast to the native enzyme. This suggests that there are thiol groups at the substrate binding sites or very near to them.

Incubation of VRS with metal ions

Table I shows the modification of the aminoacylation activity of VRS under the effect of various metal ions. Activities were measured after 25 min incubation with the metal ions at 0 °C. In all cases a strong inhibition was observed.

Table 1

Modification of the acylation activity of VRS in the presence of metal ions
Incubation time with metal ion: 25 min at 0 °C VRS concentration: 1×10^{-7} M
Enzyme activity was measured by the aminoacylation assay described in *Materials and methods*

Metal ions	Enzyme activity	
	cpm/mg protein	%
—	70 100	100
Ag^{+}, 1×10^{-5} M	9 640	14
Cu^{2+}, 1×10^{-5} M	6 905	10
Hg^{2+}, 1×10^{-5} M	3 810	5.5

Fig. 3 shows the VRS activities assayed by amino-acylation and by the formation of valyl-AMP-enzyme complex as a function of Cu^{2+} concentration and of the incubation time. It can be seen that the inhibition takes place in 1–2 minutes and does not change any more. Furthermore, at low metal ion concentration, there is no difference between the two reactions; at higher Cu^{2+} concentrations, however, the formation of tRNA is inhibited more strongly than the activation of valine.

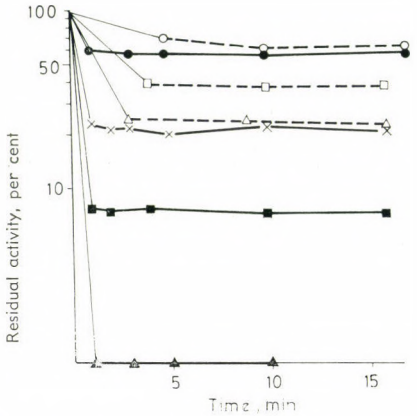

Fig. 3. *Effect of Cu^{2+} on the two stages of valyl-tRNA formation.* ○- - -○, amino acid activation in the presence of 2×10^{-6} M Cu^{2+}; □- - -□, amino acid activation in the presence of 1×10^{-5} M Cu^{2+}; △- - -△, amino acid activation in the presence of 2×10^{-5} M Cu^{2+}; ●——●, aminoacylation in the presence of 2×10^{-6} M Cu^{2+}; ×——×, aminoacylation in the presence of 5×10^{-6} M Cu^{2+}; ■——■, aminoacylation in the presence of 1×10^{-5} M Cu^{2+}; ▲——▲, aminoacylation in the presence of 2×10^{-5} M Cu^{2+}; VRS concentration 5×10^{-7} M. 2-ME was removed before assaying

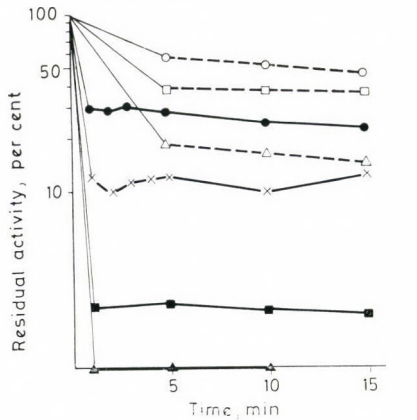

Fig. 4. *Effect of Hg^{2+} on the two stages of valyl-tRNA formation.* For more details see Fig. 3

Fig. 4 represents the effect of Hg^{2+} on the enzyme activity. The formation of valyl-tRNA seems to be more sensitive in this case, too.

Fig. 5 gives a comparison between Cu^{2+} and Hg^{2+} with regard to their inhibiting effect on VRS activity.

It can be seen that 5×10^{-7}M VRS reacted with various amounts of the inhibitors (4, 10, 20 and 40-fold, related to the molar concentration of the enzyme). In the case of a tenfold excess of the inhibitor (5×10^{-6}M), when all of the free thiol groups were blocked, the exchange reaction was inhibited by Cu^{2+} and Hg^{2+} to 50 and 55 per cent, respectively.

On the other hand, the formation of valyl-tRNA as the second stage was inhibited to 80 and 90 per cent respectively, under the same conditions. A complet blocking can be observed in the case of a twentyfold ion excess of the inhibitors. This shows that the first stage of enzyme reaction is less sensitive to metal ions than the whole reaction.

It can be seen from Fig. 5 that some SH-groups which are essential for tRNA acylation are not required for the activation of valine.

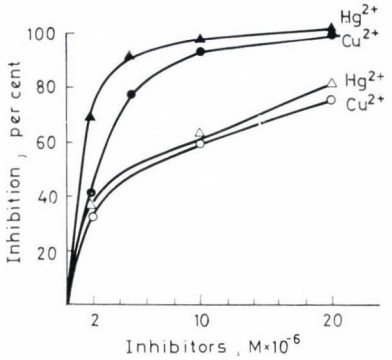

Fig. 5. *Inhibitory effect of Cu^{2+} and Hg^{2+} on the two stages of valyl-tRNA formation.* Inhibition of aminoacylation activity by Hg^{2+}, ▲ — ▲; by Cu^{2+}, ● — ●. Inhibition of amino acid activation by Hg^{2+}, △ — △; by Cu^{2+}, ○ — ○. Incubation time with metal ions was 10 min

Incubation of VRS with thiol reagent

Fig. 6 demonstrates the inhibition by pCMB of both enzymatic activities of VRS. A complete inhibition could be observed at 2×10^{-5} M pCMB concentration. There was no significant difference between the inhibition of amino acid activation and that of the formation of valyl-tRNA.

The effect of ^{60}Co-γ-irradiation on thiol groups

Previously, we have reported (Bölöni, Szabó 1978) that VRS was inactivated by ^{60}Co γ-irradiation and that the inactivating effect on the ATP-PP_i exchange reaction was of a lower degree than that on the formation of valyl-tRNA. Therefore, it seemed of interest to study the possible correlation between inactivation by irradiation and the number of thiol groups.

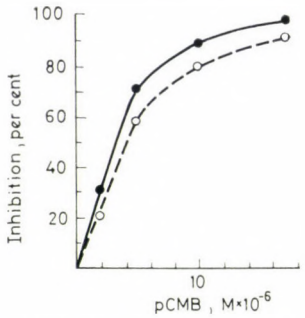

Fig. 6. *Inhibitory effect of pCMB on VRS activity.* ● — ● Aminoacylation; ○ - - - ○ Amino acid activation. VRS concentration was 2.5×10^{-7} M

VRS samples were irradiated by different dose of γ-ray after removing 2-ME and glycerol by gel filtration on Sephadex-G-25 and the enzyme activity as well as the number of thiol groups was determined.

Fig. 7 shows a decrease of the number of SH-groups with increasing dose rate.

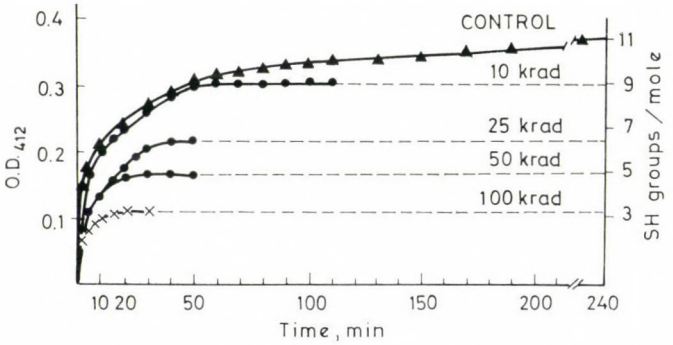

Fig. 7. *Titration of free SH-groups of VRS after irradiation in vitro.* Before irradiation VRS was freed from protecting 2-ME by gel-filtration. Enzyme concentration during the exposure 0.5 mg/ml

Fig. 8 represents the number of SH-groups related to enzyme activity. The aminoacylation activity of the VRS was measured as a function of the dose and expressed, on a logarithmic scale, in per cent of the unirradiated control.

While at low doses the decrease of free SH-groups runs parallel with the inactivation of the enzyme, this is not case at doses higher than 50 krad.

Effect of γ-irradiation in vivo

Chicken embryos in eggs were irradiated with doses less than 500 rad. From this material the enzyme was isolated in the same way as from the non-irradiated control. The preparations were controlled by gel electrophoresis and UV spectrophotometry.

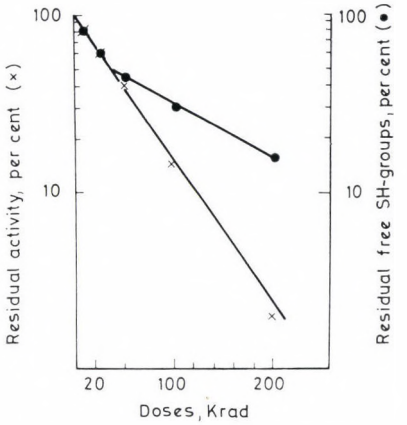

Fig. 8. *Change in activity and in the number of SH-groups of VRS after irradiation in vitro.* VRS activity was assayed by registering the formation of ^{14}C-valine-tRNA. × — ×, Remaining activity expressed in per cent of non irradiated control; • — •, Remaining free SH--groups, estimated by titration with DTNB, in per cent of the control

In irradiation experiments carried out *in vivo* (e.g. in eggs) three main parameters have to be considered (Bölöni, 1978, in press):

i) dose of irradiation,

ii) age of embryos and

iii) postirradiation time.

Therefore, in our experiments two different arrangements were used:

i) 18-day-old chicken embryos were irradiated with various doses and killed 24 hours later, and

ii) 15-day-old chicken embryos were irradiated with various doses and killed 4 days later.

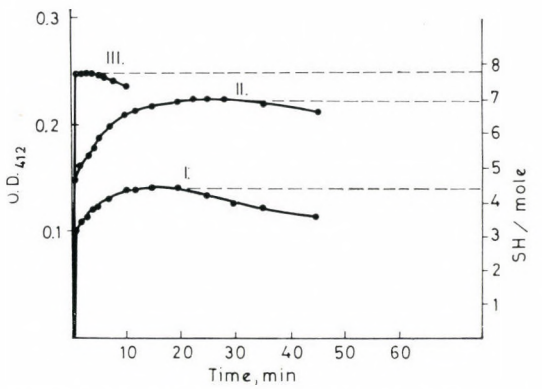

Fig. 9. *Titration of SH-groups of VRS irradiated with 200 rad in vivo on day 18 of the embryonic life.* I, Enzyme alone; II, enzyme with substrates; III, enzyme with 8 M urea

SH-groups were determined in both cases in the enzymes samples isolated from chick embryo brains.

The number of total SH-groups in VRS of chick embryo brain irradiated on day 18 with 200 rad, decreased to 7.8 while that of the free SH groups to 4.4 (Fig. 9). In the presence of substrates, however, 7 SH-groups could be titrated with DTNB. The reaction took place very rapidly in all cases.

All SH radicals of VRS from chick embryo brain irradiated with 400 rad on day 15 could be titrated in the presence of 8M urea, the number of free SH-groups, however, was only 7. In the presence of substrates 9 SH-groups could be titrated (Fig. 10). Table 2 summarizes the effect of irradiation *in vivo* on VRS activity. A marked difference in the aminoacylation activity can be seen. The first group shows a dose dependent decrease in activity, while the activity of the second one exceeds the control value.

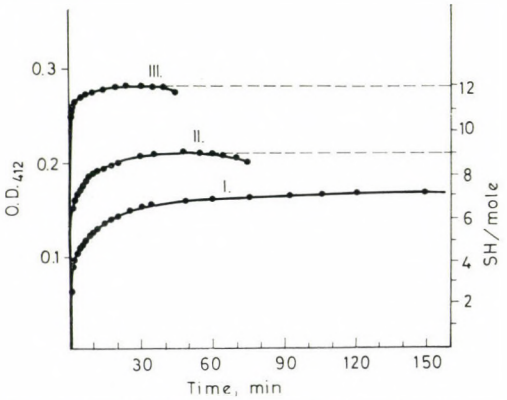

Fig. 10. *Titration of SH-groups of VRS irradiated with 400 rad in vivo on day 15 of the embryonic life.* VRS from the brain of irradiated embryos was titrated with DTNB by the method of Ellman. The production of TNB was estimated by measuring the increase in absorbance at 412 nm after the addition of DTNB at a final concentration of 2×10^{-4} M. I, Enzyme alone (0.3 mg/ml); II, enzyme with 4 μM tRNA + 2 mM L-valine + 2 mM ATP; III, enzyme with 8 M urea. Before the titration 2-ME was removed by gel filtration

Table 2

Effect of irradiation in vivo on the activity of VRS and on the number of its SH-groups
Chick embryos were killed and VRS prepared from brains on day 19 in all experiments

Day of irradiation	Dose (rad)	Total SH	Free SH	SH in the presence of substrates	Enzyme activity cm/mg protein	
18	100	7.5	5.0	7.2	106 000	94%
18	200	7.8	4.4	6.9	78 980	70%
18	400	7.0	4.4	5.7	60 880	54%
Control	—	12	11	8.2	112 750	100%
15	400	12	7	8.9	143 960	128%
15	500	10	7.2	7.0	135 400	120%

Discussion

Although the exact role of SH-groups in catalysis has not been demonstrated yet, they are generally required for the function of aminoacyl-tRNA synthetases. Free and buried SH-groups in aminoacyl -tRNA synthetases of different species and specificity were studied by a number of authors. Fasiolo et al. (1970) demonstrated 17 free SH-groups in phenylalanyl-tRNA synthetase from yeast and only 10 of these could be titrated in the presence of substrates. De Lorenzo et al. (1974) found 2 free and 2 buried SH-groups in histidyl-tRNA synthetase from Salmonella typhimurium. Lemarie et al. (1969) detected 14 free and 2 buried thiol groups in tryptophanyl-tRNA synthetase isolated from bovine pancreas. This was confirmed by Iborra et al. (1973) who found a decrease of thiol groups in the presence of substrates. On the other hand, Joseph and Muench (1971) found 2 free thiol groups in tryptophanyl-tRNA synthetase from E. coli.

Valyl-tRNA synthetase prepared from E. coli K12 contained 4 titratable SH-groups according to Yaniv and Gros (1969) and only 2 of these in the presence of valine and ATP. George and Meister (1967) detected 16 SH-groups in VRS isolated from E. coli strain B. In our experiments 11 free and 1 buried SH-groups were found in VRS isolated from chick embryo brain. Free SH-groups in the VRS molecule from chick embryo brain may be divided into three classes with respect to their reaction capacity. The first class contains 5 SH-radicals, the second class 2 radicals and the third one 4 SH-radicals. In the presence of the substrates 7 – 7.5 SH-radicals become accessible in a few minutes and no further increase in their number can be observed. Both the increase in the number of the immediately reacting SH-groups and the lack of any further change in the molecular structure show that the binding of the substrate(s) induces the alteration in conformation and further the stabilization of the new conformation of the molecule.

Correlation between enzyme activity and number of SH-groups was studied in two different ways:
1./SH-groups in VRS were reacted with various metal ions and thiol reagents and the enzyme activity was determined, and 2./The number of SH-groups was measured as a function of γ-irradiation, *in vitro* is known to result in a decrease of VRS activity (Bölöni, Szabó, 1978). In both cases we found a linear correlation between the decrease of enzyme activity and that of the number of thiol groups. By assaying separately the two phases of the VRS enzyme reaction, the valine dependent ATP-PP_i exchange reaction was found to be significantly less sensitive than the aminoacylation reaction. This observation is in good agreement with those previously described by Kuo and Deluca (1969), Cassio (1968), Rouget and Chapeville (1971) and Murayama et al. (1975). Modifications of thiol groups in several tRNA synthetases caused a decrease of the aminoacylation reaction but only a minimum or no concomitant in the ATP-PP_i exchange reaction. This suggests that there are SH-groups that are not important for the amino acid activation but indispensable for the formation of aminoacyl-tRNA.

Irradiation *in vivo* on day 18 caused a decrease of enzyme activity which ran parallel to that of the number of SH-groups. After irradiation of 15-day-old chick embryos with 400, 500 rad the number of SH-radicals of VRS reached that of the control and the enzyme activity surpassed the control. This relates to the function of some restoring mechanism in the organism of the chick embryo.

I am indebted to Prof. L. Boross for valuable discussions and critical reading of the manuscript, as well as to Mrs Hanna Sinay and Mrs Ildikó Batke chemical engineer for their excellent technical assistance.

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X-ray Microanalytical Studies on Native Myofibrils and Mitochondria Isolated by Microdissection from Honey-Bee Flight Muscle

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The distribution of elements was studied by means of energy dispersive X ray microanalysis in the honey-bee flight muscle. Semi-quantitative analysis of bulk muscle specimen, isolated mitochondria and isolated myofibrils either in resting or stretched state was performed.

High K peak was observed in the spectra of bulk muscle specimen resembling the spectra of myofibrils and high P peak resembling the spectra of mitochondria. In the myofibrils no substantial difference was found between the spectra of A bands and Z lines, but they contained much higher K peak than the spectra of I bands.

Introduction

In order to better understand the physiological role of elements in muscle contraction, studies on the subcellular distribution of elements in the muscle tissue and the elemental composition of the contractile apparatus- i.e. the myofibrils-are important. These problems are amenable to X ray microanalysis. Recently attempts were made to study these problems with the method of X ray microanalysis using different preparatory techniques:

a) Analysis of freeze-dried cryo sections.

Sjöström and Thornell (1975) gave a semi-quantitative estimation of the elements found in the A bands, Z lines and mitochondria of the frog toe extensor muscle. Somlyó et al. (1977) performed a quantitative analysis of the complete fibres, the extracellular space, the cytoplasm, the sarcoplasmic reticulum and the terminal cisterna of the frog toe and semitendinosus muscle. Although this preparatory technique is suitable for the analysis of the contractile apparatus, unfortunately, neither of two very excellent works dealt with the myofibrils in details.

b) Analysis of cryofractured bulk specimens with freeze-dried surfaces.

Zeerold et al. (1978) described the applicability of this preparatory technique to study the distribution of elements in the skeletal-muscle tissue depending on its physiological state. However, the poor ultrastructural details and the uncertainty of depth and spread of probe did not make the analysis of a single myofibril possible.

c) Analysis of freeze-dried and plastic embedded tissue.
Edelmann and Hubert (1978) studied dried-cut sections of K, Cs or Tl loaded muscles with this technique. Since this preparatory technique had maintained the recognizable ultrastructural organization, detection of the distribution pattern of K, Cs and Tl *in a sarcomer* was initiated. These elements were preferentially accumulated at specific sites of the A band and in the Z line.

In connection with the latter findings the purpose of this paper is to describe the applicability of X ray microanalysis of myofibrils and mitochondria isolated without any chemical treatment in order to study the distribution of elements along the myofibrils and to compare the elemental composition of myofibrils and mitochondria in the flight muscle of honey-bee.

Material and methods

The dorsoventral flight muscle of honey-bee (Apis mellifica) was used. The myofibrils were prepared according to the method of Ernst et al. (1956) with a slight modification. The preparation of the specimen was done in the following manner: head, abdomen, protothorax, legs and wings were removed from the thorax. The thorax was bisected and few fibres were put on a copper grid covered with carbon coated Formvar films. The fibres were quickly desintegrated between two needles on the surface of the Formvar film without using any liquid. All the preparative procedures were carried out under stereo microscopic control. Isolated myofibrils of different striated patterns and isolated mitochondria were produced by desintegration. After the desintegration the specimens were separated;

1. for X ray microanalysis.

The specimens were dried at room temperature and immediately transferred to the electron microscope. Examination and analysis were performed in a JEOL TEMSCAN-100 C electron microscope equipped with a goniometer stage and liquid nitrogen cooled anticontamination device. An energy dispersive ORTEC Si (Li) detector was fitted directly into the microscope and interfaced with an ORTEC 6230 multichannel analyser. The results were obtained by using an accelerating voltage of 80 KV. The specimen tilt was 45°, the counting time was 200 sec and the spot size was 0.5 μ in diameter. The beam current was the same throughout the study. The window display vertical scale was chosen to be 500 and the energy per channel was 10 eV. Elements of Na to Ca were expected therefore the analysis was performed in the energy range of $1-5$ KeV.

2. for the scanning electron-microscopy.

For the best visualization the desintegrated muscle was prepared for the scanning electron-microscope parallel with the analysis. After the desintegration the specimens were fixed with glutaraldehyde dehydrated by ascending alcohol series and dried at room temperature. Prior to the examination in a TEMSCAN-100 C microscope, operated at 20 KV, the specimens were coated with a thin conductive film of gold, in a JEOL vacuum evaporator.

Results

The contractile material of the flight muscle is arranged in separated myofibrils running parallel to each other in bulk specimen (Fig. 1). The myofibrils are separated by mitochondria. Fig. 2 shows X ray spectrum of similar bulk specimen. The spectral peaks corresponding to Na, Mg, P, S, Cl, K and Ca are clearly seen. The peaks of P, S and K are prominent. The relative magnitude of the P and K peaks varied, depending on the place analysed. Analyses were taken over the mitochondria and over the A bands of unstretched myofibrils. The spectrum taken over the mitochondrium indicated higher amount of P than that over the A band, whereas the peak of K was lower (Fig. 3). The structure of isolated myofibrils cor-

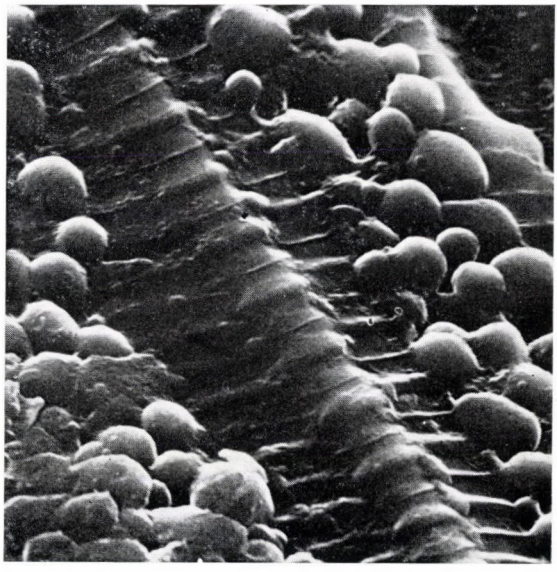

Fig. 1. Bulk muscle specimen containing sonie myofibrils and mitochondria (x 6.000)

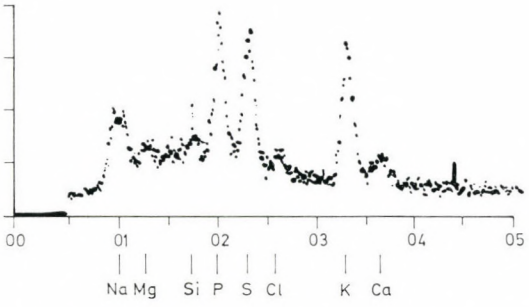

Fig. 2. X-ray spectrum of bulk muscle specimen with prominent P, S and K peaks

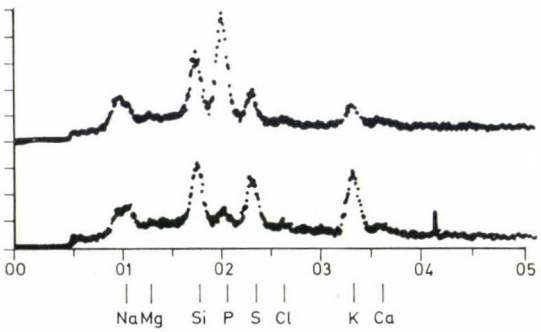

Fig. 3. X-ray spectra of isolated mitochondrium (top) and A band from resting length myofibril (bottom). Note the considerable difference of P and K peaks. The window display vertical scale was selected to be 1000

responds well to the structure of native myofibrils observed in phase contrast microscope (Garamvölgyi et al. 1964). The myofibrils isolated in resting length contain only A bands, M and Z lines (Fig. 4 a,b). In the stretched myofibrils, besides the above mentioned parts, I bands can also be seen (Fig. 5 a,b).

Paired analysis of an A band (bottom) and an I band (top) are shown in Fig. 6. In both spectra the same elements – Mg, P, S, Cl, K – were observed. Comparing the two spectra, we found a considerable difference between the magnitude of the K peaks. It seems that the A band contains much more K than the I band. On the other hand, no substantial difference was found between the spectra obtained from A bands of unstretched myofibrils, A bands of stretched myofibrils and the Z lines. Special care was taken in the selection of myofibrils analysed. We used only that myofibril which had a very clear environment and where the analysis taken over the Formvar film just outside the myofibril showed no indication of K "spilled" from the myofibrils. In such arrangement paired spectra are shown in Fig. 7, which were taken over an A band and over the Formvar film just outside the myofibril. A prominent Si peak can only be seen in the spectrum of the Formvar film. The presence of a varying, but frequently high Si peak can be due to the contamination caused by pump oil and vacuum grease used in the electron microscope and vacuum evaporator, and signal originating from the dead layer of the detector.

Discussion

The spectra taken occasionally over the bulk muscle specimen generally show high P and K peaks (Fig. 2). On the other hand, the spectra of mitochondria indicate higher amounts of P than those of the A bands, whereas the peak of K is lower (Fig. 3). Therefore, it can be supposed that a considerable part of P may

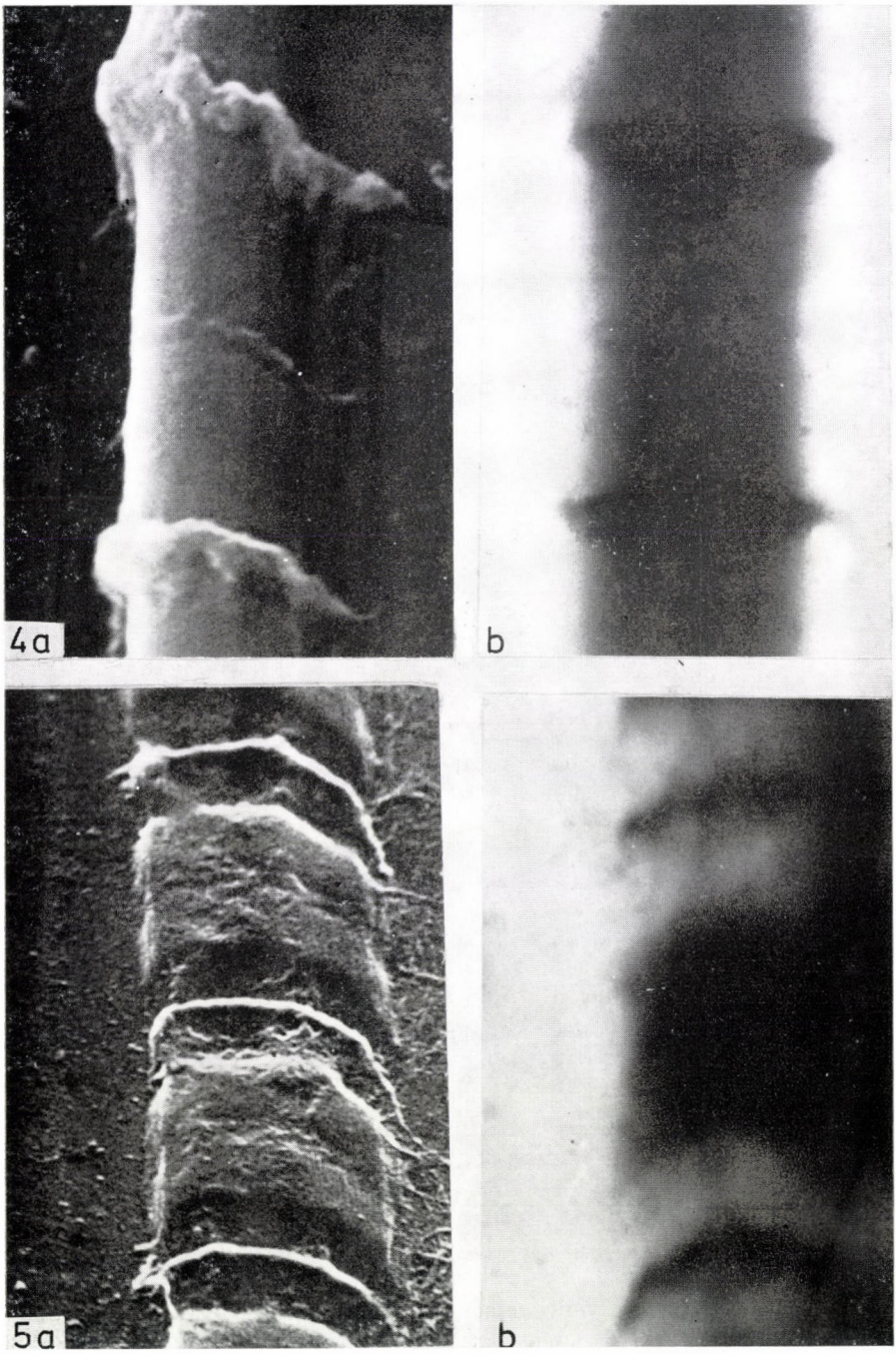

Fig. 4. Isolated myofibrils in resting length (× 20,000). a. scanning micrograph; b. transmission micrograph

Fig. 5. Isolated myofibrils in stretched state (× 10,000). a. scanning micrograph; b. transmission micrograph

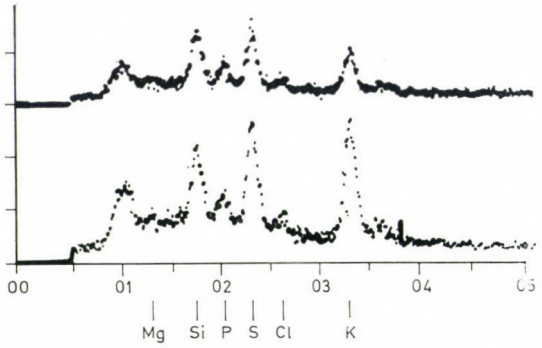

Fig. 6. X-ray spectra from two areas of stretched myofibril. The spectrum of the I band (top) indicates lower amount of K than that of the A band (bottom)

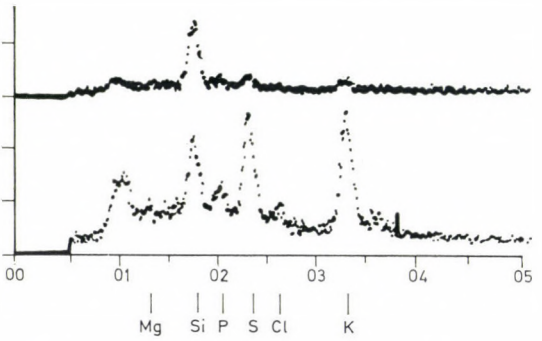

Fig. 7. X-ray spectra taken over an A band (bottom) and over the Formvar film just outside the myofibril (top)

arise from the mitochondria in the insect flight muscle, and large part of the muscle K may arise from the myofibrils.

The greatest advantage of this preparatory technique is that it makes the analysis of the complete myofibril possible. The analyses of stretched myofibrils gave some remarkable results. It was found that the A bands and Z lines contained a large amount of K. No substantial difference was detectable between the two K contents, which corresponds with the findings of Sjöström and Thornell (1975) who used freeze-dried cryo sections. The most striking observation, however, was the uneven distribution of K in the stretched myofibrils. K is much more accumulated in the A bands than in the I bands (Fig. 6). This finding corresponds with the work of Edelmann and Hubert (1978) who used Cs and Tl loaded frog sartorius in freeze-dried and plastique embedded preparations. They pointed out that K is preferencially accumulated at specific sites of the A band and in the Z line. The uneven K distribution along the stretched myofibrils of the insect flight

muscle supports the earlier results, which were observed by Ernst (1963) and reaffirmed by his co-workers (Tigyi–Sebes, 1962; Nyesterov, Tigyi–Sebes, 1965; Kállay, Tigyi–Sebes, 1969). According to Ernst, " . . . most of the potassium is localised in the anisotropic parts of the muscle-fibrils" (quoted from Ernst, 1963) as "bound"-potassium. Furthermore, our results are also in good agreement with the recent works of Ling (1977) and Edelmann (1977), who demonstrated that the bulk of intracellular K is located primarily in the A bands and Z lines of living frog muscle cell in adsorbed state on the β- and γ-carboxil groups of cell protein.

Since the volatalization of potassium from the specimen due to the energy from the electron beam (Hodson and Marschall, 1971) decreases the height of potassium peak, and since the air-drying technique is not suitable for wide application in X ray microanalysis (e.g. Appleton, 1972) in any further work, it will be necessary to stabilize the elements under the electron beam by cooling the specimens, and on the other hand to examine frozen hydrated muscle specimens and to compare the results.

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The Passive Electrical Characteristics of Giant Neurones Identified in the Central Nervous System of Lymnaea stagnalis

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The passive electrical characteristics of four giant neurones in the CNS of *Lymnaea stagnalis* were studied. The parameters were calculated on the basis of potential transients evoked by hyperpolarizing square impulses. The examined neurones can be classified in two groups, which show significant differences considering the time constant and membrane resistance. This classification is in correlation with the functional properties of the cells. The differences in the passive electrical characteristics of the giant neurones were compared to some data obtained by using voltage clamp method. The role of these parameters in the generation of the single spike and the rhythmic discharge is discussed.

Introduction

Neurones of the central nervous system (CNS) of Molluscs have a wide variety of rhythmic activity. There are significant differences in the mechanisms of spike generation of the individual neurones, which have been studied extensively on the pond snail Lymnaea stagnalis (Kiss, Salánki, 1973; 1974; Kiss, Vadász, 1977). Some of the neurones show action potential of short duration, and others of longer duration. This difference is in part a function of the time-course of spike generation.

In the central sensory cells of leech Zhuravlev, Safonova, 1975, further in Planorbis neurones Magura et al., 1972 have found that cells generating action potentials of different time-courses differed also in respect of their passive electrical properties.

For the present experiments four giant neurones were choosen in the CNS of Lymnaea identified (Kiss, 1973; Kiss, Salánki, 1976), which showed pronounced differences in the parameters of the spontaneous activity. We intended to investigate the passive electrical characteristics of these neurones in order to clear up the role of these parameters in the generation of the spontaneous activity.

Materials and methods

Experiments were carried out on the cells A2, A6, A10 and P13 (Kiss, Salánki, 1976) located in the visceral and right parietal ganglia of the isolated CNS of *Lymnaea stagnalis*. Membrane and action potentials were recorded by glass

microelectrodes of 3 – 5 MOhm resistance filled with 2.5 M KCl. For recording and current application the same electrode connected with a FET input amplifier (Véró, 1971) and a square-wave generator (Véró, 1972) was used.

The passive electrical parameters of the membrane were calculated on the basis of potential transients evoked by hyperpolarizing square pulses. For calculating the total input resistance (R) 5 nA pulses were used. The value of impulse duration allowed the potential transient to reach a steady maximal value (V_∞). The total input resistance was determined as $R = \frac{I}{V}$. The value of this is always smaller than the somatic input resistance (r_s), as the above consists of the resistance of soma (r_s) and that of the axonal and dendritic processes (r_a). The axo-somatic conductance ratio (ρ) gives the relation between r_s and r_a. This relation was first described by Rall (1960) and was applied to the unipolar neurones of molluscs by Gorman and Mirolli (1972) and Marmor (1971). Practically we obtained its value with graphical differentiation of the potential transients at the moment $t = 0$. For calculation of r_s and r_a we used the equation described by Magura et al. (1972) and Zhuravlev and Safonova (1975):

$$r_s = R(1 + \rho) \qquad \text{and } r_a = \frac{r_s}{\rho}$$

The membrane capacity (C_s) is given by the equation $C_s = \frac{\tau}{r_s}$ (Magura et al., 1972). The time constant (τ) of the membrane was measured directly from the potential transients: τ is defined as the time required for the potential reach 63% of V_∞.

Results

Some important parameters of the spontaneous activity of the examined neurones are presented in Table 1. The most regular rhythmic discharge and the highest firing rate can be recorded from neurone A10. From time to time this discharge is interrupted by inhibition of long duration (ILD), which is obviously of synaptic origin (Kiss, Salánki, 1971). The other neurones show lower firing rate and an irregular frequency distribution. The duration and latency of the action potentials evoked by depolarization in neurones A10 and P13 exceed those in the neurones A2 and A6. Neurone A2 was the only one that generated more than one spike as a reaction to the depolarizing impulse of given duration.

The action potentials recorded from neurones A10 and P13 have longer after-hyperpolarization, as compared to the other two neurones examined. When using depolarizing pulses of longer duration (50 – 100 msec) on neurones A10 and P13 another type of long after-hyperpolarization (LAHP) can be observed after switching off the applied current (Table 1., Fig. 1), the duration of which can reach more than 10 sec in the case of neurone A10. The duration and the maximal amplitude

Table 1

Cell	Spike duration (msec)	Duration of after-hyperpolarization (msec)	Characteristics of spontaneous activity	Average frequency (cps)	LAHP following depolarizing impulse
A2	6—7	10—15	silent or irregular spontaneous activity	0.53 ± 0.43*	no
A6	7—8	10—15	silent or irregular spontaneous activity	0.33 ± 0.27	no
A10	12—23	250—300	regular pacemaker activity	1.15 ± 0.6	10—15 mV
P13	12—13	40—50	synaptically influenced latent pacemaker	0.72 ± 0.46	10—15 mV

* SD.

of this LAHP appeared to be strongly depending on the intensity of the depolarizing current pulses. (Fig. 1). After swithing off the hyperpolarizing current each examined cell generated action potential.

Table 2 demonstrates some passive electrical characteristics of the cells examined. The smaller neurones (A6 and A10) show higher input resistance, than

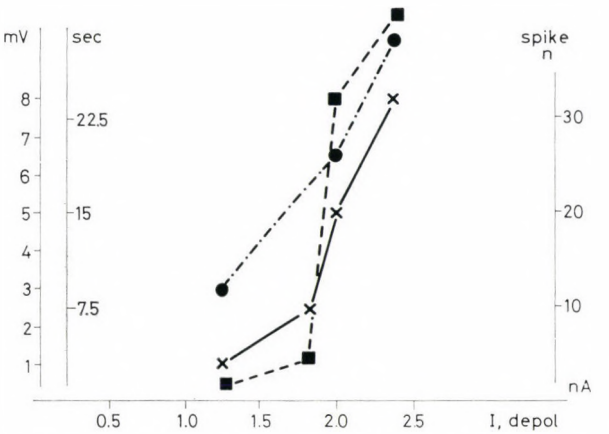

Fig. 1. Changes in some electrophysiological properties of neurone A10 depending on the depolarizing current applied to the soma (I, depol, nA). ■ — — ■ duration of the afterhyperpolarization recorded after switching off the depolarizing pulse, (sec); ×———× maximal amplitude of the same after-hyperpolarization, (mV); ● — — ● number of action potentials generated during the maintained depolarizing pulse (spike, n)

Table 2

Cell	d (μm)	R Ohm ($\times 10^6$)	τ (msec)	ϱ
A_2	170	4.30 $\pm$ 0.14*	34.50 $\pm$ 0.31	5.40 $\pm$ 0.25
P_{13}	130	3.50 $\pm$ 0.30	71.40 $\pm$ 1.41	5.01 $\pm$ 0.10
A_6	110	7.30 $\pm$ 0.17	42.00 $\pm$ 1.08	1.13 $\pm$ 0.04
A_{10}	100	7.20 $\pm$ 0.12	82.25 $\pm$ 1.65	7.68 $\pm$ 0.41
Cell	d (μm)	r_a Ohm ($\times 10^6$)	r_s Ohm ($\times 10^6$)	C- F ($\times 10^{-9}$)
A_2	170	26.91 $\pm$ 1.49	4.98 $\pm$ 0.10	1.30 $\pm$ 0.12
P_{13}	130	21.03 $\pm$ 1.57	4.23 $\pm$ 0.27	3.18 $\pm$ 0.27
A_6	110	15.17 $\pm$ 0.56	13.42 $\pm$ 0.12	2.86 $\pm$ 0.11
A_{10}	100	63.40 $\pm$ 4.70	8.22 $\pm$ 0.46	1.32 $\pm$ 0.11

d — diameter of the cell; R — total input resistance; ϱ — axosomatic conductance ratio; τ — membrane time constant; r_s — somatic input resistance; r_a — axonal input resistance; C_a — total membrane capacity.
* S.D.

the greater ones (A2 and P13), as it is to be expected. It is characteristic of all examined cells that the axon contributes to the total input resistance in a high degree (see ρ values), thus r_s appears to be significantly higher than R. In this respect it should be taken into consideration that the method we used for calculation of ρ involved some simplifying supposition (Rall, 1960; Lux, Pollen, 1966).

On the basis of the membrane time constants the neurones can clearly be classified into two categories:
(1) A2 and A6: the value of τ varies between 34.5 and 42 msec
(2) A10 and P13: the value of τ varies between 71.4 and 82.2 msec.
The difference between the two groups proved to be significant ($P < 0.001$).

At the same time no significant differences were found between the r_s values characterizing neurones A2, A6 and P13, only neurone A10 showed an extreme value presumably owing to its electrical coupling with an other giant neurone (Kiss, 1977).

Discussion

Some of the passive electrical characteristics of the examined neurones show considerable differences. These are especially pronounced in relation of the time constant of the membrane. We classified the examined neurones in two groups:

neurones A2 and A6 may be considered as "fast" cells, while neurones A10 and P13, as slow ones on the basis of τ values. There is a 2–2,5-fold difference between the τ values characteristic of the two groups. Considering the other parameters such pronounced differences cannot be observed. This classification is in correlation with the functional properties of the cells (cf. Table 1). In earlier investigations (Kiss, Salánki, 1976; 1977) the "fast" cells proved to be synaptically driven, while the "slow" ones clear (A10) or latent (P13) pacemaker neurones. The cells, which generate action potential of longer duration and of considerable after-hyperpolarization are characterised by greater time constant and input resistance. Similar findings have been described on the neurones of leech (Zhuravlev, Safonova, 1975) and Planorbis corneus (Magura et al., 1972).

The differences in the values of τ obviously cannot completely account for the time-dependent characteristics of the excitation process, nevertheless the greater time-constant may contribute for instance to the prolongation of the after-hyperpolarization (if the recovery of the membrane potential to the resting level after excitation is simplified as being a passive process). It may partly explain, why the LAHP can only be observed in case of the "slow" neurones. Similar relationship was observed on the neurones of Hirudo sanguisuga (Zhuravlev, Safonova, 1975).

The relationship between the duration of the action potential and the time course of the change in the membrane potential during the ionic current flowing through the membrane is much more complicated. Presumably, the differences in the time course of depolarization – repolarization of the membrane can hardly be connected with the capacitive parameters. Rather the membrane currents measured directly have an importance, as far as they play a decisive role in determination of the somatic input resistance. Consequently making a comparison between the present results and the data obtained earlier by voltage clamp method (Kiss, Vadász, 1977) may have some interest: the inactivation kinetics of the delayed outward current also showed significant differences when "fast" and "slow" cells were compared.

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Absorption and Fluorescence of Fluram-labelled Lysozyme and Peroxidase Solutions

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The absorption spectra of fluram, lysozyme, horse-radish peroxidase, and mixtures of lysozyme + fluram and peroxidase + fluram and the fluorescence and fluorescence excitation spectra of the mixtures in 0.05 M phosphate buffer with 1 per cent dioxane are determined. Due to formation of a protein-fluram compound, the absorption spectra of the mixtures are not algebraic sums of the components. From the fluorescence intensities the number of bonding sites is found 6 in both cases.

The fluorescence spectrum of the peroxidase-fluram compound has maxima at 305, 350, 400, 450 nm due to peroxidase and at 480 nm originating from fluram.

In mixtures of 10^{-5} M lysozyme + 10^{-4} M fluram, 3/4 of the excitation energy is transferred from lysozyme to fluram within the compound under 280 nm excitation. Under similar conditions 4/5 of the excitation energy is transferred from peroxidase to fluram.

Introduction

Weigele et al. (1972) were the first to synthesize fluram (fluorescamine)*; this reacts with primary amines to give substances fluorescing intensely, with a maximum at 475 nm. Fluram and its hydrolysis products do not fluoresce (Udenfriend et al., 1972). This reaction provides a rapid and sensitive assay for primary amines. The reaction proceeds in some seconds in aqueous medium (optimally at pH = 8), and the products obtained are stable for 6–8 hours. The intensity of fluorescence is proportional to the concentration of the amino acid and permits the detection of picomole quantities of primary amines. Böhlen et al. (1973) demonstrated that fluram is bound only to the ε-amino group of lysine and to the N-terminal atom groups of proteins. Weigele et al. (1973) showed that secondary amines can be transformed into primary amines, which can then also be detected with fluram. Stein et al. (1973) used fluram in automatic amino acid analysers. On the basis of the correlation between electrophoretic mobility and molecular weight, proteins labelled with fluram may be used for determining the molecular weights of the proteins (Pace et al., 1974). With respect to various important applications based upon fluorescence analysis, it seemed justified to study the light absorption and fluorescence characteristics of the new reagent with special emphasis on the changes caused by its incorporation into the protein.

* 4-phenilspiro [furan-2(3H), 1'phtalan]-3,3'-dione

Materials and methods

Fluram (a product of Roche, Schweizerhalle) was purified to constancy of the absorption spectrum by repeated recrystallization. Lyophilized, acid-free, powdered horseradish peroxidase (HRP) and ovalbumin-lysozyme recrystallized three times, both prepared by the Nutritional Biochemical Corporation (Cleveland) were used. The fluram-protein solution was prepared according to Böhlen et al. (1973) in 0.05 M phosphate buffer (pH = 8), with the modification that fluram dissolved in dioxane was added to the aqueous medium to give a final dioxane was added to the aqueous medium to give a final dioxane content of 1 per cent. The solutions were prepared by dilution of a 10^{-4} M stock solution.

The absorption spectrum $k(\lambda)$ of the solutions ($k(\lambda) = 2.3\, E/l$, where $E = \log I_0/I$ is the extinction of the solution and l the layer thickness) was measured with a spectrophotometer Unicam SP 1800; by using different layer thicknesses. $E(\lambda)$ was adjusted to fall into the region 0.2–0.8. The fluorescence spectra $f_q(\lambda)$ and the excitation spectra $f_{exc}(\lambda)$ were determined with a Perkin–Elmer–Hitachi spectrofluorimeter Type MPF-3 at room temperature. Fluorescence was observed right-angle or front-surface observation, depending on the absorption of the solution. True spectra were obtained from the measured ones by applying corrections with respect to the spectral sensitivity of the apparatus, to spectral distribution of exciting light and by correcting for the reabsorption of fluorescence and for secondary fluorescence. (Vigny and Duquesne, 1974; Budó and Ketskeméty, 1956; Várkonyi, Szalay, 1974a). For the comparison of fluorescence spectra measured at different times, with different concentrations and exciting wavelengths, reference standards were used.

Results and discussion

1. The absorption spectra of lysozyme, fluram and lysozyme + fluram solutions are shown in Fig. 1, curve *a*, *b* and *d*, respectively. The spectrum of the lysozyme + fluram solution (curve *d*) lies above the sum of the component spectra (curve *c*), pointing to the formation of a protein + dyestuff compound.

A similar difference is also observed in the absorption spectrum of the HRP + fluram compound (Fig. 2, curve d) compared to the sum (curve c) of the component spectra (curve a and b, respectively). The absorption spectrum of the compound shows at about 400 nm the band characteristic of the absorption of the haem group of HRP and at 390 nm a shoulder originating from the long wave absorption band of fluram.

The fluorescence spectra of both mixed solutions were measured with excitation at 275–280 nm (where the absorption of both components leads to fluorescence and at 390 nm (where only the absorption of fluram will result in fluorescence).

In the case of $1 \cdot 10^{-5}$ M lysozyme + $1 \cdot 10^{-4}$ M fluram solution (Fig. 3) with excitation at 280 nm, fluorescence bands appear at 345 and 485 nm (curve *b*),

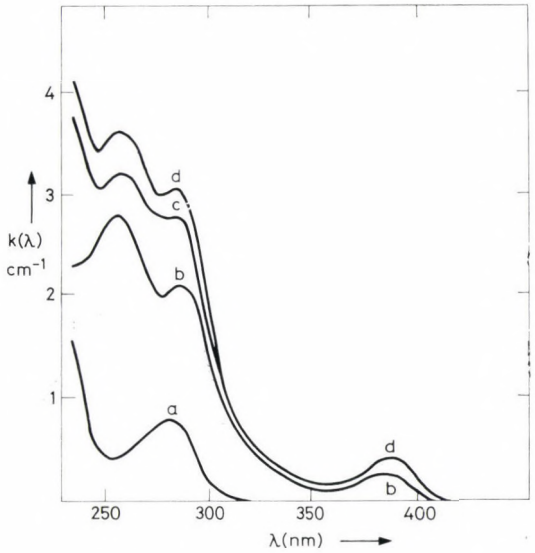

Fig. 1. Absorption spectra of 1.10^{-5} lysozyme (a), 1.10^{-4} M fluram (b); sum of spectra (a) and (b), (c); absorption spectrum of the mixture (d), measured in phosphate buffer (pH = 8.0) with 1 per cent dioxane

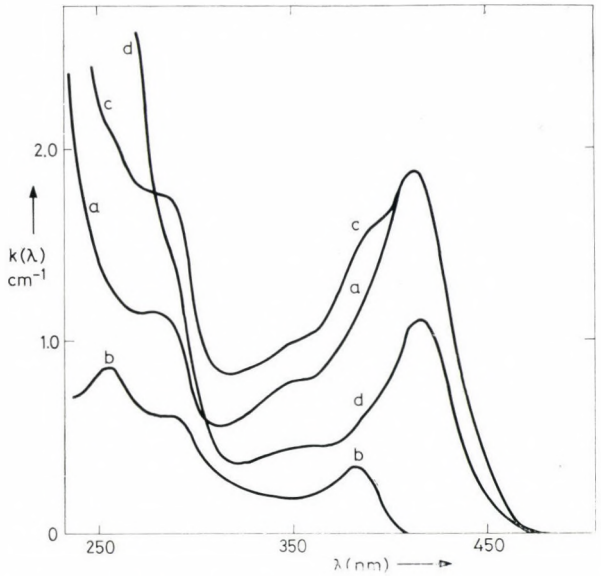

Fig. 2. Absorption spectra of 1.10^{-5} M peroxidase (a); 1.10^{-4} M fluram (b); sum of spectra (a) and (b), (c); absorption spectrum of the mixture (d), measured in phosphate buffer (pH = 8.0) with 1 per cent dioxane

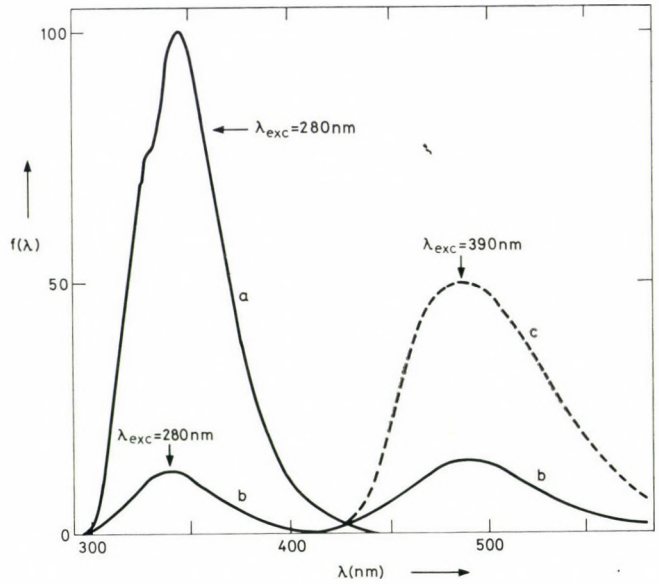

Fig. 3. Fluorescence spectra of 1.10^{-5} M lysozyme (a), mixture of 1.10^{-5} M lysozyme and 1.10^{-4} M fluram in phosphate buffer (pH = 8.0) with 1 per cent dioxane, excited at 280 (b) and 390 nm (c)

characteristic of lysozyme and fluram fluorescence, respectively. The fluorescence intensity of the compound is 12 if that of lysozyme without fluram (curve *a*) is taken arbitrarily 100. This decrease can be attributed partly to the transfer of excitation energy from lysozyme to fluram and partly to the trivial "inner-filter" effect. The inner-filter effect was taken into account by calculating the fluorescence intensity at the maximum (340 nm) as follows:
$F(340) = \text{const}\ [1-\exp\ k(280)_{LYS}.\ l]/[1-\exp\ k(280)_{mix}.l] = \text{const}.\ 0.46$. Here $k(280) = 0.56$ is the absorption coefficient of lysozyme and $k_{mix}(280) = 2.8$ is that of the mixed solution. (In absence of this effect, when $k_{mix}(\lambda) = k(\lambda)$, $F = = \text{const}\ 1.00$). As the measured fluorescence intensity is lower by a factor of $46/12 = = 3.83$ than the fluorescence of lysozyme without fluram, about 3/4 of the excitation energy is transferred to the fluram within the compound. Exciting the system at 390 nm (Fig. 3 curve c) a maximum around 480 nm – characteristic of fluram – is obtained.

Exciting a $1 \cdot 10^{-5}$ M HRP + 1.10^{-4} M fluram solution at 276 nm (in the absorption range of tyrosine in HRP) (Fig. 4), the 305 nm fluorescence band of HRP appears (Várkonyi, Szalay, 1974a), while the band of fluram fluorescence can be seen at 485 nm. The fluorescence intensity of the 305 nm HRP band of the mixed solution decreases to 15 compared to that of the HRP solution without dye, arbitrarily 100. Calculating the fluorescence intensity F(305) and taking into account the inner-filter effect with $k_{HRP}(276) = 1.02$ and $k_{mix}(273) = 1.64$, the value $F(305) =$

= const. 93 is obtained. Thus the measured fluorescence intensity of the compound is lower by a factor of 93/15 = 6.2 than the calculated value. Accordingly, about 4/5 of the exciting energy is transferred to fluram. In addition to the bands at 305 and 485 nm a further band at 400 nm appears (Fig. 4).

2. According to Böhlen et al. (1973) the F fluorescence intensity of the mixed protein + dye solution, measured in the region of fluorescence of fluram (for practical reasons at the maximum of the spectrum) is proportional to the number (concentration) of the bound fluram molecules ($[D]_{bound}$):

$$F = \text{const. } [D]_{bound}.F',$$

where F' means the fluorescence intensity in case of $[D]_{bound} = 1$. As according to Weigele et al. (1972) fluram excited in the 390 nm long wave absorption band fluoresces only bound to protein, its intensity of fluorescence increases up to a value F_0 where all bonding sites of the protein are occupied by fluram. Denoting the number of bonding sites and the initial protein concentration by N and $[P]$, respectively,

$$F_0 = \text{const } N.\ [P].\ F'.$$

The ratio of partically and totally occupied bonding sites is:

$$x = \frac{F}{F_0} = \frac{[D]_{bound}}{N[P]}. \qquad (1)$$

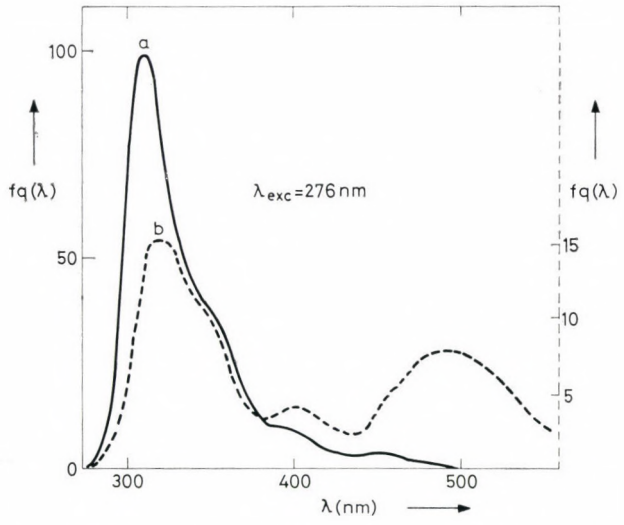

Fig. 4. Fluorescence spectra of 1.10^{-5} M peroxidase (a), mixture of 1.10^{-5} M peroxidase and 1.10^{-4} M fluram (b) in phosphate buffer (pH = 8.0) with 1 per cent dioxane, excited at 276 nm

Fig. 5 shows F as a function of the initial fluram concentration $[D]$ at $1 \cdot 10^{-6}$ M lysozyme concentration. When $F = F_0$, $[D]_{bound} = 6. 10^{-6}$ M and from Eq. (1) $N = 6$. Similar measurements showed that in the case of HRP + fluram systems

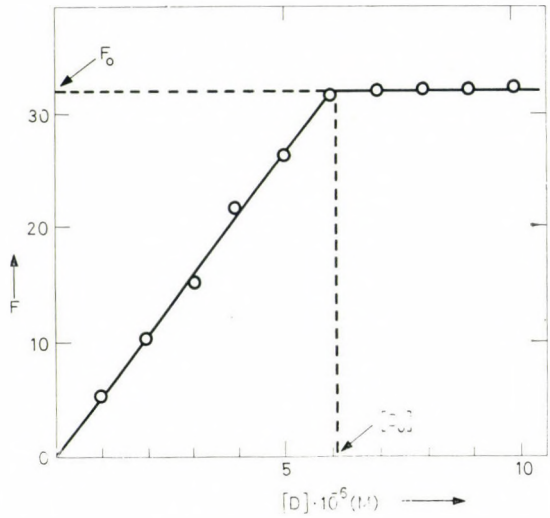

Fig. 5. Relative intensity of fluorescence of fluram in 1.10^{-6} M lysozyme solution in phosphate buffer (pH = 8.0) with 1 per cent dioxane, as a function of the fluram concentration (excitation 390 nm, observation 480 nm)

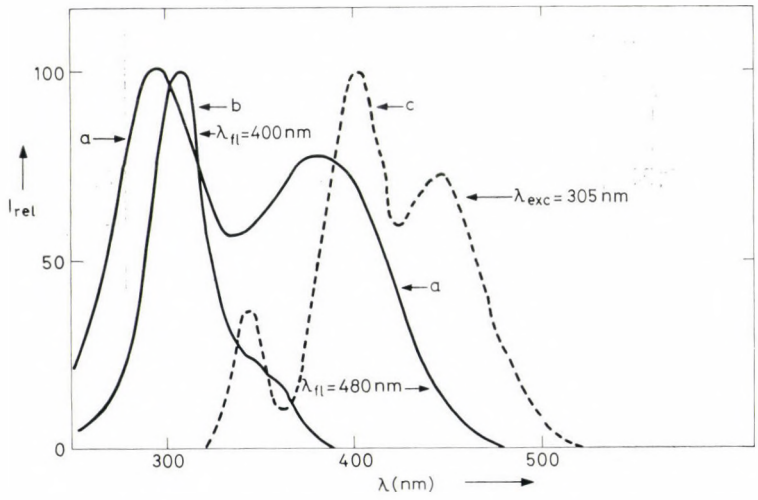

Fig. 6. Relative fluorescence excitation spectra of mixture of 1.10^{-4} M fluram and 1.10^{-5} M peroxidase in phosphate buffer (pH = 8.0) with 1 per cent dioxane observed at 480 nm (a) at 400 nm (b). Fluorescence spectrum of the mixture excited at 305 nm (c)

$N = 6$, too. As the number of bonding sites ought to be 7 for both lysozyme and HRP, one of the 7 bonding sites is unaccessable for fluram.

3. We tried to clear up the factors producing the fluorescence bands by studying the excitation spectra of $1 \cdot 10^{-5}$ M HRP + $1 \cdot 10^{-4}$ M fluram mixture (Fig. 6). The excitation spectrum of the 480 nm fluorescence (Fig. 6 curve a) has maxima at 290 nm and 380 nm, which points to fluram as causing the fluorescence. The broadened 290 nm band corresponds to the joint 265 and 285 nm bands of fluram and the 276 nm band of HRP. According to the excitation spectrum observed for 400 nm fluorescence (Fig. 6, curve b), major part of the fluorescence is due to absorption at 305 nm with a minor contribution of the absorption at about 350 nm. To obtain further information concerning the 305 nm absorption band, the fluorescence was excited also with 305 nm. The fluorescence spectrum (Fig. 6, curve c) shows bands with a maximum at 340 nm (tryptophan fluorescence), and with maxima at 400 nm and 450 nm. The 450 nm fluorescence band appears in form of a weak shoulder also in the case of 276 nm excitation (see Fig. 4). This band is characteristic of the fluorescence of HRP and is probably an excimer fluorescence band of tyrosine (Várkonyi, Szalay, 1974). The fluorescence band at 400 nm is characteristic of HRP (Fig. 4, curve *a*), enhanced by energy transfer from tyrosine (curve *b*).

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Changes in the Structural Ordering of F-Actin Filaments Induced by Interaction with Myosin Fragments

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In the recent theories of muscle contraction it is believed that in the process of force generation the actin filaments play an important role concerning the attachment of cross-bridges to actin (Huxley, 1969). Regulation of the contraction in striated muscle is suggested to be coupled to the thin filaments and it is controlled by the level of Ca^{2+} (Ebashi, Endo, 1968). When the muscle is activated, conformational changes are expected to occur in actin and myosin filaments. It was recognized that conformational changes were induced in the contractile protein system, when tension was generated (Ernst, 1963). Fluorescence and spin label methods have also shown that the myosin heads undergo conformational changes on interaction with substrates and actin and the mobility of the attached labels reflects the dynamics of the protein motion (Mendelson et al., 1973; Stone, 1973; Seidel, Gergely, 1973; Thomas et al., 1975 a, 1975 b; Thomas, 1978; Harvey et al., 1977).

Muscle is highly organized structurally, therefore, concerning the contraction mechanism it is more relevant to study the molecular motion in oriented supramolecular complexes. In the present paper we report on EPR experiments on myosin-actin interaction in oriented F-actin filament system. It is an important question whether or not the F-actin filament itself has the ability for undergoing conformational changes on interactin with myosin heads (HMM) or subfragment-1 (S–1).

Heavy meromyosin, subfragment-1 and actin were prepared by standard methods. Actin was spin-labelled in F-form with the maleimide spin label. One mole of label was reacted with one mole of actin at 0 °C for 30 min. The labelled group is suggested to be at the cysteine-373 residue in actin (Lin, 1978). Actin was decorated in F-form with different amount of HMM or S–1. The EPR measurements were performed on aligned F-actin pellett on the surface of a flat cell using an ER 9 spectrometer (Zeiss, GDR). The samples were controlled by scanning electron microscopy (JEOL Temscan 100 C, Japan).

Spin labelling of actin results in an EPR spectrum which is the superposition of EPR spectra from strongly immobilized labels and from a small amount of weakly immobilized labels. The rotational correlation time of actin bound spin label using the method of McCalley et al. (1972) was calculated to be 13 ± 2 ns for the five-membered maleimide spin label. From the plot of τ_2^{-1} against T/η it can be derived that the label is rigidly bound to the G-actin molecule (Belágyi et al.

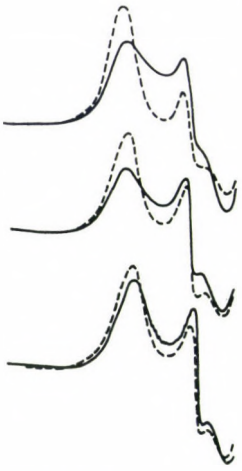

Fig. 1. Low-field region of the EPR spectra of oriented F-actin filaments at different molar ratio of actin to HMM. a) 10 : 1; b) 7 : 1; c) 2 : 1 mol actin to HMM. Solid line shows the spectra where the filament axis was perpendicular to the magnetic field, whereas dotted line shows the spectrum of the same sample where the filament axis was parallel to the magnetic field. Actin is free of regulatory proteins. The label used in the experiments was the six-membered maleimide spin label

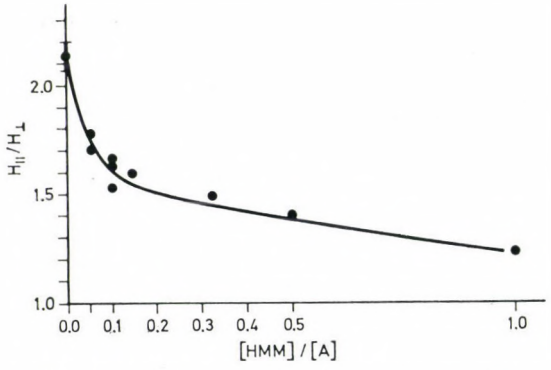

Fig. 2. The dependence of $\alpha = H_{||}/H_{\perp}$, a parameter from spectra as the molar ratio of HMM to actin. $H_{||}$ is defined as the ratio of the heights of the low-field peaks for strongly and weakly immobilized labels in parallel orientation of filament axis to magnetic field whereas $H_{\perp}$ is the same ratio in perpendicular orientation of the filament axis to the magnetic field

Fig. 3. EPR spectra of iodoacetamide spin labelled HMM attached to F-actin in oriented filament system. The molar ratio of actin to HMM was 3 : 1 (- - - - $H_{\perp}k$, ——— $H_{||}k$)

1978). However, estimating the rotational correlation time by the method of Goldman et al. (1972), $\tau_2 = 17{,}4$ ns was obtained assuming that the hyperfine splitting constant in the EPR spectra for F-actin pellet represents the rigid limit for the labels on the time scale of the conventional EPR spectroscopy. Both values are reasonable for a protein, like G-actin, having a molecular weight of 42.000 D, low asymmetry and a moderate degree of protein hydratation. The polymerization of actin reduces the mobility of spin label by a factor of 10^3 measured by saturation transfer EPR technique (Thomas et al., 1975 a).

Moreover, the EPR spectrum of actin bound maleimide spin label exhibits a strong dependence on orientation measured on aligned samples of F-actin filaments (Burley et al., 1972). From the orientation dependence of EPR spectra it can be derived that the Z principal axis of the molecular reference system for nitroxide labels is oriented more nearly parallel than perpendicular to the long axis of the F-actin filaments. It follows that the EPR spectra of spin labels are sensitive to the intrinsic order in the filaments. The intrinsic order is determined by the conformational state of the entire F-actin filament. The orientation dependence of EPR spectra is strongly influenced by the effect of binding of myosin heads to actin (Fig. 1). The change in the intrinsic order of F-actin filaments depends on the molar ratio of HMM to actin (Fig. 2). Similar results were obtained on actin decorated with S−1.

It can be suggested that heavy meromyosin molecules attached to the F-actin cause a change in the local conformation of actin monomers. The effect induced by the binding of myosin fragments propagates by longrange forces to other monomers producing changes in the intrinsic order of the F-actin filament, which reflect as a decrease in the degree of order of spin labels. The effect seems to be cooperative because the relation between the spectral parameter α (see legend to Fig. 2) and the molar ratio of myosin fragments to actin shows a non-linear character. The maximum spectral change was observed at a molar ratio of about 0.1 moles of HMM to actin.

It should be noted that the orientation dependence of the EPF spectra with moderate degree of order can be observed on aligned F-actin-myosin complexes containing iodoacetamide spin labelled HMM (Fig. 3). Similar orientation dependence of EPR spectra were obtained on glycerol-treated muscle fibres labelled with maleimide spin label (Belágyi, 1975).

To sum up the experimental data provide evidence that the conformational changes in the actin monomers induced by the interaction with HMM are not restricted to one actin monomer, but the effect propagates to other actin monomer as well producing changes in the intrinsic order of the entire filament. The effect may be important in the tension generation process of muscle contraction.

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Book Reviews

Endorphins' 78 Proceedings of the International Workshop Conference held in Budapest, 2—6 October 1978. by L. Gráf, M. Palkovits, A. Z. Rónai (eds.). Publishing House of the Hungarian Academy of Sciences, Budapest and Excerpta Medica, Amsterdam, 1978. 336 pages.

As a recent branch of neurotransmitter research, investigation of endorphins and enkephalins started in the fall of 1975 when from pig brain *Huges* and *Kosterlitz* isolated methionine enkephalin and leucine enkaphalin, the two pentapeptides considered as endogenous ligands for opiate receptors, the mediators of analgesia. On the basis of these findings other isolated endorphins, substances of even greater opiate activity, from the degradation products of the pituitary hormone β--lipotropin. This hormone contains enkephalin sequence within its structure. Later the occurrence of endorphins was demonstrated also in living organisms. In the hope of exploring the mechanism of physiological relief of pain, research of enkephalins and endorphins has been greatly stimulated. The results of these investigations, divided into five subject areas, were summarized by internationally known experts of the field, in Budapest.

Under the heading "*Structure and Action*" *C. H. Li* deals with the relationship of chemical structure and biological function, whereas S. Bajusz discusses problems of structure and biodegradation.

A. A. Goloyan reviews his results on the issue proteinase enzymes obtained from different brain regions.

R. E. Mains provides novel data concerning the biosynthesis of endorphin produced in the pituitary. He concentrates on the structure and degradation of the macromolecular precursor that contains ACTH sequence within its molecule. In two subsequent lectures *L. Gráf and his coworkers* outline the mechanism of β-lipotropin degradation, the last step in the biosynthesis of endorphin.

The chapter "*Distribution and Release*" is introduced by *J. Hughes* discussing the neurotransmitter role of enkephalins. *J. Borvendég* and *M. Palkovits* report on the distribution of enkephalins, endorphins and ACTH in different areas of the central nervous system (CNS).

A comparison of opioid receptors assayed in different pharmacological test systems is given in the three lectures of *H. W. Kosterlitz*, *R. Simantov*, and *A. Z. Rónai*, respectively, in the chapter "*Receptor and Function*".

In the last chapter ("*Pharmacology and Clinical Aspects*") *D. T. Krieger* gives an account of his measurements of the β-lipotropin and endorphin levels in human plasma while data on the endorphin levels in the human brain and in the pituitary under normal and pathological conditions are reported by *A. Herz* and associates. At least, *J. L. Székely and his coworkers* describe detailed pharmacological assays of D-Met^2, Pro^5 enkephalin amide.

In addition to the text of lectures and discussions the book contains the printed material of the round table conference entitled "*Perspectives of Endorphin Research*".

It was generally agreed that although endorphin and the different enkephalins cannot

solve the problem of pain relief devoid of morphine-like side effects, their investigation gave rise to a considerable progress in understanding the physiological porcesses of the CNS. It seems within reach today that these substances may acquire a direct application in the therapy of certain neuronal diseases. Another conference on these topics is going to be organized in two or three years.

M. Lőw

Iron and Copper Proteins by Kerry T. Yasunobu, Howard F. Mower and Osamu Hayaishi (eds.) Plenum Press, New York and London, 1976.

The book contains collected papers from the participants of the "Fe- and Cu-protein Symposium", held in December 1975 at Hawaii.

The book is divided into four parts: I. Iron-sulfur proteins (non-mitochondrial electron transport proteins), II. Iron-sulfur proteins and heme proteins of the mitochondrial electron transport system, III. Other heme and non-heme Fe-proteins, IV. Copper proteins.

The Fe- and Cu-proteins play a fundamental role in various electron transport phenomena. These, in general, involve more complex molecular systems than other biochemical reactions catalyzed by single enzymes in cytoplasmic metabolism. The investigation of the structure-function relationship, one of the fundamental questions of biochemistry in the last thirty years, gave many interesting results concerning the mechanism of action of simple enzyme molecules. At the present time, this type of investigation is extended to more complicated biological processes, such as electron transport in complex biochemical systems. The fine molecular architecture of the electron transferring macromolecules, their clusters and the environment around the clusters, the molecular mechanisms of the electron transfer are under very intensive investigation today. The book gives an excellent cross-section of this research.

Since the authors of the 48 papers of the book are specialists working in different fields, the book not only shows the development in the research of iron- and copperproteins but also reflects the problems from different points of view.

Therefore the book is interesting and useful not only for scientists who are studying one of the electron transport processes but also for many other specialists who are working in the field of organic, inorganic or physical biochemistry. Since the microsomal and mitochondrial dytochrome P-450 oxidizing system plays an important role in the biosynthesis of steroids, in the metabolism of various drugs, poisons and carcinogens, and the papers in Part III. deal with the properties and reactions of these iron-proteins, the book is of particular importance from the point of view of modern health sciences.

L. Boross

Animal, Plant and Microbial Toxins. Volume 1. Biochemistry. by Akira Oshaka, Kyozo Hayashi and Yoshio Sawai (eds) Plenum Press, 1976, New York and London

This first volume of a two-volume set, which contains original reports and review articles presented at the Forth International Symposium on Animal, Plant and Microbial Toxins (Tokyo, 1974), concentrates on the biochemistry of toxins.

The book consists of three parts. The first deals with the biochemistry of animal toxins; this part contains 29 papers on different biochemical characteristics of various animal toxins, such as citocidal activity, hemagglutinating activity, enzymic properties, enzyme inhibition etc. The second part deals with the biochemistry of plant toxins; this part consists of four papers on the mechanism of the anticancer activities of abrin and ricin, on the isolation and characterization of the hemolysins in the green alga Ulva pertusa, on a dermatitis-producing blue-green alga M. lyngbyaceus, and on the mechanism of the lytic action of prymnesin. The third part, biochemistry of microbial toxins, contains 16 papers. They deal with various molecular and biochemical properties of toxins of Clostridium species, Staphylococcus aureus, Flavobacterium aquatile, Vibrio parahemolyticus, Pseudomonas aeruginosa, and present experimental data on some effects of endotoxins.

Our knowledge of toxins, chemical tools in the war for life, has been developed practically during the last two decades, when bio-

chemistry has advanced enough to understand such complex phenomena as the action of toxins at a molecular level. The book, including a quite broad field of toxin research, presents many interesting experimental results. Not only these individual data are useful for a biochemist working in toxin research, but also the description of the various methods used, and the literature references increase the usefulness of the articles. Therefore this book is of valuable help to young research students who want to investigate the structure and/or function of biologically active compounds (not only toxins): they can get an overall survey of the laboratory methods in this kind of research, from isolation procedure through the various ways of the biochemical characterization of compounds to kinetic studies of binding to receptor proteins. From this point of view, the "Index" of the book is relatively poor. For instance, it does not refer to methods used in the chemical modification or labelling of single amino acid residues in polipeptides, or in the measurement of receptor-binding, although these are described in some individual articles of the book.

L. Boross

Pathobiochemie, Ein Lehrbuch für Studierende und Ärzte by Buddecke, E., Walter de Gruyter, Berlin – New York, 1978. 445 pages, 188 Figures, 91 Tables and formules, DM 39.–

About 25 years ago biochemistry entered clinical laboratories and has become eversince a more and more indispensable tool in diagnostics, especially in differential diagnostics. This means not only the clinical use of biochemical methodology but also the prevalence of a molecular-biological view and an increasingly effective utilization of theoretical results for practical, diagnostical and therapeutic purposes.

Instead of biochemical journals, biochemical results utilized in medical practice are most often published in various medical papers. To review these results is therefore laborious and time-consuming for the reader. The review-like summaries published usually cover only one or another group of problems. For this reason E. Buddecke's new work is of great help for those who are intrigued by the connection *in general* between the anomalous, pathological phenomena of the human organism and biochemistry, and by the possibility to interpret the pathomechanism of certain diseases in exact, molecular terms and to cure them on this basis.

The summarization of the research carried out more and more intensively all over the world is in itself a respectable effort. The other work of the author, Grundiss der Biochemie is a well-known and reputed textbook in the German linguistic area; its 5th edition was published in the last year which is also a proof for Buddecke's successful activity as a specialist.

According to our present-day knowledge, the diseases characterizable from a biochemical aspect are related to some anomaly of the biosynthesis of the informational macromolecules. One group of these diseases is characterized by a defect of the synthesis of a certain enzyme. Therefore – owing to the failure of a given step of the *metabolism* – most of them result in the pathological accumulation of a metabolite or an intermediate, or in the absence of a necessary metabolite. Depending on the importance of the metabolic step affected, the defectiveness may have mild, grave or often lethal consequences. In the other group of diseases the anomaly primarily manifests itself in processes not classified as metabolic (e.g. haemoglobins – oxygen transport, anomalous fibrinogen or other blood clotting factors – blood clotting, myeloma multiplex – immunoglobulin synthesis, etc.). Buddecke discusses mainly the anomalies of the first group, i.e. the congenital or acquired malfunctions of the metabolism and only touches upon the second group.

The merit of Pathobiochemie is therefore the summarization from a biochemical point of view of those phenomena which are commonly referred to as pathophysiology. The volume is characterized by stress being laid on giving practicable information, and for this reason the first part makes the reader acquainted with data that are necessary for the quantitative characterization of biochemical processes i.e. units of measurement, SI-units, enzyme nomenclature.

The next chapter on metabolism describes enzymes, their normal and anomalous features

according to the classification of standard biochemistry textbooks. Important problems from the diagnostic point of view, such as the characteristic isoenzyme pattern of the different organs, the origin and types of enzymopathies, the enzymes occurring in the plasma and their immunological use are discussed in detail. The part dealing with nuclei acids is especially interesting. The various disturbances or nucleic acid metabolism and their consequences are easier to approach. However, the informational functioning of nucleic acids and the evolvement of the various anomalies in the information transfer can be discussed at present only at a theoretical level. This question is doubtlessly one of the most intriguing ones to be solved in the future.

The disturbances of amino acid metabolism have been known for the longest time and studied in greatest detail. According to our present-day knowledge, the most often occurring metabolic defect of genetic origin is the disturbance of phenylalanine metabolism, phenylketonuria (besides lactose intolerance, the consequences of which are unpleasant but usually not grave). Lately more and more attention has been paid to the results of the malfunctioning of carbohydrate metabolism — although several of these disturbances have been recognized for as long as a quarter of a century —, especially to the disturbances of glycoprotein synthesis which take the majority of their victims at newborn and infant age. The last two parts of the chapter deal with the metabolism of lipids, salt and water.

The second part discusses the regulation of metabolism, the hormones and the vitamin-coenzyme relationship. The thyroid hormone, the catecholamines, the central nervous system, the adrenal gland and the gonads, the so-called tissue hormones and the biogenic amines are discussed in great detail. In not a quite unambiguous way, prostaglandins are also included in the latter group, together with the kinins, serotonin and histamine. In accordance with the modern biochemical way of discussion, water-soluble vitamins are now considered as coenzyme precursors, although the connection between avitaminosis and coenzyme function can be satisfactorily explained only in a few cases.

The third part covers the pathobiochemistry of cells, tissues and organs. The biochemical background so far established for the pathological processes of red blood cells, leukocytes, the liver, the stomach and the intestines, the kidney, the skeleton, cardiac and skeletal muscle, the connective tissue and finally tumorous processes, is analyzed in altogether nine chapters.

The last part bears the title "Dynamic systems". Here the author discusses haemostasis problems of inflammation and wound healing, furthermore problems of immunochemistry. It is evident from the list of the titles that the volume endeavours to cover a very wide range of problems. For the inquiter an introduction is given to each of the above mentioned topics and the bibliography at the end of the volume enables the reader interested in one or another special field to fruther extend his knowledge on the given subject.

P. ELŐDI

Mechanismen der Zellevolution (Grundriss einer moderner Zelltheorie) by Werner Schwemmler/Walter de Gruyter, Berlin, New York, 1979. p. 275

The rapid accumulation of new scientific data in biology requires new synthesis both in biology in particural and natural sciences in general. Several attempt have been made to achieve such a synthesis (systemstheory, synergetics) but none of them seem to be as promising as those which use the natural organizational hierarchy of matter as a basis of approach. The book of Werner Schwemmler follows the historical route of the evolution of matter, from the evolution of micromolecules to that of human culture. The task is not easy at all and naturally it is impossible to expect full solution to all problems. This book, however, gives very interesting solutions to some details.

The first two chapters give an essentially popular scientific outline of the orders of magnitude of the Universe, followed by a short chapter on chemical evolution. The fourth chapter is entitled "Devition des Lebens". Unfortunately, it does not discuss at all the problem of what a living system is, and gives only a short popular outline of some

biological and molecular biological properties of living systems.

These chapters are the least valuable part of the book.

From the fifth chapter on biological evolution is discussed in detail. For this purpose the cells are classified into four groups. These are: Präzyten (Protocells, early cells), Prozyten (Prokaryotes), Euzyten (Eukaryotes) and Myzetozyten (special Eukaryotes with Prokaryote endosymbionts). The evolution of the Präzyten is discussed mostly on the basis of works by H. Kuhn. An attempt is made to outline the general sequence of events from chemical evolution to the appearance of the prokaryotes. The origin of the genetic code is discussed in detail, but almost nothing is ritten about the origin of metabolism, although the author's compatriot, Peter Decker has some really interesting results in this field. The evolution of the prokaryotes is quite a good chapter, only the evolution tree of prokaryotes (originally published by L. Margulis, 1970) needs some corrections. The evolution of the eukaryotes is discussed on the basis of the well-known endosymbiosis theory.

At the end of the book an overview about "Allgemeine Evolutionsmechanismen" is presented, unfortunately only at a very popular level. The "Baukastenprinzip" is accepted as being valid for the living world, too. This part contains a really remarkable attempt: the author designs a periodic system of cells, arranging them into seven horizontal periods and eight vertical groups. This system seems to be quite plausible. It shows the natural relations between the functional and constructional properties of cells.

The book is a good summation of our present knowledge of the evolution of cells with remarkable new concepts, but a weak summation of the mechanism of evolution in general.

T. GÁNTI

Transport by Proteins. Proceedings of the Symposium held at the University of Konstanz, German Federal Republic, July 9 – 15, 1978, FEBS Symposium No. 58. by Gideon Blauer and Horst Sund (editors). Walter de Guyter, Berlin, New York, 1978. 420 pages.

The book provides a brief survery about transport phenomena displayed by proteins. Papers presented cover a wide range of different transport systems: some of them are working in aqueous media others carrying out, transport through membraneous structures.

In the first section, out (of five), the reader gets the necessary thermodynamic basis. This includes relevant topics of both reversible and irreversible thermodynamics. After this introductory "brushing up", the transport of oxygen, heme, metal ions, hormones, metabolites, charges, and drugs are considered. Transporters are dealt with such as hemoglobin, serum albumin, ligandin, protein-A, mitochondrial transport systems, Na - K channels in excitable membranes, bacteriorhodopsin, ferritin, transferrin, hemoplexin, and ceruloplasmin. Transport within a multienzyme sstem is shown on the fatty acid synthetase complex. The last part of the book is devoted to the physiological and pharmacological significance of transport by proteins. This part also gives hints to clinical and industrial applications.

Some relevant subjects could inevitalby not be dealt with within the framework of the congress. The aim of the book is, however, well achieved: we get a nice guide line in this rapidly growing field. The records of discussions annexed to each invited lecture are to the reader's help and pleasure in the orientation.

J. HAJDU

Molecular Biology, Biochemistry and Biophysics. Vol. 27. *Effects of Ionizing Radiation on DNA* (Physical, Chemical and Biological Aspects). by A. J. Bertichamps (Coordinating Editor) Springer Verlag, 1978, XXII + 383 pages.

This book presents the culmination of collaborative efforts among specialists of several disciplines concerned with the effects of ionizing radiation on nucleic acids.

The first section devoted to physical aspects consists of seven chapters comprising the following topics:

1. Structure and electronic properties of DNA Questions of nomenclature, primary structure of DNA, electronic properties and

interactions (in-plane and vertical interactions) are covered.

2. "Interactions of ionizing radiations with matter". Besides the effects of ionizing radiations, those of activation spectra, superexcited states, inner-shell ionizations, direct and indirect effects and energy transfer are dealt with.

3. "Structure of radicals from nucleic acid constituents". Molecular orbital calculations and radical formation mechanisms are discussed.

4. Structure of radicals from nucleic acids in frozen solution and dry state.

5. Radical yields with special focus on the effect of moisture content and the molecular weight.

6. Radiomimetic radical production and mechanism of radical formation.

7. Transfer phenomena.

The second section deals with chemical aspects, as primary events of the radiolysis of aqueous solutions of nucleic acids and radiation induced degradation of the bases and the sugar component in DNA as well as related substances. A brief survey is given on the changes in the secondary and tertiary structure of DNA after irradiation.

In the third section biological aspects are summarized: methods of testing the biological function of DNA before and after irradiation and methods of modification of the radiation damage are described. Repair processes for radiation induced DNA-damage and molecular aspects of mutagenesis due to ionizing radiation get a special emphasis.

The inclusion of the up-to-date definitions of radiation dose-units in the Appendix seems to be a very useful idea. The detailed listing of references further increases the usefulness of this work.

L. D. SZABÓ

Principles of Protein Structure by G. E. Schulz and R. H. Schirmer. Springer-Verlag; New York, Heilderberg, Berlin, 1979

The last few years have witnessed an explosion of information about protein structure. Today, the structure of more than 70 proteins is known. Principles of Protein Structure, the first of the Springer Advanced Texts in Chemistry (Ed. Ch. R. Cantor), provides a comprehensive overview of this massive set of information.

The book consists of eleven chapters and an appendix about "Statistical Mechanics of the Helix-Coil Transition". All chapters are self-contained and can be read in any order.

In the first chapter the authors introduce the 20 standard amino acids, the building blocks of proteins. They show the colinear relationship between nucleic acids and polypeptides and the empirical similarities among amino acids.

Chapter 2 gives a very good survey of peptide bonds including dimensions, configuration and conformational energy.

The next two chapters review the noncovalent forces that determine protein structure and the covalent structure of proteins.

The following three chapters make us acquainted with the different patterns of folding of polypeptide chains, the prediction of secondary structure from the amino acid sequence and the documentation and display of protein structure.

Chapter 8 discusses the way the three-dimensional structure of a native globular protein is formed. The authors consider both thermodynamic and kinetic aspects of folding.

The next chapter describes protein evolution showing the specialization and differentation of proteins, as well as their convergent evolution.

Chapter 10 discusses protein-ligand interactions and interactions of proteins with other macromolecules.

The last chapter shows the structural basis of protein function.

In general, protein structures provide biochemists with models for the design and interpretation of experiments. The aim of this book is to consider principles that have emerged from comparisons of known structures. The authors use the known structures to illustrate the forces which determine protein structure in general and the mechanisms by which proteins perform their biological functions.

This book surveys not only what is known, but also how structural data can be organized and systematized and what they can be used for in biochemical research.

Each topic of this textbook is developed from general principles, so it can be read by

readers with different backgrounds. Since the book includes the most comprehensive published bibliography on protein structure it will be an important text also for experts in this field.

I. SIMON

Hemopoietic Colonies. In Vitro Cloning of Normal and Leukemic Cells. (Recent Results in Cancer Research 61) by D. Metcalf. Springer-Verlag Berlin – Heidelberg – New York, 1977. 227 pages

This volume is the 61st in the series Recent in Cancer Research and includes our present knowledge about in vitro cloning of normal and leukemic cells. The review contains 14 chapters and is well documented with 28 tables, 52 figures and 428 references.

In the first chapter the author gives an overview about the history of the cloning of hemopoetic cells in semisolid agar. The "cloning Story" began some 15 years ago, and when colonies of T and B lymphocytes were grown by several groups in 1975, the last major subclasses of hemopoietic cells had been successfully cultured in vitro. The second and third chapters of the volume are the most essential parts of the work: the former deals with the current terminology of hemopoietic populations, and the latter is a very exact and detailed description of the methodology for culturing hemopoieitic cells in semisolid medium. In the fourth chapter features shown in common by the various hemopoietic populations during colony formation are described.

In the next section the formation by mouse cells of neutrophilic granulocytic and macrophage colonies (GM-colony-forming cells, GM-CFCs) is discussed, the first cloning system developed and the one about which the most is known. This chapter has some biochemical aspects: the proliferation of GM-CFCs to form colonies is under the control of a specific glycoprotein, the GM-colony-stimulating factor (GM-CSF). GM-CSF occurs in more than one molecular form varying in size from 23,000 to 100,000 daltons, and, as it seems, the target cells are different subpopulations of the GM-CFCs. The most important conclusion of this chapter is that agar cultures permit the determination of the number of granulocytic and monocyte progenitor cells in tissues, the assessment of their proliferative capacity or activity, the measurement of GM-CSF levels, and the detection of other factors influencing the action of GM-CSF on granulopoietic and macrophage-forming cells. Chapter 6 is of particular clinical and partical interest. Leukemic cells obtained from two mouse strains and humans could proliferate and form colonies in semisolid agar. The chronic myeloid leukemic cells and acute myeloid leukemic cells were dependent on GM-CSF. Abnormalitis have been demonstrated, however, in serum and urine GM-CFS as well as in inhibitor levels in CML and AML patients: it is possible that these abnormal regulator levels are responsible for the emergence and progressive proliferation of the leukemic clones.

In Chapters 8 – 11 the formation of eosinophil colonies (consisting of eosinophil-colony-formig cells, EO-CFC), megakaryocyte colonies (consisting of megakaryocyte colony forming cells, MEG-CFC) erythroid colonies (consisting of B-lymphocyte colony forming cells, BL-CFC) is discussed. From a biochemical point of view the detection of such factors is very remarkable, of although the molecular structure the eosinophil colony-stimulating factor (EO-CSF) and that of the megakaryocyte colony-stimulating factor (MEG-CSF), are unknown yet. Section 12 has some clinical aspects again. It deals with neoplastic lymphoid and plasmacytoma colonies.

Metcalf's review is a well-documented, excellent book both for experts in this field and for young gradzates with an interest in hemopoiesis or in cell biology. The enthusiastic words of the finishing sentences are not only for hematologists, but for biochemists too, who want to study the biophysical and biochemical aspects of cell proliferation, including the action of stimulatory and inhibitory factors.

P. KERTAI

Pathology of Laboratory Animals Vols. I. – II. pp. 2171. by K. Benirschke, F. M. Garner, T. C. Jones (eds.). Springer Verlag New York, Heidelberg, Berlin, 1978.

This book published by Springer-Verlag supplies a great want. Publication of such a summarizing work is particularly justified, because lately a number of new species came into use as laboratory animals, and new laboratory strains have been developed.

The two volumes consist of twenty three chapters written by specialists of the given fields. The individual chapters begin with a short introduction — embryological, anatomical, physiological — and end with an abundant bibliography.

Chapter one dealt with the pathology of the cardiovascular system. It describes in detail the inflammatory and degenerative alternations of the endo-, myo-, and pericardium, the developmental disorders of the vascular system, its obliterative, degenerative and proliferative diseases.

Chapter two deals with the respiratory system. After an anatomical, histological and ultrastructural survey it describes the alterations of the respiratory system of various laboratory animals caused by bacteria, viruses, fungi, protozoa and parasites. The chapter ends with a description of different tumors of the respiratory system.

Diseases of the urinary system are described in Chapter three. It begins with a detailed study of different kinds of glomerulonephritis including autoimmune nephritis of certain mouse strains. After the various types of interstitial nephritis it analyses different kinds of pyelonephritis, then degenerative kidney alterations and those of vascular origin. Congenital malformations of the urinary system are also dealt with in detail.

In Chapter four the pathology of the digestive system is described. Starting with the mouth cavity, the inflammatory, parasitic, tumorous and miscellaneous lesions of different parts of the intestinal canal are describe. The pathology of liver and pancres is particularly well worked out.

Chapter five is on the nervous system. Its first part deals with the diseases of viral origin. A great merit of this chapter, in addition to a review of pathological alterations, is that it includes the most important clinical symptoms and diagnostical possibilities of the above diseases. Furthermore, it comments upon the bacterial, fungal and parasitic infections of the nervous system. It touches upon the neoplasmic and degenerative diseases and alternations of the nervous system that are due to toxic agents.

Chapter six deals with the pathology of the endocrine system. The chapter begins with the description of the pituitary gland. It surveys the histology of the pituitary gland of various laboratory animals, the hormones produced by certain types of cells. After a review of the consequences of the hypo- and hyperfunctioning of the pituitary it analysies the alterations of the adrenal, thyroid and parathyroid glands. In my opinion this chapter is somehow out of proportion. The part about the parathyroid gland makes up more than half the chapter, its elaborateness surpasses the requirements of a book like this.

Chapter seven deals with the pathology of reproductive organs. In its first part the developmental desorders of different parts of the female reproductive system as well as its inflammatory and neoplastic diseases are described. In its second part the diseases of the male reproductive system can be found.

The skin diseases of various laboratory animals are treated in Chapter eight. After a survey of the structure and function of certain parts of the skin it deals with inflammatory and tumorous alterations. It describes the parasitic and fungal skin alterations in compliance with their importance.

Chapter nine is on the pathology of the sense organs. The chapter begins with the diseases of the ear. Both histological alterations and the most important clinical symptoms are dealt with. The second half of this chapter is about the pathology of the eye. In this part the classification eye tumors is of particular interest.

Chapter ten is on the musculosceletal system. After a slightly disproportionate normal histological description the author produces an excellent summary of the inherited bone and joint diseases of laboratory animals. Then the effects of hormonal and alimentary factors on bones follow. It is a short coming of this chapter that there is no reference to bone tissue alterations of animals suffering from auto immune diseases and immunodefficiency. The second part of the chapter is about the pathology of the muscular system.

Chapter eleven deals with hematologic disorders. Ways of taking blood as well as different parameters of the animal blood are

described. Finally the diseases of the erythron, leukocytes and platelets are discussed.

Chapter twelve contains a detailed description of tumors of different organs. It offers a survey of the macro- and microscopical characteristics of both non-malignant and malignant tumors in different species of laboratory animals.

Chapter thirteen comprises viral diseases of laboratory animals.

Chapter fourteen treats bacterial diseases. Clinical symptoms, possibilities of diagnosis and pathological alterations are described.

In Chapter fifteen the diseases due to mycoplasmas and rickettsiae, in Chapter sixteen fungal and in Chapter seventeen protozoal and metazoal diseases are discussed.

Chapter eighteen deals with cytogenetics. Chromosome numbers, characteristics of the chromosomes as well as chromosome abnormalities of different animal species are included.

Chapter nineteen deals with biochemical aspects. An excellent Appendix is included in this relatively short part. It contains several thousands of laboratory parameters.

Chapter twenty deals with developmental abnormalities.

Chapter twenty-one is on immunopathology. It stresses the pathogenetic mechanisms of immune injury as well as specific immunologic lesions. The immunopathological importance of the thymus seems to be somewhat under-estimated.

Chapter twenty-two contains a description of hereditary diseases and chapter twenty-three nutritional and metabolic diseases of different animal species.

It is the editor's special merit that the work has been published in excellent quality. It containg 465 black and white figures.

The book will certainly be useful for specialists in different fields.

B. MÁNDI

Membrane Functions, by C. Nicolau and A. Paraf (eds.). Springer-Verlag Berlin, Heidelberg, New York, 1977, 204 pages

The proceedings of the meeting held in Grignon (France) in 1976 is a brilliant and up-to-date summing-up of the interactions between membrane structure and function. Although the chapters representing 13 lectures cover a considerably large field of membrane research, the matter reported can be devided into two groups, depending on whether structural problems or functional aspects of membranology have been given particular emphasis.

The individual contributions were made by authors of various training (physicists, biochemists, physico-chemists, medical scientists), demonstrating the complexity of present-day membrane research. Among the structural studies it is worth mentioning cooperative state transitions, lipid-protein interactions, demain formation, modulation of plasma membrane architecture, freeze facture and spin label studies, lipid organization of plasma membrane, structure and shape of membrane proteins, etc. The functional aspects of membrane include problems of high significance, such as coupling between ATP hydrolysis and $Na^+ + K^+$ transport, mechanism of activation of vasopressin sensitive adenylate cyclase, changes courring in membrane structure and functions upon tumor-virus transformation, role of membrane enzymes in the modulation of transmembrane control, as well as the molecular architecture of reconstituted calcium pump.

The book is equally useful for those desirous of getting latest information on the subjects here outlined, and for those interested in the up-to-date solution of problems in some special field. The leading principle — that the complexity of membranology can be approached only by combined, and extensive research work — is emphasized throughout the 200 pages of the volume. Fundamental into dependence of structure and function, especially with biomembrans, is another basic concept that emerges of from this very informative volume.

I. SOMOGYI

Geometrical Probability and Biological Structures: Buffons 200th Anniversary. Proceedings of the Buffon Bicentenary Symposium on the Geometrical Probability, Image Analysis, Mathematical Stereology, and Their Relevance to the Determination of Biological Structures, Held in Paris, June 1977, by R. E. Miles and J. Serra (editors). The book appeared in the series "Lecture Notes in

Biomathematics". Managing editor S. Levin. Published by Springer-Verlag, Berlin, Heidelberg, New York, 1978. 338 pages

The present volume contains lectures delivered at the anniversary meeting. This meeting was attended by about 60 scientists of mathematical and biological disciplines. Papers appear in chronological order.

After a review on Buffon's pioneering work in geometrical probability, topics of mathematical stereology, mathematical morphology, geometrical probality, geometrical statistics, and epistemology are considered. There are applications to biological problems such as the clustering and layering of neurons in the central nervous system, the morphological analysis of connective tissues and cirrhotic liver tissues, or the growth of the folliculum. One gets useful hints in particle counting, quantitative image analysis, sampling problems, and in "automated" cytology. On the other hand, the reader finds good reviews on up to date geometry such as e.g. fractal geometry.

Papers are written in a good everyday manner avoiding intrigning technicalities. There are however two points where the editorial policy of the publishers may be questioned:

1) 5 of the 28 papers are written in French. The majority of readers prefers unilingual books.

2) The inclusion of discussions would have helped insight in this amazing field.

J. HAJDU

G. Adam, P. Läuger, G. Stark: Physikalische Chemie und Biophysik. Springer-Verlag, Berlin, Heidelberg, New York, 1977. 465 pages

It is becoming more and more evident nowadays, that physical chemistry considered formerly as a purely chemical discipline — has to penetrate, and has already penetrated into different, originally not chemical fields of science. In this process it has become an indispensable help of engineers and physicists. and at least as much, or even more indispensable for research workers and practical experts of biology, examining the most tomplicated system, the living organism.

It is also obvious, that physical-chemical training of such experts can not include the whole physical chemistry like the training of a chemist or a chemical engineer does. Therefore the physico-chemical manuals and study-aids of sciences not of a chemical direction have to be far too tendentious besides the treatment of general basic principles, and though they have to comprise a short and intelligible expounding of general basic principles of physical chemistry, they also have to treat the material which is important for its special application in the given professional field in a much greater volume and depth.

With the rapid development of biophysics, and urged by its needs for the methods of physical chemistry and its applications in the interpretation and expedient influencing of the processes of living organism it grains ground more and more nowadays in biology as well.

The manual written by the three authors greatly considers this, so a very good summarizing work and — as a result of their evidently long experience in education — a really tendentious good study-aid was produced for the thorough training of students of biology which is really oriented to the physico-chemical aspects of biology.

The material of the book, and the good correlation of the volumes of the parts of the material is well reflected by the following ennumeration of the main chapters (the numbers in brackets refer to the total page number of the chapters): Basic notions of thermodynamics (38); Main theses of thermodynamics and the bases of their application for biological systems (34); Thermodynamic potentials and equilibria (22); Systems of several components, ideal and real mixtures, activity, decrease of steam-pressure, osmotic characteristics, phase equilibria (33); Chemical equilibria, law of mass-effect, solubility, pH, hydrolysis, buffer mixtures (27); Bases of electrochemistry, ion migration, redox processes, equilibria of ions in electrodes and membranes (35); Phenomena of edge plane, surface tension, adsorption, surface layers (32); Transport-phenomena, viscosity, diffusion, precipitation, electrokinetic movement phenomena (50). Biological membranes, structure, characteristics, transport of material through the membrane (74); Kinetics, speed of reaction, mechanism of reaction,

enzyme-kinetics (74); Radiation biophysics (38).

The reviewer is glad, when a book gets into his hand, the conception of which corresponds to his own, and the presentation of the material on its basis is successful. In this case this has been realized.

The book is pronouncedly a manual, a good manual, with lots of examples, well demonstrating figures, in a nice make-up and in a good paper-back at a reasonable price, obtainable for every university student.

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