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J. BIELAWSKI and BARBARA KWINTO

THE INFLUENCE OF GRAMICIDIN A AND VALINOMYCIN ON THE PERMEABILITY OF MAMMALIAN ERYTHROCYTES

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1. The K^+ transport in erythrocytes induced by gramicidin A or valinomycin is a first-order reaction. The activation energy of the induced transport is low and amounts to 6 and 10 kcal/mole, respectively. The indirect method for calculation of the driving force of diffusion, *c*, is given; in pig erythrocytes the *c* value for gramicidin A is 21.7, and for valinomycin 35.4 mM-KCl.

2. The valinomycin-induced transport was found to be the same in erythrocytes and bimolecular lipid membranes. The gramicidin A-induced transport corresponds to that of a single gramicidin channel, and not to the multichannel transport observed in the model systems.

3. Erythrocytes of various mammals show large differences in sensitivity to the ionophores. No correlation could be found between membrane lipids and the induced permeability. The role of membrane proteins in ionophore-induced permeability is discussed.

Introduction of ionophorous antibiotics into biological systems induces, in addition to the bacteriostatic action (Shemyakin *et al.*, 1965), several other effects: uncoupling of oxidative phosphorylation and stimulation of ion transport in mitochondria (Neubert & Lehninger, 1962; Pressman, 1965; Henderson *et al.*, 1969), haemolysis and stimulation of cation exchange in erythrocytes (Chappell & Crofts, 1966; Bielawski, 1968). The mechanism of ionophore action is usually explained by an increase in the permeability of the membrane to ions.

A convenient system for investigation of the mechanism of the ionophore-induced ion transport is the lipid bimolecular membrane. Basing on investigations of this model, two mechanisms are postulated: the carrier-mediated transport and the channel-mediated transport. The most frequently investigated carrier is valinomycin (Pressman, 1968; Eisenman *et al.*, 1968) and the best known channel-forming ionophore is gramicidin A (Goodall, 1971; Hladky & Haydon, 1972; Bamberg & Lauger, 1974).

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In a previous paper (Bielawski, 1968) the erythrocyte membrane was shown to be a convenient biological model for the study of the ionophore-induced transport. In this work, the effect of gramicidin A and valinomycin on the permeability constant of pig erythrocyte membrane is studied, and the comparative observations on the erythrocytes of various mammalian species are included.

MATERIALS AND METHODS

Pig, ox, and sheep blood was obtained from the slaughterhouse. The blood of man, dog, rabbit and horse was collected from the veins, and blood of rat and guinea pig from the heart. Clotting was prevented by addition of 5 ml of citrate solution to 20 ml of blood. The citrate solution contained 1.23 g of trisodium citrate dihydrate, 0.48 g of citric acid monohydrate, 1.47 g of glucose, and water up to 100 ml. The blood was stored at 4°C, usually overnight and never longer than 6 days. The erythrocyte suspension was obtained by centrifugation and washing three times in 160 mM-NaCl as described by Bielawski (1968).

The volume of the pellet obtained by centrifugation at 2000 *g* for 30 min was taken as the volume of erythrocytes. Osmotically active volume was determined by plotting the erythrocyte volume against reciprocal of KCl concentration and extrapolating the curve to infinite KCl concentration. In calculations the volume at 160 mM-KCl was taken as unity. During swelling, changes in absorbance of the erythrocyte suspension were measured and changes in the volume calculated therefrom (Wilbrandt, 1955). The absorbance of the erythrocyte suspension was measured with the Specol (Carl Zeiss, Jena) spectrophotometer at 589 nm. At this wavelength large changes in pH have no influence on the absorbance of haemolysed erythrocytes and the absorbance is linearly related to the volume of erythrocytes.

The correlation between molarity and osmolarity is different in KCl, NaCl and sucrose solutions. For simplicity of calculations the osmolarity of 160 mM-NaCl and KCl was taken as equal to the osmolarity of 300 mM-sucrose. No corrections for chemical properties of these substances were introduced.

Valinomycin was obtained from Calbiochem AG (Lucerne, Switzerland) and gramicidin from Sigma Chem. Co. (St. Louis, Mo., U.S.A.). The term "gramicidin D" (Bielawski, 1968) has been replaced by a more correct name "gramicidin A" (Glickson *et al.*, 1972).

RESULTS AND DISCUSSION

The ionophorous antibiotics reversibly increase the permeability of erythrocyte membranes to alkali metal cations (Bielawski, 1968). To compare change in permeability of erythrocytes of different species and of other membranes, the permeability constant has to be determined. For calculation of this value, the rate of salt penetration across the membrane and the driving force of diffusion must be known.

Driving force of diffusion. In a simple diffusion, the driving force, *c*, corresponds to the difference in concentration of the penetrating salt on either side of the mem-

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brane. In erythrocytes, the intracellular substances which do not penetrate the membrane, complicate the measurement. Some of these substances undergo electrolytic dissociation and hence the Donnan effect is superimposed on the osmotic effect. Therefore, in the present paper c is calculated indirectly as the difference in salt concentration which corresponds to the resultant of all the forces acting on the penetrating salt. In the previous paper (Bielawski, 1968) it was shown that swelling of erythrocytes induced by gramicidin A can be prevented by substituting an appropriate part of the KCl (or NaCl) solution in the external medium by isotonic sucrose which does not penetrate the membrane. Thus, the amount of KCl spared by sucrose can be taken as a measure of c. To estimate this amount of KCl, isotonic sucrose solution was added to the KCl-containing medium, and the volume of erythrocytes was measured by monitoring the absorbance of the erythrocyte suspension (Fig. 1) in the presence of gramicidin A and valinomycin. The concentrations corresponding to the intersection points of the experimental plots with the control

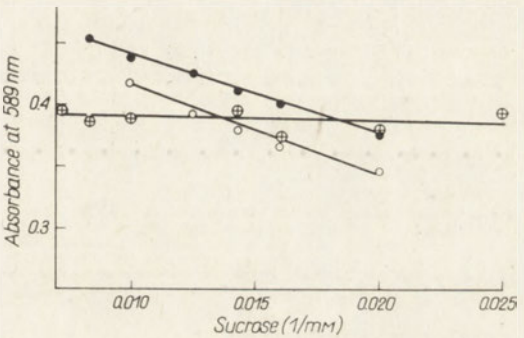

Fig. 1. Effect of sucrose concentration on absorbance of pig erythrocyte suspension in the presence of 5 nM-gramicidin A (●) and 0.9 μM-valinomycin (○); control, without ionophores (⊕). The system contained: sucrose from 50 to 200 mM and KCl from 133 to 53 mM, respectively; erythrocytes, 1.6 μl/ml. The absorbance was measured after 3 h of incubation at 20°C.

one (without ionophores) were taken as the values of c. In pig erythrocytes at the KCl concentration of 160 mM and pH 7.0, c amounts to 21.7 mM-KCl in the presence of gramicidin A, and 35.4 mM in the presence of valinomycin. The difference between the effect of gramicidin A and valinomycin can be explained by the fact that in the presence of the latter compound Na^+ does not penetrate the membrane. If this reasoning is correct, the concentration of Na^+ within the erythrocytes can be calculated by subtracting c values measured in the presence of gramicidin A from those measured in the presence of valinomycin. The Na^+ concentration calculated in this way amounts to 13.7 mM and 82.9 mM in pig and ox erythrocytes, respectively. These values are in good agreement with those measured directly by Coldman & Good (1967) and Evans & Phillipson (1957) proving the correctness of our calculations.

The pK of several substances inside the cells is close to neutral pH. Hence, it could be expected that c depends on pH of the suspension. The effect of pH on the absorbance of the suspension in the presence and absence of gramicidin A is shown in Fig. 2. The appropriate pH was obtained by substituting a part of the NaCl in the incubation medium by the same amount of NaOH or HCl. The time of incubation was long enough for equilibration of ions inside and outside of the cells.

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In the absence of gramicidin A, the erythrocyte membrane is permeable to Cl^- and OH^- but almost impermeable to Na^+. On alkalization of the medium OH^- enters the cell in exchange for Cl^-. The rise in internal OH^- concentration decreases the dissociation of the alkaline groups of the intracellular non-penetrating substances and, as the result, the positive charge of theses substances decreases and shrinkage occurs (Jacobs & Steward, 1947). At high pH, the membranes are destroyed and haemolysis is observed. On the other hand, the increase in acidity raises the positive charge of the substances which do not penetrate the membrane, and causes the swelling of erythrocytes. The swelling is relatively small because the hidden acidic groups of haemoglobin are liberated (Steinhardt & Beychok, 1964) and a part of the positively charged groups is neutralized.

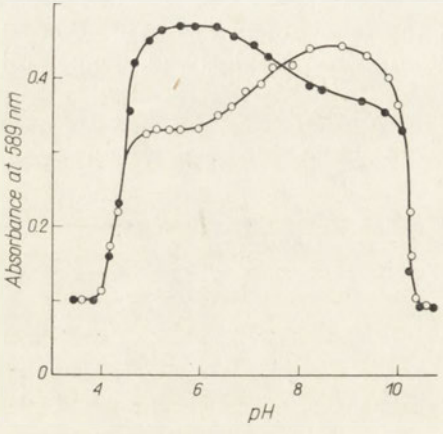

Fig. 2

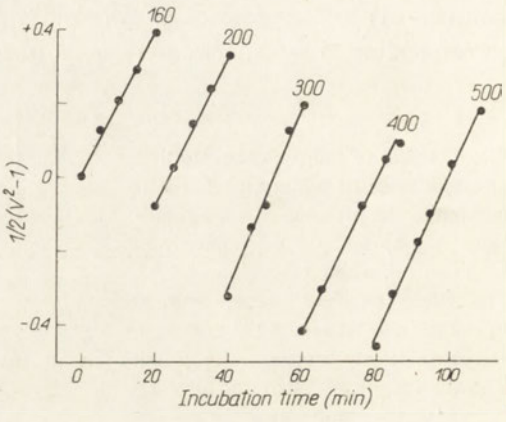

Fig. 3

Fig. 2. Effect of pH on absorbance of pig erythrocyte suspension in the absence (○) and presence of 0.5 nM-gramicidin A (●). The system contained: 120 mM-NaCl, 75 mM-sucrose, and erythrocytes, 1 µl/ml. The measurements were taken after 3 h of incubation at 20°C.

Fig. 3. Effect of KCl concentration on the rate of swelling of pig erythrocytes in the presence of 2.5 nM-gramicidin A. The system contained: 20 mM-potassium phosphate buffer, pH 7.0; erythrocytes, 1.5 µl/ml; temperature, 20°C. The volume of the erythrocytes in the buffered 160 mM-KCl was taken as unity.

In the presence of gramicidin A, the erythrocyte membrane is permeable to Cl^- as well as to Na^+. The erythrocyte volume is therefore the smallest when the sum of charges of the non-penetrating substances within the erythrocytes is zero. At this pH, the osmotic concentration of these substances within the cells is considered equal to the osmotic concentration of sucrose in the incubation medium. Swelling occurs on alkalization as well as on acidification. In the latter case a broad plateau between pH 5.5 and 6.5 is observed (Fig. 2). This plateau can be explained by unmasking of the hidden acidic groups of haemoglobin. To eliminate the influence of pH on c, the routine measurements were taken at pH 7.0.

From the results shown in Fig. 2 several properties of the erythrocytes, such as: osmotic concentration and electrolytic dissociation of the non-penetrating substances

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within the cells, and the electric potential across the erythrocyte membrane, can be calculated. The erythrocytes are in osmotic equilibrium with the incubation medium, hence:

$$S_i + K_i^+ + Cl_i^- = S_e + K_e^+ + Cl_e^- \quad (1)$$

where K_i^+, Cl_i^-, K_e^+, and Cl_e^- are the osmotic concentrations of K^+ and Cl^- in the internal and external media, respectively. S_e is the osmotic concentration of sucrose in the external medium, and S_i the osmotic concentration of all the intracellular non-penetrating substances. Relation (1) is independent of the dissociation of these substances.

The erythrocytes are in the Donnan equilibrium and therefore the following relation can be written:

$$\frac{K_i^+}{K_e^+} = \frac{Cl_e^-}{Cl_i^-} \quad (2)$$

S_i at a given pH can be easily calculated if the change in volume of erythrocytes on shifting pH from 6.0, at which $S_i = S_e$, to the given pH is known. The volume of erythrocytes is very small in comparison with that of the external medium and therefore the exchange of ions across the cell membranes has a negligible effect on K_e^+ and Cl_e^-. K_i^+ and Cl_i^- can be calculated from the equations (1) and (2). The ratio of K_i^+/K_e^+ determines the electric potential difference across the membrane.

In four experiments similar to that shown in Fig. 2, S_i, K_e^+ and Cl_e^- were determined in pig erythrocytes incubated in 160 mM-KCl at pH 7.0, and Cl_i^- and K_i^+ were calculated. The electric potential difference across the membrane was further calculated and found to be 7.0 mV. This value is similar to that obtained with other methods by Coldman *et al.* (1970) and Cotterrell & Whittam (1971), and is again a proof that the evaluation of c is correct.

The rate of ionophore-induced salt uptake by pig erythrocytes. The erythrocyte membrane is permeable to water (Siedel & Solomon, 1957; Rich *et al.*, 1967), Cl^- (Love & Burch, 1953; Tosteson, 1959) and almost impermeable to alkali metal cations.

Permeability to cations is considerably increased by gramicidin A and valinomycin but it is still much lower than the permeability to water. During ionophore-induced swelling, uptake of salt at isotonic concentration takes place and therefore the rate of swelling can be a measure of the rate of salt penetration.

The dependence of erythrocyte volume on time during swelling could be expressed by a simple equation provided that the c value, the osmotically active volume and surface of the erythrocyte are taken as units (Wilbrandt, 1955). The relationship discussed takes the form:

$$K \cdot t = \frac{1}{2}(V^2 - 1) \quad (3)$$

where t is the time of incubation, V is the volume of erythrocytes and K is the cell constant. The constant K depends on permeability of the cell membrane to salts

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and on geometry of the cells. As it can be seen in Fig. 3, the swelling of erythrocytes in the presence of gramicidin A conforms with the equation (3).

Since in 160 mM-KCl the swelling occurring before haemolysis is but slight, to increase swelling 200 mM-KCl was included in the standard incubation medium. The osmotically active volume of erythrocytes in 160 mM-KCl was introduced to equation (3) as unit volume.

K in equation (3) is the constant specific for the given cells. To estimate the permeability constant from the K values, the fraction units should be recalculated to moles, cm^3, cm^2 and seconds. The permeability constant P is therefore:

$$P = K \cdot H$$

and

$$H = \frac{KCl_e}{60 \cdot c \cdot A}$$

where A means the surface of the erythrocytes with the osmotically active volume equal to 1 cm^3. KCl_e is potassium chloride concentration in the external medium. The surfaces were calculated from the critical cell radius according to Wessels & Veerkamp (1973).

Effect of KCl concentration. In lipid bimolecular membranes carrier-mediated transport is linearly related to K^+ concentration. In channel-mediated transport the rate of transport can increase much faster than the increase in K^+ concentration (Mueller & Rudin, 1968). The influence of KCl concentration in the incubation medium on the increase in volume induced by gramicidin A, was therefore tested (Fig. 3). The slope of all the curves is the same which means that at the volume equal to unity the increase in volume is the same at different KCl concentrations. This means further that in erythrocytes the rate of gramicidin A-induced transport is linear with the concentration of KCl in the external medium.

Effect of ionophore concentration. Data given in Fig. 4 show that the increase in ionophore concentration raises the permeability of erythrocytes till the saturation point for transport is achieved. This saturation results from the limited solubility of ionophores (Kemp *et al.*, 1972) and occurs at concentrations of 10 nM-gramicidin A and 1.0 μM-valinomycin. To minimize the precipitation, the incubation medium with the ionophore added was stirred vigorously prior to the addition of erythrocytes. Below the saturation point the ratio of the increase in log of permeability to the increase in log of valinomycin concentration is close to unity, and the transport is a first-order reaction. The first-order reaction in carrier-mediated transport was also observed in chloroplast membranes (Junge & Schmid, 1971), in mitochondria (Haynes *et al.*, 1974) and in bimolecular lipid membranes (Andreoli *et al.*, 1967; Eisenman *et al.*, 1968; Stark & Benz, 1971). The same mechanism of transport can be therefore assumed for all the systems.

Some differences are observed in the channel-mediated transport. In bimolecular lipid membranes in the presence of gramicidin A the ratio of Δlog conductivity/Δlog ionophore concentration equals 2, and in the presence of alamethicin even 6. The high order of the reaction is explained by the formation and breakdown of the chan-

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nels involving two molecules of gramicidin A (Goodall, 1970; Bamberg & Lauger, 1974) and six molecules of alamethicin (Mueller & Rudin, 1968). In erythrocytes the ratio of Δlog permeability/Δlog concentration of gramicidin A amounts to 1 (Fig. 4). The rate of transport is also linear with concentration of the salt transported (Fig. 3).

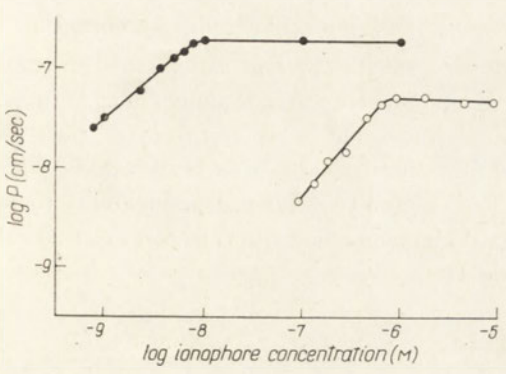

Fig. 4

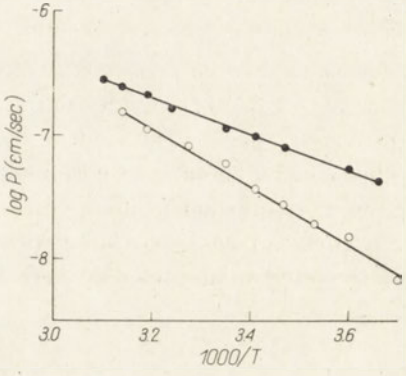

Fig. 5

Fig. 4. Effect of ionophore concentration on the permeability constant (P) of pig erythrocyte membranes. The system contained: 200 mM-KCl; erythrocytes, 1.5 µl/ml; and gramicidin A (●) or valinomycin (○). Temperature, 20°C.

Fig. 5. Arrhenius plots of the effect of temperature on the ionophore-induced permeability of pig erythrocyte membranes. The system contained: 200 mM-KCl; erythrocytes, 1.6 µl/ml; and 2.5 nM-gramicidin A (●) or 0.45 µM-valinomycin (○).

It is difficult to compare directly the results obtained on bimolecular lipid membranes with those concerning erythrocytes. The important component of the latter are proteins which can modify the properties of lipids. One may assume that gramicidin A forms in the erythrocyte membranes relatively stable channels. Their formation and decomposition have little effect on the transport; however, gramicidin A can be easily washed out of the erythrocytes, and impermeability to cations is restored on washing (Bielawski, 1968).

Effect of temperature. In bimolecular membranes with a well-defined temperature of the liquid/solid state transition a sudden drop in the valinomycin-induced transport occurs on transition to the solid state. However, the gramicidin A-induced transport is not markedly changed by this transition (Krasne *et al.*, 1971). The Arrhenius plots representing the relation between ion transport induced by these antibiotics in erythrocytes and temperature, are given in Fig. 5. The concentration of ionophores at about half saturation at 20°C was chosen to avoid the possible effect of temperature on their solubility in the incubation medium. As it is evident from Fig. 5, the plots are linear over the range of 0° to 45°C for both antibiotics. Thus it may be concluded that lipids in the erythrocyte membranes do not undergo transition of the liquid/solid state of the type observed in bimolecular membranes (Krasne *et al.*, 1971) over this temperature range. The low activation energy (10 kcal/

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mole for valinomycin and 6 kcal/mole for gramicidin A) supports the assumption that diffusion is the rate-limiting step in the transport induced by the antibiotics.

The activation energy for gramicidin A-induced transport in erythrocytes is close to that obtained for a single gramicidin A channel, and is significantly lower than that for multichannel system in bimolecular membranes (Hladky & Haydon, 1972; Bamberg & Lauger, 1974). This result also supports higher stability of gramicidin A channels in erythrocytes than in the bimolecular lipid membranes.

Ionophore-induced permeability in erythrocytes of different mammals. The results presented in Table 1 show large differences in sensitivity to ionophores of erythrocytes from various species. The highest induction is found in pig erythrocytes, their permeability being about ten times higher than that induced in ox erythrocytes, which are the most resistant to ionophores. The valinomycin-induced permeability to K^+ in sheep erythrocytes, measured with a different method by Tosteson *et al.* (1967), is close to the value presented in Table 1.

Table 1

Permeability to KCl *induced by valinomycin and gramicidin A in erythrocytes of various mammals*

The system contained 200 mM-KCl, 1 μM-valinomycin or 10 nM-gramicidin A, and erythrocytes, 1.5 μl/ml; the temperature was 37°C. V, volume of erythrocytes; V_F, osmotically active volume as the fraction of the total volume of erythrocytes suspended in 160 mM-KCl (a mean of three experiments); A, surface area of the erythrocytes; P, permeability constant (a mean of five experiments, ±S.D.). Volume and surface of erythrocytes were taken from Wessels & Veerkamp (1973) except those for guinea pig taken from Gruber & Deuticke (1973).

Animal	V (10^{12} cm^3)	V_F	A (10^8 cm^2)	P	
				Valinomycin (10^8 cm/sec)	Gramicidin A (10^8 cm/sec)
Pig	61.5	0.55	102	9.96±5.34	25.45±9.33
Rat	62.7	0.55	105	7.50±1.85	8.40±0.40
Dog	65.1	0.52	89.6	3.64±2.04	5.69±1.52
Guinea pig	77.0	0.50	129	3.24±1.21	3.59±1.12
Man	87.7	0.56	129	2.15±1.04	2.03±1.57
Rabbit	64.3	0.47	107	1.51±0.36	1.38±0.57
Horse	49.7	0.54	72.4	1.39±0.58	1.45±0.88
Sheep	35.3	0.60	68.8	1.00±0.37	1.39±0.25
Ox	51.1	0.47	104	0.83±0.29	1.19±0.74

Among the different species a close correlation was found between the valinomycin and gramicidin A-induced permeability. This means that the same properties of the membranes are responsible for the sensitivity to both ionophores. However, no correlation could be found between the lipid composition of the membranes (Nelson, 1967) and the sensitivity to the antibiotics. This confirms the conclusion that the non-lipid components of the membranes, mainly proteins, modify the ionophore action.

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From semiquantitative analysis of erythrocyte membrane proteins (Kobylka *et al.*, 1972) it appears that the content of the amphipatic proteins belonging to "fraction III" in disc-gel electrophoresis, increases in the following order: pig<dog <man<ox. This is consistent with the order of decreasing sensitivity to ionophores presented in Table 1. The protein of fraction III is situated in the membrane in such a way that it can diminish the mobility of ionophores or ions within the membrane.

Proteins, as the potent surface-active material, can modify the ionophore activity also by changing the properties of the surface of erythrocyte membranes.

The presented paper clearly shows the advantages of the use of ionophores in the studies on physico-chemical properties of biological membranes and the cell interior.

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WPŁYW GRAMICYDYNY A I WALINOMYCYNY NA PRZEPUSZCZALNOŚĆ ERYTROCYTÓW SSAKÓW

Streszczenie

1. Transport K^+ indukowany w erytrocytach przez gramicydynę A i walinomycynę jest reakcją pierwszego rzędu. Energia aktywacji wynosi odpowiednio 6 kcal/mol i 10 kcal/mol. Przedstawiono pośrednią metodę obliczania siły napędowej dyfuzji, c; w erytrocytach świni c wynosi w odniesieniu do gramicydyny A 21.7 i do walinomycyny 35.4 mM-KCl.

2. Transport indukowany przez walinomycynę jest zasadniczo taki sam w erytrocytach i w bimolekularnych błonach lipidowych. Transport indukowany w erytrocytach przez gramicydynę A wykazuje cechy transportu przez pojedynczy kanalik gramicydynowy, a nie transportu przez układ wielokanalikowy obserwowany w systemach modelowych.

3. Wrażliwość błon erytrocytów różnych ssaków na antybiotyki jonoforowe jest bardzo różna. Brak jest korelacji między składem lipidowym tych błon a wrażliwością na jonofory. W pracy omówiono wpływ białek błon na transport indukowany jonoforami.

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ISOLATION AND PROPERTIES OF tRNA NUCLEOTIDYLTRANSFERASE FROM WHEAT EMBRYOS*

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From wheat embryos, tRNA nucleotidyltransferase (EC 2.7.7.25) was isolated. By chromatography on Sepharose 6B, DEAE-cellulose and affinity chromatography on tRNA-hydrazyl-Sepharose 4B, 7000-fold purification of the enzyme was achieved.

The enzyme required for its activity Mg^{2+} or Mn^{2+} ion.

ATP inhibited incorporation of CMP from CTP into lupin tRNA, and CTP acted as a competitive inhibitor of AMP incorporation from ATP.

The regulatory role of ATP in incorporation of terminal CMP into tRNA is discussed. The incorporation of terminal CMP into tRNA deprived of terminal CCA or CA, was also studied.

tRNA nucleotidyltransferase, the enzyme attaching terminal CMP or AMP to incomplete or immature tRNA, has been so far isolated from bacteria (Carre *et al.*, 1970; Miller & Philipps, 1971a; Best & Novelli, 1971a), yeast (Morris & Herbert, 1970; Sternbach *et al.*, 1971), and animal tissues (Deutscher, 1972a). Although the enzymes from these sources are similar, some differences in their properties were observed. The enzymes have a different number of active centres (Anthony *et al.*, 1963; Deutscher, 1972b), and the effect of ATP and CTP on the incorporation of CMP and AMP, respectively, is also different (Carre *et al.*, 1970; Deutscher, 1972b).

Since no data are available on tRNA nucleotidyltransferase from higher plants (except the report by Cudny *et al.*, 1975, concerning lupin seed), an attempt was made to isolate and purify the enzyme from wheat embryos. Properties of the purified preparation were studied and compared with those of the enzyme from other sources. A preliminary account of this work has been presented (P. Dullin *et al.*, 1974, IX FEBS Meeting, Budapest, Abstr. no. f 9b18).

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MATERIALS AND METHODS

Reagents. [^{14}C]CTP (spec. act. 480 μCi/mmole) and [^{14}C]ATP (spec. act. 570 μCi/mmole) were from the Radiochemical Centre (Amersham, England); Tris from Koch-Light Lab. Ltd (Colnbrook, Bucks., England); Sepharose 6B from Pharmacia Fine Chemicals (Uppsala, Sweden); DEAE-cellulose Whatman DE-11, Folin-Ciocalteu phenol reagent, POPOP and PPO from Merck (Darmstadt, G.F.R.); membrane filters BA-85 from Schleicher & Schuell A. G. (Dassel, G. F. R.). Yeast tRNA-X (tRNA without terminal CCA) and tRNA-XC (tRNA without terminal CA), were prepared in this Institute by T. Dzięgielewski, and tRNA-hydrazyl--Sepharose 4B and lupin seed tRNA by H. Jakubowski. The tRNA preparation was partly degraded but the degree of degradation of 3′-terminal has not been estimated.

Plant material. Wheat embryos were kindly supplied by Państwowe Zakłady Zbożowe, Poznań. The embryos were stored at room temperature for one year, and their humidity did not exceed 15%.

Determination of tRNA nucleotidyltransferase activity. The standard assay mixture contained in a final volume of 100 μl: 160 μM-[^{14}C]CTP (20 μM for determinations of activity in chromatographic fractions), 80 mM-Tris-HCl buffer, pH 7.6, 6 - 10 mM-$MgCl_2$, 5 μg of tRNA, and 0.4 μg of protein when the purified enzyme preparation was used. The reaction was carried out at 30°C for 20 min, and was stopped by addition of 3 ml of 10% trichloroacetic acid containing 0.9% sodium pyrophosphate. The acid-insoluble precipitate was collected on membrane filter, washed with 5% trichloroacetic acid, the filter was dried, placed in a scintillation vial to which 5 ml of scintillation fluid (0.1 g of POPOP and 4 g of PPO in 1 litre of toluene) was added, and the radioactivity was measured in Packard Tri-Carb scintillation counter. The enzyme activity is expressed as pmoles of CMP or AMP incorporated per sample during 20 min incubation at 30°C.

Determination of the activity of other enzymes. Ribonuclease was assayed according to Tanaka (1967), DNA-dependent RNA polymerase as described by Fabisz-Kijowska *et al.* (1975), poly(A)-nucleotidyltransferase according to Twu & Bretthauer (1971), poly(C)-nucleotidyltransferase by the method of Edmonds (1965) and polynucleotide phosphorylase according to Singer (1966).

Electrophoresis of enzyme at pH 8. The enzyme was layered on 7.5% polyacrylamide gel and subjected to electrophoresis at 5 mA/gel. Coomassie Brilliant Blue G-250 was used for staining as suggested by Tadeusz Rorat, M. Sc.

Protein was determined by the method of Lowry *et al.* (1951) or spectrophotometrically (Warburg & Christian, 1942), in the preparations with low protein content.

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

Isolation of the enzyme. All steps of the procedure were performed at 0°C. Wheat embryos, 20 g, were ground in a mortar with 60 ml of 0.05 M-Tris-HCl buffer, pH 8.0, with 5 mM-$MgCl_2$, 0.1 mM-EDTA, 4 mM-2-mercaptoethanol and 30% glycerol (BS-30 buffer). After 30 min, the homogenate was filtered through 4 layers of gauze,

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then ammonium sulphate (pH 7.0) added to 0.2 M concentration, and the very viscous suspension was sonicated in MSE ultrasonic generator for about 1.5 min till disappearance of viscosity. Next, 0.135 M-$LaCl_3$ in a volume corresponding to 0.1 that of the extract, was added to precipitate nucleic acids and deoxynucleoproteins (Yoshida & Shimura, 1972) and the mixture centrifuged at 110 000 *g* for 1.5 h. The clear supernatant was diluted with 2 vol. of 0.05 M-Tris-HCl buffer, pH 8.0, with 5 mM-$MgCl_2$, 0.1 mM-EDTA and 4 mM-2-mercaptoethanol (so that glycerol concentration decreased to 10%) and was fractionated with ammonium sulphate. The 0.4 - 0.7 saturation fraction was collected, suspended in BS-30 buffer containing 0.2 M-KCl, and applied to a Sepharose 6B column (16 × 700 mm) equilibrated with the same buffer solution. The active fractions were pooled (Fig. 1), dialysed against

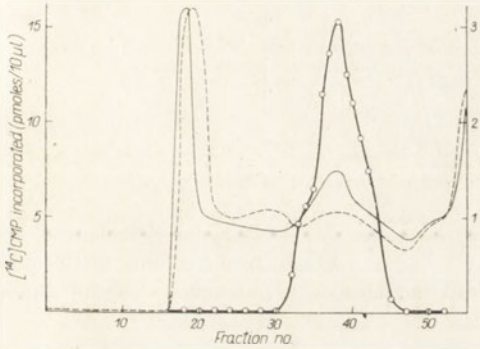

Fig. 1

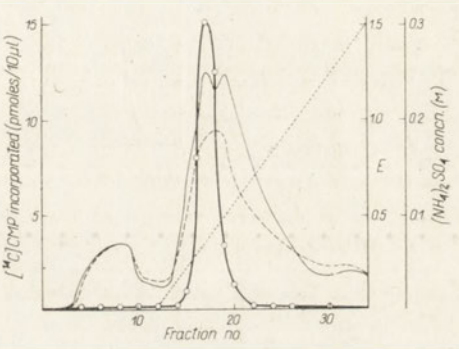

Fig. 2

Fig. 1. Sepharose 6B chromatography of the nucleotidyltransferase. The ammonium sulphate fraction, 2 ml, in BS-30 buffer containing 0.2 M-KCl, was applied to the column (16 × 700 mm). Fractions of 2 ml were collected, and the enzyme activity determined in 10 µl samples. — — —, $E_{260\ nm}$; ———, $E_{280\ nm}$; ○, activity.

Fig. 2. DEAE-cellulose chromatography. The active fractions from Sepharose 6B gel filtration were dialysed and applied to the column (10 × 100 mm). Fractions of 2 ml were collected, and the enzyme activity determined in 10 µl samples. — — —, $E_{260\ nm}$; ———, $E_{280\ nm}$; ○, activity; · · ·, $(NH_4)_2SO_4$ concentration gradient.

BS-30 buffer and applied to a DEAE-cellulose column (10 × 100 mm) which was equilibrated and eluted first with the same buffer alone and then with ammonium sulphate concentration gradient up to 0.3 M, in BS-30 buffer. One active peak was obtained at 0.09 M-$(NH_4)_2SO_4$ concentration (Fig. 2); the active fractions were pooled, dialysed against BS-30 buffer and applied to a tRNA-hydrazyl-Sepharose 4B column (9 × 50 mm), equilibrated and washed with 10 ml of the same buffer. The enzyme was eluted with BS-30 buffer containing 0.2 M-KCl (Fig. 3). The active fractions were pooled and concentrated with Aquacide II. The obtained preparation separated on polyacrylamide-gel electrophoresis into one major and some minor protein bands (Fig. 4).

The concentrated enzyme preparation was stabilized with bovine serum albumin, fraction V (1 mg/ml) and tRNA (10 µg/ml). The preparation stored under these

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conditions at −12°C lost usually about 50% of the activity within a fortnight, and that is why for each experiment the time of storage of the enzyme is given.

The tRNA-hydrazyl-Sepharose 4B column was repeatedly used after being washed with 50 ml of 1 M-KCl and stored in 0.02% sodium azide at 4°C.

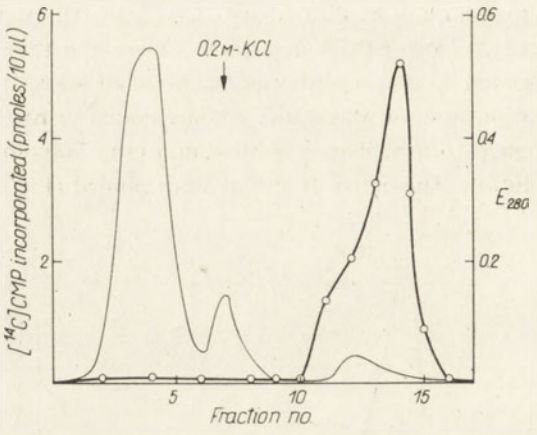

Fig. 3. Affinity chromatography of nucleotidyltransferase on tRNA-hydrazyl-Sepharose 4B. The active fractions from DEAE-cellulose were dialysed and applied to the column (8 × 50 mm). Fractions of 2 ml were collected and enzyme activity determined in 10 µl samples. ———, $E_{280\ nm}$; ○, activity.

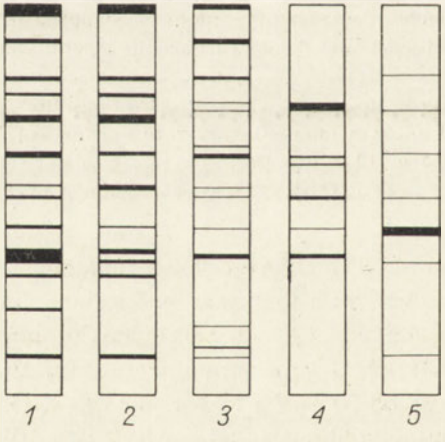

Fig. 4. Diagram of polyacrylamide-gel electrophoresis at pH 8. Acrylamide concentration 3% and 7.5% as stacking and resolving gels, respectively. The electrophoresis was conducted at 5 mA/gel, with toluidine blue as indicator. Coomassie Brilliant Blue in methanol - acetic acid - water (70 : 50 : : 880, by vol.) was used for staining. The gels were destained in a mixture of methanol, acetic acid and water. To the gels was applied 100 µg of protein: *1*, after $LaCl_3$ treatment; *2*, ppt. at 0.4 - 0.6 ammonium sulphate sat.; *3*, after Sepharose 4B; *4*, after DEAE-cellulose chromatography; and *5*, 75 µg of protein after chromatography on tRNA-hydrazyl-Sepharose 4B.

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RESULTS AND DISCUSSION

Isolation and purification of tRNA nucleotidyltransferase from wheat embryos. The course of the purification procedure is summarized in Table 1. The cytoplasmic enzyme was extracted from wheat embryos with a buffer of low ionic strength; after raising ionic strength by adding ammonium sulphate to 0.2 M concentration, the extract was sonicated to disrupt residual cell fragments and deoxyribonucleoprotein, thus lowering its viscosity. Deoxyribonucleoproteins were removed with $LaCl_3$ by the method of Yoshida & Shimura (1972), elaborated for isolation of non-histone chromosomal protein from chromatin, except that high ionic strength was not applied, treatment with $LaCl_3$ was carried out in the presence of 0.2 M-$(NH_4)_2SO_4$, and the precipitate was spun down by high-speed centrifugation. In this way about 80% of DNA present in the homogenate was removed and 5 - 6-fold purification of the enzyme was achieved. Attempts to remove DNA with protamine sulphate (Carre *et al.*, 1970; Best & Novelli, 1971a) were unsuccessful, as this treatment led to complete inactivation of the wheat embryo enzyme.

Table 1

Purification of tRNA nucleotidyltransferase

For isolation of the enzyme, 20 g of wheat embryos was used. For details see Methods. The enzyme activity is expressed as pmoles of [^{14}C]CMP incorporated/20 min, and specific activity, per 1 mg of protein.

Preparation	Total protein (mg)	Total activity	Specific activity	Purification factor
Homogenate	2520	9 576	3.8	—
After $LaCl_3$ treatment	761	16 379	21.5	5.6
Ppt. at 0.4 - 0.7 $(NH_4)_2SO_4$ sat.	164	7 239	44	11.5
Sepharose 6B	40	22 800	570	150
DEAE-cellulose	5.8	12 656	2 182	574
tRNA-hydrazyl-Sepharose 4B	0.02	540	27 000	7122

Ammonium sulphate fractionation resulted in a considerable loss of the total enzyme activity but after Sepharose 6B gel filtration a threefold increase in the total activity was observed; the specific activity was raised by a factor of 13, and the E_{280}/E_{260} ratio was 1.5. The purification of the enzyme achieved at this step was higher than that obtained by Carre *et al.* (1970) with the enzyme from *E. coli* after Sephadex G-200 chromatography.

The preparation obtained by Sepharose 6B filtration, on DEAE-cellulose column chromatography lost about a half of the total activity but was free from RNase. This preparation was further purified by affinity chromatography on tRNA-hydrazyl-Sepharose 4B.

The tRNA-hydrazyl-Sepharose 4B was found to have rather low capacity, probably due to a decrease in affinity of the nucleotidyltransferase to the tRNA

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bound with the terminal CCA sequence to the gel through hydrazyl group. That is why it was necessary to apply columns of rather large volume.

Following affinity chromatography, the overall purification of the final enzyme preparation was 7000-fold. The preparation did not show any activity of DNA--dependent RNA polymerase, RNase, polynucleotide phosphorylase, poly(C)-nucleotidyltransferase or poly(A)-nucleotidyltransferase. This preparation was used for characterization of the enzyme.

Effect of incubation time, buffer concentration and pH value on CMP incorporation. The incorporation of CMP was linear with time up to 20 min incubation (Fig. 5A), and a plateau was reached after about 60 min.

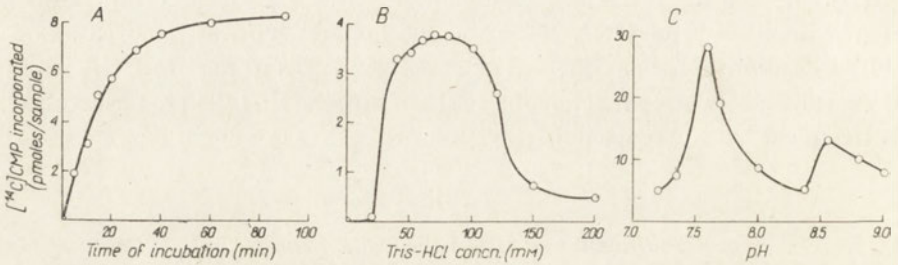

Fig.5. Effect of *A*, incubation time; *B*, buffer concentration; and *C*, pH, on CMP incorporation. The standard incubation mixture was used. *A* and *B*, Enzyme stored for 13 days; *C*, enzyme stored for 1 day.

The enzyme activity was dependent on buffer concentration (Fig. 5B); over the range 40 - 100 mM-Tris-HCl, the activity varied but slightly, with the maximum at about 80 mM.

The enzyme showed two pH optima: at 7.6 and 8.6 (Fig. 5C). At pH 7.6 the activity was twice as high as at pH 8.6, therefore all determinations were performed at pH 7.6.

The tRNA nucleotidyltransferase from wheat embryos showing two pH optima, differed in this respect from other nucleotidyltransferases which were reported to have only one optimum: at about pH 9.6 for the enzyme from bacteria (Carre *et al.*, 1970; Miller & Philipps, 1971a; Best & Novelli, 1971a) or animals (Starr & Goldhwait, 1963; Klemperer & Haynes, 1967; Deutscher, 1972a), and at pH 9.5 for the yeast enzyme (Anthony *et al.*, 1963). Nucleotidyltransferases from rat liver (Daniel & Littauer, 1963) and yeast (Morris & Herbert, 1970) at pH 7.6 acted also as pyrophosphatases.

Effect of Mg^{2+} *and* Mn^{2+} *concentration.* The requirement of the purified enzyme preparation for Mg^{2+} and Mn^{2+} was similar to that reported for tRNA nucleotidyltransferases from other sources (Miller & Philipps, 1971a; Sternbach *et al.*, 1971). The optimum concentration of Mn^{2+} was 0.7 mM , and that of Mg^{2+}, 6 mM (Fig. 6). A further increase in Mg^{2+} concentration caused a distinct drop in the enzyme activity. With the preparation obtained at an intermediate step of

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purification, i.e. after Sepharose 6B filtration, excess of Mg^{2+} had but a slight inhibitory effect.

In view of the incorporation of atypical terminal sequences in the presence of Mn^{2+} observed by Klemperer & Haynes (1967), in our experiments Mg^{2+} ion was used.

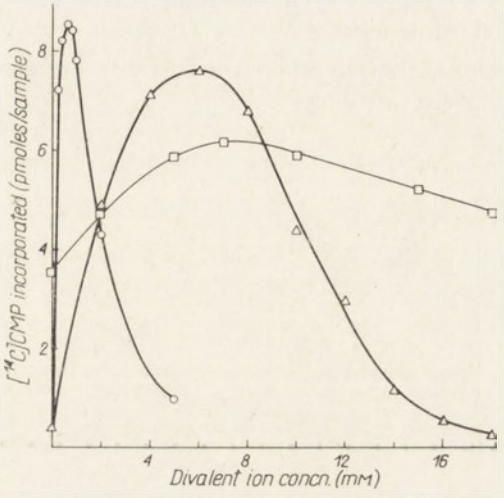

Fig. 6. Effect of Mg^{2+} and Mn^{2+} on CMP incorporation. The standard incubation mixture was used. Purified enzyme preparation, stored for 13 days, incubated with: ○, Mn^{2+} or △, Mg^{2+}; □, enzyme preparation after Sepharose 6B, with Mg^{2+}.

Effect of CTP concentration. The K_m value for CTP calculated from the Lineweaver-Burk plots was 50 - 85 μM with partly degraded lupin tRNA (cf. Fig. 8); at 20 - 30 μM-CTP concentration, an inflection point was observed on the plots, suggesting the presence of two active sites in the enzyme molecule (Deutscher, 1972b). However, as Bio-Gel A-1.5 filtration revealed the presence of aggregated form of the enzyme (unpublished experiments), it cannot be excluded that a different site is involved in the interaction of tRNA and CTP with the aggregated enzyme (Miller & Philipps, 1971b).

Table 2

Effect of components of the incubation mixture on CMP incorporation

For details see Methods.

Incubation mixture	pmoles of [14C]CMP incorporated/20 min
Complete	19.27
[14C]CTP omitted, [14C]CDP (200 μM) added	0.62
Mg^{2+} omitted, Mn^{2+} (0.7 mM) added	9.11
Complete, with Mn^{2+} (0.7 mM) added	16.96
tRNA omitted	0.36

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CDP did not replace CTP in CMP incorporation (Table 2), which is indicative of complete or almost complete absence of polynucleotide phosphorylase in the enzyme preparation.

Effect of ATP, UTP and GTP on CMP incorporation. GTP had little inhibitory effect; even at 200 μM concentration, the enzyme still retained about 80% of activity (Fig. 7). The effect of ATP and UTP was rather complex, the highest inhibition being observed at 100 μM concentration: by 73% with ATP and 84% with UTP. At higher concentrations, the inhibition considerably decreased and the activity gradually increased almost to control value.

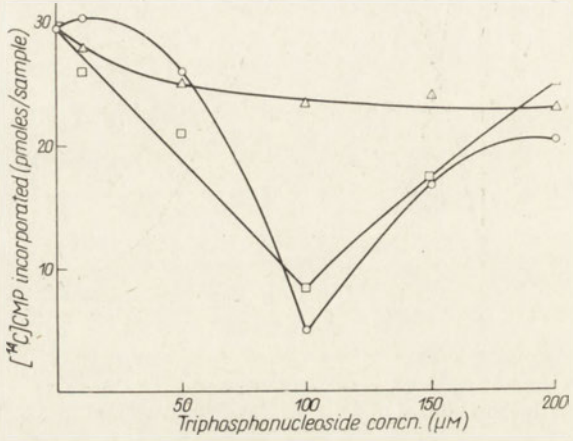

Fig. 7. Effect of: □, ATP; ○, UTP and △, GTP, on CMP incorporation. The standard incubation mixture was used; the enzyme was stored for 2 days.

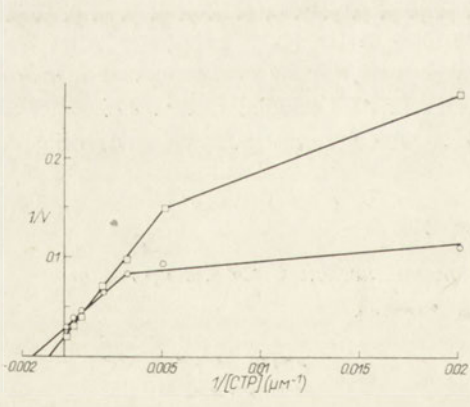

Fig. 8

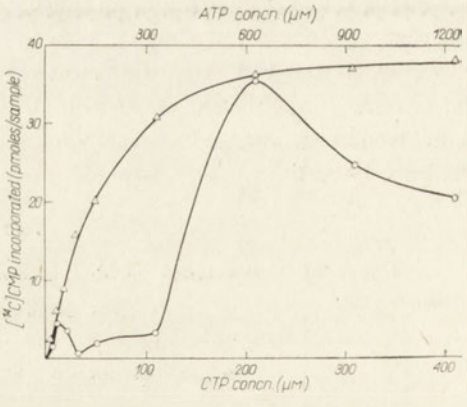

Fig. 9

Fig. 8. Double-reciprocal plots of: ○, CMP incorporation *versus* CTP concentration (10 - 400 μM), and □, in the presence of 200 μM-ATP. The enzyme was stored for 3 days.

Fig. 9. Effect of ATP on CMP incorporation at constant ATP to CTP ratio of 3 : 1. The enzyme stored for 5 days was used. △, CTP without ATP; ○, CTP+ATP.

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In view of the fact that nucleotidyltransferase catalyses the incorporation of both CMP and AMP, the effect of ATP on CMP incorporation was studied in more detail. It was calculated from the kinetic data (Fig. 8) that at a fixed ATP concentration of 200 μM, the value of K_m for CTP increased to 125 μM, indicating a decrease in enzyme affinity to CTP. The dependence of CMP incorporation on CTP concentration at a constant ATP/CTP ratio of 3:1 showed a very complex character (Fig. 9). The reaction was almost completely inhibited at 30 μM-CTP+90 μM-ATP, and no inhibition was observed at 200 μM-CTP+600 μM-ATP. These results seem to indicate that the effect of ATP is not confined to preventing the anomalous incorporation of CMP (Deutscher, 1973). Inhibition of CMP incorporation at different ATP concentrations and lack of inhibition at 600 μM-ATP at the ATP/CTP ratio of 3:1, are compatible with the regulatory role of ATP in the process of CMP incorporation into tRNA, suggested by Best & Novelli (1971b) for the *E. coli* enzyme. It is conceivable that UTP plays a role similar to that of ATP, taking into account its effect upon CTP incorporation (cf. Fig. 7).

AMP incorporation. The K_m value for ATP calculated from Lineweaver-Burk plots was 400 μM (Fig. 10), and the inhibition of AMP incorporation by CTP had a competitive character. K_i for CTP was 155 μM.

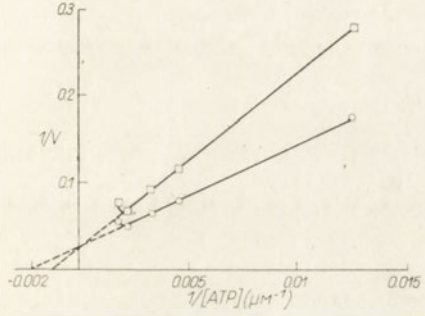

Fig. 10

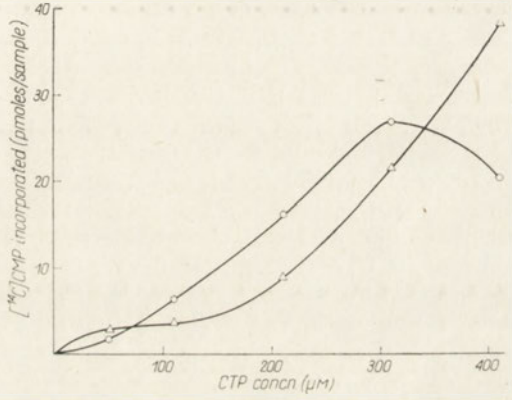

Fig. 11

Fig. 10. Double-reciprocal plot of: ○, AMP incorporation *versus* ATP concentration (50 - 500 μM), and □, in the presence of 130 μM-CTP. The enzyme was stored for 7 days.

Fig. 11. CMP incorporation into: △, tRNA-X and ○, tRNA-XC, in the presence of 200 μM--ATP, as a function of CTP concentration. The enzyme was stored for 4 days.

CMP incorporation into tRNA-X and tRNA-XC. In these experiments chemically degraded tRNA, deprived of terminal CCA (tRNA-X) or CA (tRNA-XC) was used. In the presence of 200 μM-ATP, CMP incorporation into tRNA-XC *versus* CTP concentration (Fig. 11) was different from that for tRNA-X. Incorporation of CMP into tRNA-XC was linear up to 300 μM-CTP, and at higher concentrations a certain decrease was observed. The curve for CMP incorporation into tRNA-X was sigmoid.

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It should be noted that preincubation of the enzyme with degraded lupin tRNA raised considerably its capacity as acceptor for amino acids; this points to the role of the enzyme in the repair of tRNA deprived of terminal CCA.

We are grateful to Prof. Dr. Jerzy Pawełkiewicz for helpful discussion and his precious advice. We are greatly indebted to Dr. H. Jakubowski and Mr. T. Dziegielewski for gifts of tRNA preparations and tRNA-hydrazyl-Sepharose. We thank Miss K. Przysiecka and Miss M. Skrzypek for their expert technical assistance.

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IZOLOWANIE I WŁASNOŚCI NUKLEOTYDYLOTRANSFERAZY tRNA Z ZARODKÓW PSZENICY

Przedstawiono metodę izolowania z zarodków pszenicy nukleotydylotransferazy tRNA (EC 2.7.7.25). Za pomocą chromatografii na Sepharose 6B, DEAE-celulozie i tRNA-hydrazylo-Sepharose 4B uzyskano 7000-krotne oczyszczenie enzymu.

Dla aktywności enzymu konieczne są jony Mg^{2+} lub Mn^{2+}.

ATP hamuje włączanie CMP z CTP do tRNA, a CTP jest kompetycyjnym inhibitorem inkorporacji AMP z ATP.

Rozważono niektóre sugestie dotyczące regulacyjnego oddziaływania ATP na włączanie CMP do tRNA. Badano również inkorporację CMP do tRNA pozbawionego końcowego CCA lub CA.

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INDUCTION OF LACCASE BY FERULIC ACID IN BASIDIOMYCETES

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Induction of laccase (monophenol monooxygenase, EC 1.14.18.1) by ferulic acid in *Coriolus versicolor*, *Pholiota mutabilis* and *Pleurotus ostreatus* was followed by measuring ^{64}Cu incorporation into enzymic proteins. One of the multiple forms of this enzyme was preferentially induced.

Laccase (monophenol monooxygenase, EC 1.14.18.1) is known to occur in several species of Basidiomycetes and Ascomycetes, both as an intracellular and extracellular enzyme (Ishihara & Miyazaki, 1972, 1974; Molitoris *et al.*, 1972). In *Coriolus versicolor* and *Pleurotus ostreatus*, laccase is a multiple-form enzyme induced by lignin or its phenolic monomer, ferulic acid (Leonowicz & Trojanowski, 1975). It has been shown that in *Pleurotus ostreatus* only one out of six multiple forms of laccase is inducible by ferulic acid. In the present work, our studies on the response of individual forms of laccase to this inducer have been extended to other Basidiomycetes species.

MATERIALS AND METHODS

Organisms and culture conditions. Strains: *Armillariella mellea* (Vahl. ex Fr.) P. Karst no. 14; *Coriolus versicolor* (L. ex Fr.) Quel no. 2; *Ophiostoma acoma* Naumf no. 40; *Phellinus igniarius* (L. ex Fr.) Quel no. 34; *Pholiota mutabilis* (Schaeft ex Fr.) Quel no. 1; *Piptoporus betulinus* (Bull. ex Fr.) P. Karst no. 24; *Pleurotus ostreatus* (Jacqu) Fr. no. 13; *Polyporus adustus* Fr. no. 11; *Polyporus sulphureus* (Bull. ex Fr.) no. 8; *Polystictus abietinus* (Diks ex Fr.) Cooke no. 10; *Poria crustulina* Bres. no. 22; *Tricholoma flavovirens* (Pers ex Fr.) Lund no. 32, and *Tricholoma potentosum* (Fr.) Quel no. 33, were obtained from the Department of Biochemistry, University of Lublin. All the organisms were kept on agar slants as described by Trojanowski & Leonowicz (1969) and were cultured in 20-litre bottles in the medium containing: glucose, 10 g; asparagine, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 0.45 g; $Na_2HPO_4 \cdot 12H_2O$, 0.47 g; Ca, 20 μg; Mn, 2.7 μg; Fe, Zn, Cu, 1 μg each; aneurine, 50 μg;

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distilled water to 1000 ml (Leonowicz & Trojanowski, 1975). Mycelium was harvested and washed three times with copper-free, low-glucose (2 g/litre) mineral medium. Portions of 100 g of mycelium were then transferred into 500 ml flat-bottomed flasks containing 100 ml of the same medium and 1 mCi of ^{64}Cu. In some experiments, ferulic acid (10^4 M) and actidione (5 μg/ml) were added. The cultures were incubated at 27°C for two or six hours, each flask being aerated with 10 litres of sterile air per hour.

Isolation of laccase. The mycelium was washed with cold 0.1 M-phosphate buffer, pH 6.0, suspended in 0.5 vol. of the same buffer and homogenized in a meat-grinder. The contaminating proteins were removed by centrifugation for 10 min at 20 000 *g* and subsequently for 90 min at 105 000 *g*. The supernatant obtained was saturated with ammonium sulphate and the procedure of Fahraeus & Reinhammar (1967) was followed up to the Sephadex G-25 chromatography. The laccase-rich fraction was concentrated by precipitation with ammonium sulphate, dissolved in a small volume of 0.1 M-phosphate buffer, pH 6.0, dialysed against 0.01 M-phosphate buffer, pH 6.0 (3 × 45 min), and clarified by centrifugation for 10 min at 60 000 *g*. The activity of the preparation obtained was not decreased when CO was passed through the incubation mixture for 1 h; this excludes the presence of catechol oxidase (James, 1953).

Determination of laccase activity. The enzyme activity was measured colorimetrically with *p*-phenylenediamine as described previously (Trojanowski & Leonowicz, 1969). Carbon oxide was obtained from concentrated sulphuric acid and 85% formic acid. In the determinations with the use of carbon oxide, the reaction mixture containing the enzyme was, prior to the addition of *p*-phenylenediamine, saturated with CO by passing a stream of CO (1000 ml/10 ml of enzyme for 1 h with shaking).

Polyacrylamide-gel electrophoresis. About 20 μl of a preparation containing 50 μg of protein and 2 μl of 0.1% fluoresceine was applied onto column, and the electrophoresis was carried out according to the method described previously (Leonowicz & Trojanowski, 1975).

Radioactivity determination. Radioactivity of the fraction incubated with ^{64}Cu and not subjected to electrophoresis, was measured in Isocap 300 (Nuclear, Chicago) counter; the sample contained 0.5 ml of the incubated solution, 5 ml of alcohol and 8 ml of the toluene scintillation fluid, as described by Leonowicz *et al.* (1972). In electrophoretic gels, the radioactivity was measured with the same instrument, by the method described previously (Leonowicz & Trojanowski, 1975).

Molecular weight determination. The calculation of the molecular weight of the laccase forms was carried out by means of the cationic detergent polyacrylamide-gel electrophoresis, according to Marjanen & Ryrie (1974).

Reagents. Ferulic acid and the reagents used for electrophoresis were the same as in the previous work (Leonowicz, 1974). Actidione (cycloheximide) was from Serva (Heidelberg, West Germany), $^{64}CuCl_2$ (2 mCi/mg Cu) was purchased from the Institute for Nuclear Research (Świerk, Poland).

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RESULTS AND DISCUSSION

Out of thirteen species of Basidiomycetes examined, only *Coriolus versicolor*, *Pleurotus ostreatus* and *Pholiota mutabilis* showed a distinct increase in laccase activity on addition of ferulic acid (10^{-4} M). Under the same experimental conditions, this enzymic activity remained unaltered in the following fungal species: *Armillariella mellea*, *Ophiostoma acoma*, *Phellinus igniarius*, *Piptoporus betulinus*, *Polyporus adustus*, *Polyporus sulphureus*, *Polystictus abietinus*, *Poria crustulina*, *Tricholoma flavovirens* and *Tricholoma potentosum*.

Our previous results (Leonowicz & Trojanowski, 1975) suggested that synthesis of multiple forms of laccase in *Pleurotus ostreatus* is not coordinated. This observation has now been confirmed with *Coriolus versicolor* and *Pholiota mutabilis*. The results of gel electrophoresis of laccase from mycelia incubated for 2 h with ^{64}Cu and ferulic acid (Fig. 1) show that ^{64}Cu was consistently incorporated only into one form of laccase, irrespective of the number of those forms. Prolongation of the time of incubation to 6 h had no effect on the obtained electrophoretic patterns. The inducible form of laccase from *Pleurotus ostreatus* and *Coriolus versicolor* migrated on the gel faster, and that from *Pholiota mutabilis* slower, than the other forms.

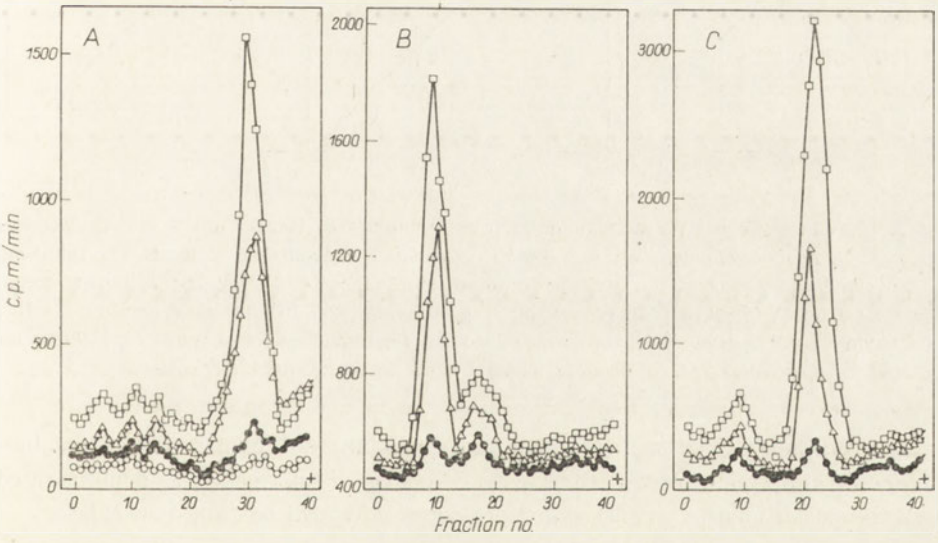

Fig. 1. Polyacrylamide-gel electrophoresis of laccase forms in *A*, *Pleurotus ostreatus*; *B*, *Pholiota mutabilis* and *C*, *Coriolus versicolor*. ^{64}Cu radioactivity was determined in the protein bands separated following 2 h incubation: without ferulic acid (●), with ferulic acid (▲), with ferulic acid and actidione (○), and following 6 h incubation with ferulic acid (□). For details see Methods.

One may assume that lack of the response of laccase to ferulic acid in the other Basidiomycetes examined, might be due to the absence of an inducible form of the enzyme in these species.

Preferential induction of a single form of an enzyme has been reported for several fungal enzymes, e.g. alcohol dehydrogenase in *Saccharomyces cheresiensis* (Fernandez

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et al., 1972), phosphodiesterase in *Physarum polycephalum* (Huttermann, 1972), and catalase and glucose-6-phosphate dehydrogenase in wheat leaves infected with stem rust (Cheung & Willetts, 1973).

To get more information on the nature of multiple forms of laccase in fungi, the molecular weight of particular enzymic proteins was determined by polyacrylamide-gel electrophoresis. The following values were obtained (Fig. 2): in *Coriolus versicolor* 6.1 and 7.1×10^4, in *Pholiota mutabilis* 6.4 and 7.2×10^4, and in *Pleurotus ostreatus* 4.7, 5.5, 5.8, 6.6, 7.2 and 7.6×10^4. The molecular weight of the inducible forms was, respectively, 6.1, 7.2 and 4.7, which implies that inducibility is not related to the molecular weight of the laccase forms. The results for *Coriolus versicolor* are in agreement with those of Konishi & Inoue (1974). The data for *Pleurotus ostreatus* show that two of the six laccase forms have practically the same molecular weight as those of *Coriolus versicolor* and *Pholiota mutabilis*.

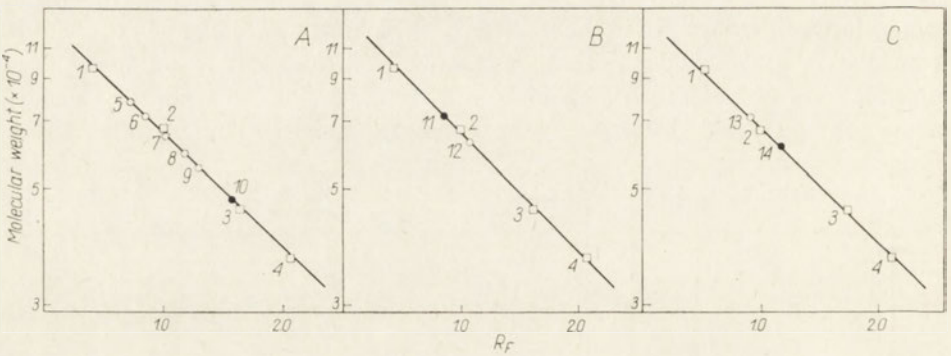

Fig. 2. Cationic detergent polyacrylamide-gel electrophoresis of laccase forms in: *A*, *Pleurotus ostreatus*; *B*, *Pholiota mutabilis* and *C*, *Coriolus versicolor*. For details see Methods. The following protein standards were used: *1*, yeast hexokinase (mol. wt. 9.6×10^4, Sigma); *2*, bovine serum albumin, fraction V (6.6×10^4, Miles-Seravac); *3*, egg albumin (4.5×10^4, Serva); *4*, pepsin (3.7×10^4, Int. Enzymes Ltd); *5* through *14*, the forms of laccase. The inducible forms were: *10*, in *Pleurotus ostreatus*; *11*, in *Pholiota mutabilis* and *14*, in *Coriolus versicolor*.

The results obtained so far in this and other laboratories may imply that biosynthesis of multiple forms of enzymes is coded by distinct genes, as demonstrated by Kobrehel & Gautier (1974) with isoenzymes of wheat seedling peroxidase.

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INDUKCJA LAKAZY KWASEM FERULOWYM U BASIDIOMYCETES

Streszczenie

Zbadano indukcję lakazy (EC 1.14.18.1) przez kwas ferulowy u *Coriolus versicolor*, *Pholiota mutabilis* i *Pleurotus ostreatus*, mierząc włączanie ^{64}Cu do białek enzymu; stwierdzono, że tylko jedna z kilku form tego enzymu ulegała wybiórczej indukcji.

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N-ACETYLMURAMYL-L-ALANINE AMIDASE OF Vi PHAGE III*

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1. A lytic enzyme was isolated from Vi phage III-induced lysate of *Salmonella typhi*, and purified about 200-fold by chromatography on IRC-50, CM-cellulose, and Sephadex G-75 columns.

2. Both *E. coli* B murein and muropeptide C6 were digested on incubation with the lytic enzyme. The main product of murein and muropeptide C6 digestion is identical with tetrapeptide Ala-Glu-DAP-Ala. The release of amino groups during digestion was not accompanied by the appearance of either reducing groups or hexosamines.

3. It is concluded that Vi phage III-induced lytic enzyme is *N*-acetylmuramyl-L-alanine amidase, which cleaves the amide bond between *N*-acetylmuramic acid and L-alanine.

Several bacteriophages are known to produce lytic enzymes which digest the host cell wall. Some of these enzymes were purified and characterized as muramidase (Mass & Weidel, 1963; Tsugita *et al.*, 1968; Eichholtz *et al.*, 1975), endopeptidase (Welker, 1967; Brehm & Welker, 1974), and amidase (Hongo *et al.*, 1974; Ogata *et al.*, 1974).

Escherichia coli or *Salmonella typhi* infected with phage λ or Vi phage II, respectively, produce an endolysin which shows the activity of: (1), endopeptidase, which cleaves the cross-linking bond of the *E. coli* B murein between the carboxyl group of D-alanine and the amino group of diaminopimelic acid (Taylor, 1970, 1971), and (2), 1,6-anhydro-*N*-acetylmuramidase, which splits the β 1 - 4 glucosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine with the concomitant dehydration; the resulting fragments contain 1,6-anhydro-*N*-acetylmuramic acid (Taylor *et al.*, 1975).

Vi phages II and III of *S. typhi* are morphologically distinct (Ackerman *et al.*, 1970) but the molecular mechanism of their primary attachment to *S. typhi* cells is the same. It consists in formation of a complex between the phage-bound Vi-polysaccharide deacetylase and Vi-polysaccharide, the latter being the bacterial

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receptor (Szczeklik *et al.*, 1974). Thus, it would be of interest to know whether the mechanism of lysis of the host cells by these two phages is also similar, i.e. to examine whether the phage Vi III lytic enzyme is identical with Vi phage II endolysin.

In the present paper we have proved that the Vi phage III lytic enzyme is an *N*-acetylmuramyl-L-alanine amidase. Specificity of its action has been determined with the 200-fold purified enzyme preparation.

MATERIALS AND METHODS

Bacteria and bacteriophage. Salmonella typhi 21802 Vi phage type A and Vi phage III were obtained from the National Center in Gdańsk, Poland.

Media. The mSB medium of Stokes & Bayne (1958) was composed of solution A (20 g of glucose in 400 ml of water), and solution B (2 g of $(NH_4)_2SO_4$, 1 g of trisodium citrate·$2H_2O$, 1 g of $MgSO_4 \cdot 7H_2O$, 25 g of $Na_2HPO_4 \cdot 12H_2O$, 4 g of KH_2PO_4, and 20 g of lactalbumin hydrolysate Difco in 1600 ml of water). The two solutions were autoclaved separately and mixed after cooling. The pH of the broth was adjusted to 7.4 with NaOH. M-9 medium (Adams, 1959) contained in 1 litre: NH_4Cl, 1 g; KH_2PO_4, 3 g; Na_2HPO_4, 6 g; glucose, 4 g, and $MgSO_4$, 0.13 g. The last two compounds were autoclaved separately and combined with the other components afterwards.

Special chemicals. Egg white lysozyme 3 × cryst. (Sigma Chemical Co., St. Louis, Mo., U.S.A.); distreptase (Warszawska Wytwórnia Surowic i Szczepionek, Warszawa, Poland); Visking tubing, Amberlite IRC-50 and CM-cellulose (Serva, Heidelberg, G.F.R.); MN-300 cellulose (Macherey & Negel, Düren, G.F.R.); Sephadex G-75 (Pharmacia, Uppsala, Sweden); Tris (Loba-Chimie, Wien, Austria). Muropeptide C6 was isolated from the lysozyme digest of *E. coli* B murein (Taylor *et al.*, 1969), as described by Primosigh *et al.* (1961) by preparative descending chromatography on Whatman no. 1 paper in the upper phase of *n*-butanol - acetic acid - water (4:1:5, by vol.) for 96 h. The substance was eluted, concentrated and further purified by electrophoresis on Whatman 3*MM* paper at 18 V/cm for 1.5 h in 2% formic acid, pH 2.0. The main fraction representing muropeptide C6 was eluted and dried in a desiccator. Muropeptide CA, an analogue of C6 containing 1,6-anhydro-*N*-acetylmuramic acid, was a gift from Dr A. Taylor. Murein from *E. coli* B was prepared as described by Taylor *et al.* (1969).

Analytical methods. Protein was determined by the method of Lowry *et al.* (1951) using egg white lysozyme as a standard. The reducing groups, amino groups and total hexosamines were determined according to Ghuysen *et al.* (1966).

Preparation of the cell walls. E. coli B cells growing to $OD_{575} = 0.5$ in M-9 medium were collected by centrifugation and washed twice with 50 mM-Tris-HCl buffer, pH 7.4. The cells were then resuspended in approximately the same volume of chloroform-saturated 50 mM-Tris-HCl buffer, pH 7.4, stirred at 37°C for 1 h, washed three times with water and lyophilized.

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Lytic activity assay. For the enzyme assay, the cell wall preparation was suspended in 50 mM-Tris-HCl buffer, pH 7.4, to obtain the solution showing OD_{470} equal to 0.300; 2 ml of this substrate was mixed with 50 µl of enzyme solution. The reaction was carried out for 10 min in a water-bath at 37°C. Transmittance was read in 1 cm cuvettes against substrate suspensions with 50 µl of buffer added. A standard curve was prepared with 1 - 10 µg/ml solutions of egg white lysozyme, one unit corresponding to the activity of 100 ng of egg white lysozyme.

RESULTS

Enzyme purification. The initial steps of enzyme purification were performed by the procedure of Tsugita *et al.* (1968), as follows: Vi phage III was grown on *S. typhi* in aerated mSB medium until complete lysis of bacteria. The clarified lysate (2 litres) was treated with distreptase (2 mg), and after 2 h incubation at 25°C left overnight at 4°C. All the subsequent operations were carried out at 4°C. To the solution, 150 ml of 0.25% rivanol (6,9-diamino-2-etoxyacridine lactate) was added and the mixture was dialysed in Visking tubing overnight against 5 vol. of water with rivanol (final concentration 0.02%). The supernatant was passed through an Amberlite IRC-50 (100 - 200 mesh) column (17 × 70 mm) equilibrated with 50 mM-phosphate buffer, pH 5.8. The column was washed with 250 ml of 10 mM-Tris-HCl buffer, pH 7.4, and the phage enzyme was eluted with the same buffer supplemented with NaCl to 0.5 M concentration. Pooled active fractions were dialysed against 1 mM--Tris-HCl buffer, pH 7.4, and concentrated by evaporation in vacuum at 0°C.

The enzyme was then passed through a CM-cellulose column (17 × 60 mm) equilibrated with 0.2 M-Tris-HCl buffer, pH 8.6 (Ghuysen *et al.*, 1969). The non--adsorbed material containing lytic activity was washed out with water. The active fractions were pooled and concentrated about fourfold by evaporation in vacuum at 0°C, and were applied to a Sephadex G-75 column (15 × 900 mm) equilibrated with 1 mM-Tris-HCl buffer, pH 7.4. The activity was eluted with the same buffer, and the pooled fractions were concentrated, as above, to a final volume of 4 - 5 ml.

This procedure, summarized in Table 1, resulted in a 200-fold purification. The bacteriolytic activity of the purified enzyme per 1 mg protein was about one--fifth that of egg white lysozyme.

Table 1

Purification procedure of the Vi phage III bacteriolytic enzyme

Preparation	Total volume (ml)	Total activity (units)	Total protein (mg)	Spec. act. (units/mg)	Purification factor	Recovery (%)
Lysate	2000	25 000	2700	9.3	1	100
After rivanol treatment	2150	22 000	1560	14.1	1	88
After IRC-50 column	180	3 400	73	46.6	5	14
After CM-cellulose column	250	3 600	5	720	80	14
After Sephadex G-75 column	40	3 750	2	1875	208	15

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Digestion of E. coli *B murein.* Enzyme solution (3000 units), dialysed against 1 mM-ammonium acetate buffer, pH 7.4, was incubated with *E. coli* B murein (25 mg), suspended in 1 mM-ammonium acetate buffer, pH 7.4 (5 ml) and the digestion was carried out with stirring at 37°C in Visking tubing immersed in 20 ml of the same buffer containing chloroform. At fixed time intervals, 1 ml samples of the diffusate were analysed. On 15 h incubation, the murein was split into soluble fragments with about 70% yield. The appearance of diffusible products was parallelled by the release of amino groups, but neither reducing groups nor hexosamines were detected (Fig. 1).

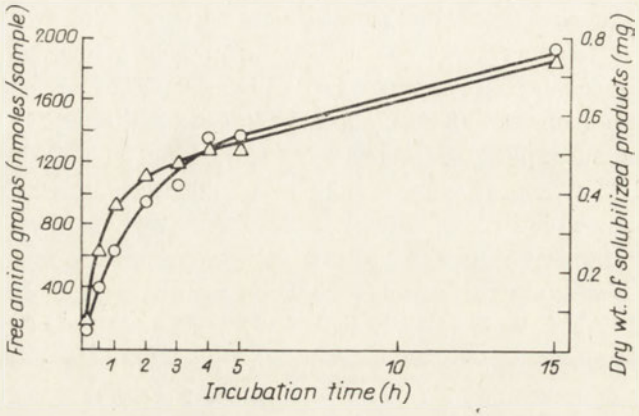

Fig. 1. Action of Vi phage III bacteriolytic enzyme on *Escherichia coli* B murein. Dry weight of solubilized products (○); amino groups calculated as alanine (△).

Chromatography and electrophoresis of the murein digestion products. The products of murein digestion by the Vi phage III lytic enzyme were compared with the products resulting from the action of egg white lysozyme and Vi phage II enzyme. The reaction mixture was composed of 200 µg of lyophilized murein suspended in 0.5 ml of a suitable buffer and 50 µl of the enzyme investigated (20 units). Digestion by egg white lysozyme or Vi phage II enzyme was carried out in 100 mM-ammonium acetate buffer, pH 6.5. For the digestion with Vi phage III lytic enzyme, 1 mM-ammonium acetate buffer, pH 7.4, was used. The reaction was carried out at 37°C for 15 h. The resultant slight sediment was removed and the supernatant was dried in vacuum over NaOH to remove the ammonium acetate. The digestion products were resuspended in 10% isopropanol and chromatographed by the descending technique on Whatman no. 1 paper, with *n*-butanol - acetic acid - water (4:1:5, upper phase) as solvent system. After 96 h at room temperature, the chromatogram was sprayed with 0.2% ninhydrin in acetone. As it can be seen from Fig. 2, the mobility of the main product of murein digestion with Vi phage III lytic enzyme was the same as that of the tetrapeptide: Ala-Glu-DAP-Ala, and different from that of the products resulting from the action of egg white lysozyme or Vi phage II endolysin on murein.

The identity of the murein peptide split off by Vi phage III enzyme with Ala--Glu-DAP-Ala was confirmed by thin-layer chromatography and electrophoresis.

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The murein peptide obtained under the same conditions by preparative chromatography on Whatman 3 *MM* paper, was applied to MN-300 cellulose plates (20 × 5 cm) and developed with *n*-amyl alcohol - isobutanol - *n*-propanol - pyridine - water (5:5:5:15:15, by vol.) as a solvent system at 30°C (Gerday *et al.*, 1968), (Fig. 3). Electrophoresis of this preparation was carried out in three buffer systems at pH 2.0, 4.0 and 6.5, on MN-300 cellulose plates (20 × 5 cm) at 18 V/cm for 1 h.

Fig. 2

Fig. 3

Fig. 2. Chromatography of the digestion products of *E. coli* B murein with egg white lysozyme and with the lytic enzymes of Vi phages II and III. Descending chromatography on Whatman no. 1 paper was used, with *n*-butanol - acetic acid - water (4 : 1 : 5, upper phase) as solvent system. *1*, Muropeptide C6; *2*, murein digest with lysozyme; *3*, Ala-Glu-DAP-Ala; *4*, murein digest with Vi phage III lytic enzyme; *5*, muropeptide CA; *6*, murein digest with Vi phage II lytic enzyme.

Fig. 3. Thin-layer chromatography of the murein peptide split off by the Vi phage III enzyme. MN-300 cellulose plates and *n*-amyl alcohol - isobutanol - *n*-propanol - pyridine - water (5:5:5:15:15, by vol.) were used. *1*, Muropeptide CA; *2*, muropeptide C6; and *3*, Ala-Glu-DAP-Ala were applied as the reference peptides; *4*, the murein peptide isolated on Whatman 3*MM* paper on a preparative scale.

In addition, the amino acid components of the murein peptide were identified. The peptide was hydrolysed in 4 M-HCl at 105°C for 14 h and the amino acids were separated by t.l.c. on MN-300 cellulose plate in *n*-butanol - pyridine - acetic acid - water (60:40:3:30, by vol.), (Primosigh *et al.*, 1961). In the hydrolysate, only three components: Ala, Glu and DAP, were detected (Fig. 4).

These results suggest that the Vi phage III lytic enzyme splits the amide bond between *N*-acetylmuramic acid and L-alanine in *E. coli* B murein.

The presence of terminal L-alanine in the murein peptide was proved by t.l.c. of the acid hydrolysate of the DNP-derivative of this peptide. DNP-alanine was identified in the ether fraction of the hydrolysate by chromatography on MN-300 cellulose plates with 1.5 M-phosphate buffer, pH 6.0 (Devenyi & Gergely, 1968) at 30°C.

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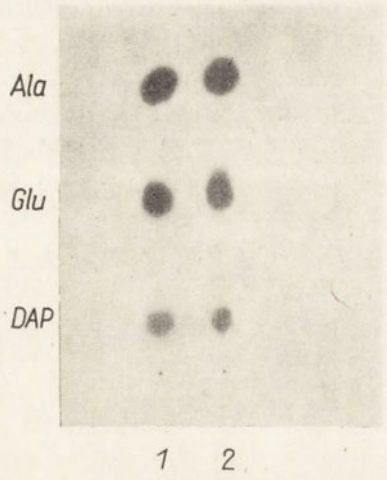

Fig. 4

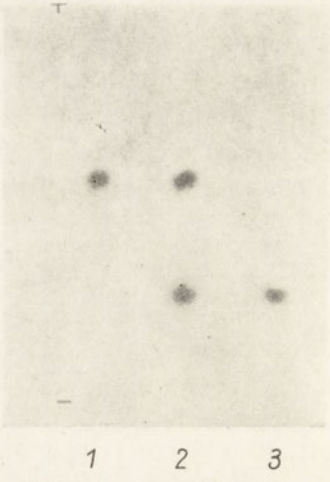

Fig. 5

Fig. 4. Thin-layer chromatography of the acid hydrolysate of *E. coli* B murein peptide. The peptide was isolated on Whatman 3*MM* paper from the incubation mixture of murein and the Vi phage III enzyme. MN-cellulose plate was developed with *n*-butanol - pyridine - acetic acid - water (60:40:3:30, by vol.). *1*, Reference amino acids; *2*, components of the peptide hydrolysate.

Fig. 5. Electrophoresis of muropeptide C6 digested by Vi phage III lytic enzyme. The electrophoresis was performed on MN-300 cellulose plate in 2% formic acid. *1*, Muropeptide C6; *2*, digestion product of muropeptide C6; *3*, Ala-Glu-DAP-Ala.

Digestion of muropeptide C6. Muropeptide C6 (100 μg) suspended in 1 mM-ammonium acetate buffer, pH 7.4, was incubated with 50 μl of the Vi phage III enzyme (20 units). The reaction was carried out for 15 h at 37°C. The sample was dried in vacuum over NaOH and resuspended in 10% isopropanol; 20 μg of the product was subjected to electrophoresis on MN-300 cellulose plate (20 × 5 cm) in 2% formic acid, pH 2.0, at 18 V/cm for 1 h. The electrophoretic pattern shown in Fig. 5 proves that the reaction product was identical with Ala-Glu-DAP-Ala.

DISCUSSION

The present paper shows that the Vi phage III lytic enzyme is an *N*-acetylmuramyl-L-alanine amidase.

The occurrence in bacteria of *N*-acetylmuramyl-L-alanine amidases hydrolysing the *N*-acetylmuramyl-L-alanine amide bond in bacterial murein, has been reported (Ensign & Wolfe, 1966; Ghuysen, 1968; Takebe *et al.*, 1970). It is believed that they are autolytic enzymes (Tipper, 1969; Csuzi, 1970, 1971) and probably participate in the biosynthesis of murein (Costerton *et al.*, 1974).

The amidases differ in substrate specificity. The amidase from *E. coli* B (Van Heijenoort & Van Heijenoort, 1971) acts only on low-molecular-weight fragments of the murein, and shows narrow specificity towards *N*-acetylmuramyl-tetrapeptide; NAc-Glu-NAcMur-tetrapeptide is not a substrate for this enzyme. The other amidases

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digest the intact murein of different bacteria (Ensign & Wolfe, 1966; Ghuysen, 1968; Ghuysen *et al.*, 1969).

Inouye *et al.* (1973) are of the opinion that the T7 amidase is a lytic enzyme. They correlate this activity with gene 3.5 product and suggest, in agreement with Studier (1972), that the enzyme is also associated with T7 DNA synthesis. Contrary to this, Jensen & Pryme (1974) suggest that *N*-acetylmuramyl-L-alanine amidase from T7 phage is not a lytic enzyme, since this enzyme did not lyse the chloroform-killed *E. coli*. However, it has been proved that T7 lysate contained, in addition to amidase, another, not yet identified, bacteriolytic activity.

For Vi phage III *N*-acetylmuramyl-L-alanine amidase the murein of *E. coli* B, similar to *S. typhi* murein (Taylor & Stępień, 1971), as well as muropeptide C6 — the essential subunit of murein — serve as substrates. Therefore it seems possible to assume the bacteriolytic nature of this enzyme.

It appears also that *S. typhi* infected with Vi phage II or Vi phage III produce different lytic enzymes, and one may speculate that these enzymes are phage-coded.

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AMIDAZA *N*-ACETYLOMURAMYLO-L-ALANINOWA FAGA Vi III

Streszczenie

1. Z lizatów *Salmonella typhi* zakażonych fagiem Vi III izolowano enzym lityczny i oczyszczono go 200-krotnie chromatografią na kolumnach IRC-50, CM-celulozy i Sephadex G-75.

2. Zarówno mureina *E. coli* B jak i muropeptyd C6 ulegały rozszczepieniu w wyniku inkubacji z enzymem. Główny produkt rozszczepienia mureiny był identyczny z czteropeptydem Ala-Glu-DaP-Ala. Rozszczepieniu mureiny towarzyszyło uwalnianie grup aminowych, nie obserwowano natomiast uwalniania grup redukujących i wolnych aminocukrów.

3. Otrzymane wyniki wskazują, że enzym lityczny faga Vi III jest amidazą *N*-acetylomuramylo-L-alaninową katalizującą rozszczepianie wiązania amidowego w mureinie między kwasem *N*-acetylomuraminowym i L-alaniną.

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THE LONG-CHAIN ACYL-CoA HYDROLASE ACTIVITY IN THE HEART OF RAT FED ON RAPE-SEED OIL

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1. Fat feeding (soybean oil or erucic acid-rich rape-seed oil) enhance after 2 to 7 days the palmitoyl-CoA hydrolase activity in the heart of weanling rats in a degree dependent on the content of fat in the diet.

2. The rise in enzyme activity between the 7th and 14th day of feeding, observed only in rats fed on rape-seed oil, coincides with the decrease in lipid infiltration in the heart.

3. The obtained results suggest that palmitoyl-CoA hydrolase may control in the heart the amount of acyl-CoA thioesters in the cell, thus decreasing the lipidosis induced by erucic acid.

Palmitoyl-CoA hydrolase (EC 3.1.2.2) which catalyses the hydrolysis of long-chain acyl-CoA thioesters to CoA and fatty acids, is widely distributed in animal tissues, and is localized mainly in the microsomal fraction (Porter & Long, 1958; Srere *et al.*, 1959; Lands & Hart, 1965; Barnes & Wakil, 1968; Barden & Cleland, 1969; Jezyk & Hughens, 1971; Kurooka *et al.*, 1972).

So far, the physiological role of palmitoyl-CoA hydrolase has not been elucidated. According to Porter & Long (1958) and Barnes & Wakil (1968) this enzyme, as a component of the fatty acids synthetase complex, may control the chain length of the synthesized fatty acids. The increase of palmitoyl-CoA hydrolase activity in liver and kidney of alloxan diabetic rats suggested that the enzyme may be involved also in the control of metabolism of extramitochondrial long-chain acyl-CoA thioesters (Kurooka *et al.*, 1971).

The amount of acyl-CoA thioesters in tissues varies in different nutritional states in the same way as the amount of acylcarnitines (Bøhmer *et al.*, 1966). It has been demonstrated by Bułhak-Jachymczyk & Hübner-Woźniak (1974) that the presence of erucic acid in the dietary fat raises the amount of long-chain acylcarnitines in rat heart, particularly during physical effort, i.e. under conditions of increased energy requirement. This increased content of acylcarnitines may point to inefficient transport of fatty acids into the mitochondrion, the site of their oxidation. Under

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these conditions, the accumulating acyl-CoA thioesters are utilized for triglyceride synthesis, which would explain the lipidosis, which is known to be induced by dietary erucic acid.

It has been reported that fatty infiltration in the heart of young rats fed on erucic acid-rich rape-seed oil is the highest after 3 to 7 days and decreases gradually on prolonged feeding (Abdellatif & Vles, 1970; Houtsmuller *et al.*, 1970; Rocquelin *et al.*, 1973). As it seemed probable that the decrease of lipid content of the heart of these animals could be dependent, at least partly, on the action of the long-chain acyl-CoA hydrolase, the activity of this enzyme in the heart of rats fed on rape-seed oil was studied in relation to the degree and changes in cardiac lipidosis.

MATERIAL AND METHODS

Animals and diets. Male weanling rats of the Wistar strain, aged 25 days, weighing about 50 g, were used for the experiment. On the day of weaning, the animals were divided into five groups of 64 animals each. One group was maintained on a low-fat (8 cal%) laboratory food (Murigran, Gorzów, Poland), the other four groups, on high-fat experimental diets. In all groups, food and water were supplied *ad libitum*.

The experimental diets contained: 23 cal% caseine, 47 cal% or 27 cal% sucrose and, respectively, 30 cal% and 50 cal% fat: soybean oil (SO-30 and SO-50 diets) or rape-seed oil (RO-30 and RO-50 diets). In the rape-seed oil, erucic acid constituted 52% of total fatty acids.

After 2, 7, 14 and 28 days, 16 animals from each group were killed by decapitation, the heart was isolated, washed with cold 0.9% NaCl solution, and frozen in liquid nitrogen.

Determination of palmitoyl-CoA hydrolase activity. The enzyme activity was assayed according to Kurooka *et al.* (1971). A part of heart muscle was thawed, homogenized in 50 mM-potassium phosphate buffer, pH 7.0, centrifuged, and the postmitochondrial fraction (16 000 *g*) was heated for 3 min at 55°C; after cooling, the fraction was centrifuged at 16 000 *g* and the enzyme activity assayed in the supernatant with palmitoyl-CoA as substrate. The reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) by the thiol group of released CoA was recorded at 412 nm in a Unicam model SP 700 spectrophotometer. One unit of the enzyme was defined as the activity which hydrolyses 1 μmole of palmitoyl-CoA per minute at 38°C under conditions decribed by Kurooka *et al.* (1971).

Determination of total lipids. From the remaining part of the heart muscle total lipids were extracted as described by Folch *et al.* (1957). The estimations of lipids were performed according to Ilinow (1967), and lipid content expressed in milligrams of palmitic acid per 1 g of fresh tissue.

Reagents. The palmitoyl-CoA thioester was prepared by the method of Seubert (1960). Coenzyme A (free acid), 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) and Tris were purchased from Sigma Chem. Co. (St. Louis, Mo., U.S.A.); other reagents were of analytical grade, from POCh (Gliwice, Poland).

Statistical analysis was performed by Student's *t* test.

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RESULTS AND DISCUSSION

The activity of palmitoyl-CoA hydrolase in the heart of rats which, after weaning, were fed on different diets, is presented in Table 1. In rats kept on the control laboratory diet, the enzyme activity on the second day after weaning amounted to about 0.8 unit per 1 g tissue weight, then decreased somewhat and remained practically constant till the 28th day.

Table 1

Activity of palmitoyl-CoA hydrolase in the heart of rats fed on normal laboratory diet and experimental diets

The activity is expressed as μmoles of palmitoyl-CoA hydrolysed/min/g fresh heart tissue. Mean values ±S.D. are given from 8 separate estimations (each on tissue samples from 2 animals).

Diet	Time of feeding (days)			
	2	7	14	28
Normal	0.81 ±0.12	0.65 ±0.08	0.70 ±0.10	0.67 ±0.08
Soybean oil				
SO-30	0.89 ±0.12	1.05* ±0.16	0.91* ±0.13	0.81* ±0.08
SO-50	0.95 ±0.09	1.22* ±0.08	1.21* ±0.12	1.12* ±0.09
Rape-seed oil				
RO-30	0.95 ±0.11	1.06* ±0.13	1.23* ±0.20	1.25* ±0.18
RO-50	1.01* ±0.09	1.20* ±0.15	1.52* ±0.35	1.58* ±0.12

* Difference statistically significant ($P=0.01$) in relation to the results for the control group on the same day.

In animals fed with high-fat diets the enzyme activity was increased and the differences, as compared with the respective controls, were statistically significant for all groups after 7, 14 and 28 days, whereas after 2 days only for the group fed on RO-50 diet.

The increment in enzyme activity in relation to the activity of the control group on the same day, is shown in Fig. 1. In all experimental groups the activity was higher on the 7th than on the 2nd day. On the 7th day, the enzyme activity was the same in the two groups fed on diets containing 50 cal% of fat (SO-50 and RO-50) and lower, but again the same in the two groups kept on 30 cal% of fat (SO-30 and RO-30).

On more prolonged feeding with rape-seed oil, the enzyme activity increased between the 7th and 14th day, the increase being especially pronounced in group RO-50.

In groups fed on soybean oil, after the 7th day the activity was lowered; for the group SO-50 the observed slight decrease was not statistically significant.

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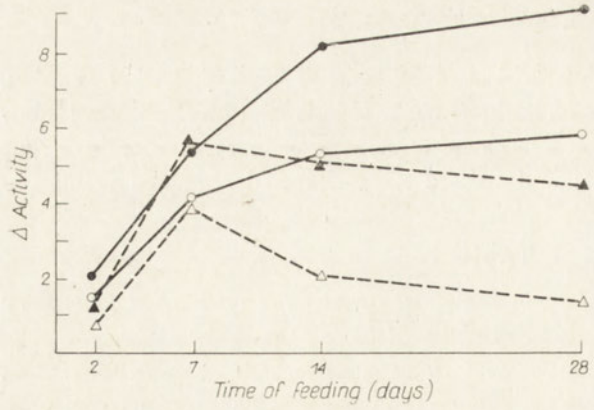

Fig. 1. Changes in palmitoyl--CoA hydrolase activity in the heart of rats fed on diets: △, SO-30; ▲, SO-50; ○, RO-30; ●, RO-50. The results are expressed as the increment in the activity (10^{-1} × units/g fresh tissue) over the respective control values.

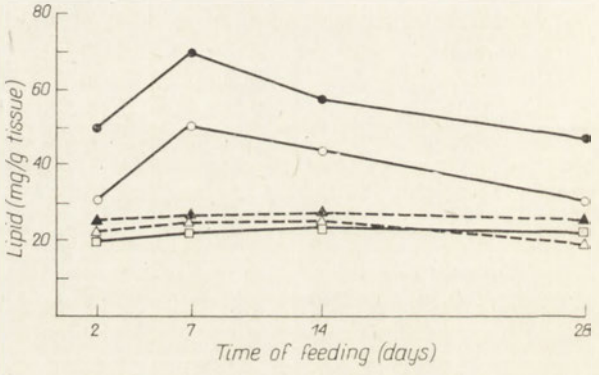

Fig. 2. Total lipid content in the heart of rats fed on diets: □, normal (control); △, SO-30; ▲, SO-50; ○, RO-30; ●, RO--50. The content of lipids is expressed as milligrams of palmitic acid/g fresh tissue. Mean values are given from 4 separate estimations (each on tissue samples from 4 animals).

The effect of high-fat feeding on the content of lipid in heart muscle is presented in Fig. 2. In the animals receiving soybean oil, the amount of lipid was practically the same as in the control group, whereas in animals fed on rape-see oil the content of lipids was increased after 2 days by a half in group RO-30, and more than twofold for RO-50. After 7 days the increase was even greater (2.5- and 3.5-fold, respectively), then the content of lipids decreased gradually to values corresponding to those observed after 2 days of rape-seed oil feeding.

The decrease in lipid content beginning with the 7th day, was concomitant with the increased activity of palmitoyl-CoA hydrolase. After 28 days, the content of lipids continued to decrease; at the same time the activity of palmitoyl-CoA hydrolase remained almost unchanged.

The above results indicate that palmitoyl-CoA hydrolase can play an important role in adaptation of the heart muscle to the increased supply of dietary fat. In the case of animals fed on erucic acid, the enzyme can prevent the imbalance of lipid metabolism in the heart cells induced by this acid.

The rise in the amount of acyl-CoA thioesters in the tissues of rat fed on high-fat diet is assumed to indicate increased oxidation of fatty acids (Tubbs & Garland, 1964), but when erucic acid is the quantitatively dominating component of the

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high-fat diet, its less efficient oxidation in the heart (Christophersen & Bremer, 1972; Lemarchal *et al.*, 1972; Bułhak-Jachymczyk & Hübner-Woźniak, 1974; Swarttouw, 1974), together with its inhibitory effect on the oxidation of other fatty acids (Christophersen & Bremer, 1972; Christophersen & Christiansen, 1975) lead to enhanced use of acyl-CoA thioesters for lipid synthesis.

The observed relation between the increase in palmitoyl-CoA hydrolase activity and the amount of fat in the diet, confirms the role of this enzyme in the control of the pool of long-chain acyl-CoA thioesters in the extramitochondrial compartment of the heart cell.

It seems that the increased activity of palmitoyl-CoA hydrolase makes possible to maintain such a concentration of acyl-CoA thioesters in cytoplasm at which excessive synthesis of triglycerides does not occur. This assumption would explain the gradual decrease in fat infiltration in the heart muscle of rats during prolonged feeding with erucic acid, although, as demonstrated by Clouet *et al.* (1974), there is no increase under these conditions in the ability of heart mitochondria to oxidize erucic acid.

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AKTYWNOŚĆ HYDROLAZY ACYLO-CoA W SERCU SZCZURÓW ŻYWIONYCH OLEJEM RZEPAKOWYM

Streszczenie

1. Spożywanie tłuszczu (oleju sojowego i oleju rzepakowego o wysokiej zawartości kwasu erukowego) przez okres 2 do 7 dni, zwiększa aktywność hydrolazy palmitoilo-CoA w sercu młodych szczurów w stopniu zależnym od ilości tłuszczu w diecie.

2. Wzrost aktywności enzymu w okresie pomiędzy 7 a 14 dniem żywienia, obserwowany tylko u szczurów otrzymujących olej rzepakowy, nastąpił równocześnie ze zmniejszeniem się stłuszczenia serca.

3. Uzyskane wyniki sugerują, że hydrolaza palmitoilo-CoA może kontrolować poziom acylo-CoA w cytoplazmie komórek mięśnia serca szczura i w ten sposób uczestniczyć w zaniku stłuszczenia, indukowanego przez kwas erukowy.

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NON-SPECIFIC ACETYL-CoA CARBOXYLASE AND METHYLMALONYL-CoA CARBOXYLTRANSFERASE IN *STREPTOMYCES NOURSEI* VAR. *POLIFUNGINI****

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1. Acetyl-CoA carboxylase (EC 6.4.1.2) and methylmalonyl-CoA carboxyltransferase (EC 2.1.3.1) have been isolated from mycelia of *Streptomyces noursei* var. *polifungini*, and purified about 50-fold.

2. Both enzymes carboxylate acetyl-CoA and propionyl-CoA; the respective K_m values are 1.1 and 1.6 mM with acetyl-CoA carboxylase and 2.5 and 1.25 mM with carboxyltransferase.

3. The activities of both enzymes are inhibited by free fatty acids. Almost total inhibition of methylmalonyl-CoA carboxyltransferase was observed by 0.1 mM-butyrate or 0.1 mM-C_{14} - C_{18} acids. Acetyl-CoA carboxylase was affected to the same extent by these compounds at concentration of about 1 mM.

4. The role of both carboxylating enzymes in biosynthesis of the antibiotic is discussed.

The occurrence of two systems carboxylating acetyl-CoA and propionyl-CoA has been proved in *S. noursei* var. *polifungini*: ATP- and Mg^{2+}-dependent acetyl-CoA carboxylase (EC 6.4.1.2), and methylmalonyl-CoA carboxyltransferase (EC 2.1.3.1) which uses oxaloacetate as a carboxyl group donor (Rafalski & Raczyńska-Bojanowska, 1972). The latter enzyme has been so far reported for propionic bacteria (Swick & Wood, 1960) and traces of this enzymic activity could be detected in *S. erythreus* in logarithmic phase of growth (Raczyńska-Bojanowska *et al.*, 1970). It has been found that in mutants of *S. noursei* var. *polifungini* the activity of both carboxylating systems is associated with higher antibiotic-synthesizing ability (Roszkowski *et al.*, 1972). This indicates a similar regulatory role of these carboxylating systems in the synthesis of macrolide antibiotic as that of acetyl-CoA

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carboxylase in the synthesis of fatty acids. The mechanism of polymerization of acyl-CoA units in both processes is the same (Lynen, 1967).

In the present work, non-specific acetyl-CoA carboxylase and non-specific methylmalonyl-CoA carboxyltransferase have been isolated from *S. noursei* var. *polifungini*, purified, and general characteristics of both enzymes is given.

MATERIAL AND METHODS

Reagents used were the same as in the previous work (Rafalski & Raczyńska-Bojanowska, 1972).

Streptomyces noursei var. *polifungini* (ATCC 21581), polifungin-producing mutant, was obtained from Mrs. D. Kotiuszko, M. Sc., from the Division of Microbiology. The organism was grown in 500 ml Erlenmeyer flasks on a rotary shaker (240 strokes/min) at 28°C in a medium containing per 100 ml: corn steep liquor, 1 g; $CaCO_3$, 0.8 g; $(NH_4)_2SO_4$, 0.4 g; sonicated brewer's yeast, 0.3 g; soluble starch, 3 g; and glucose, 4 g. The 48 h cultures were used for preparation of the enzymes.

Carboxylase activity was determined according to Wawszkiewicz & Lynen (1964) as described previously (Rafalski & Raczyńska-Bojanowska, 1972). The incubation mixture contained in a volume of 0.5 ml: Tris buffer, pH 8.0, 100 µmoles; ATP, 2.5 µmoles; $MgCl_2$, 5 µmoles; NaF, 10 µmoles; $NaH^{14}CO_3$, 5 µCi (spec. act. 6 µCi/µmole), the enzymic extract containing 1 - 2 mg of protein, and acetyl-CoA or propionyl-CoA, 0.5 µmole. After 60 min incubation at 30°C, acid-stable radioactivity was determined.

Methylmalonyl-CoA carboxyltransferase activity was measured according to Wood & Stjernholm (1961) in the modification described by Rafalski & Raczyńska-Bojanowska (1972), by determining pyruvate formed from oxalacetate. The reaction mixture contained in 1 ml: Tris buffer, pH 7.6, 100 µmoles; glutathione, reduced, 1 µmole; oxaloacetate, 2 µmoles; propionyl-CoA or acetyl-CoA, 0.5 µmole, and enzymic protein, 3 - 5 mg. After 15 min incubation at 30°C, the pyruvate formed was determined with lactate dehydrogenase. The activity is expressed in nmoles/h.

RESULTS

Isolation and purification of acetyl-CoA carboxylase and methylmalonyl-CoA carboxyltransferase. In isolation of carboxylase (Table 1) advantage was taken of the localization of the enzyme exclusively in the "cell wall" fraction obtained from mycelium by the lysozyme-EDTA treatment (Rafalski & Raczyńska-Bojanowska, 1972). Washed cells suspended in 2 vol. of 20% saccharose containing 0.05 M-Tris-HCl buffer, pH 8.0, and 0.01 M-2-mercaptoethanol, were incubated with lysozyme and EDTA (2 mg and 9.3 mg per gram of wet wt. of the cells, respectively) for 1 h at 30°C. Spheroplasts were spun down, washed with 20% saccharose and discarded. The supernatant and the washings were combined and dialysed against 0.01 M-K,Na-phosphate buffer, pH 7.0, containing 5 mM-2-mercaptoethanol. From the dialysed solution (200 ml) containing 1 - 2 mg protein/ml, the protein was absorbed on calcium

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Table 1

Purification of acetyl-CoA carboxylase

For determination of enzyme activity, acetyl-CoA was used as a substrate. For details see Methods.

Step	Total activity (counts/ /100 sec)	Specific activity (counts/100 sec/mg protein)	Purification factor	Yield (%)	Acetyl-CoA: :propionyl- -CoA carboxylation rat.o
1. "Cell wall fraction"	1 270 000	1 020	—	100	1.3
2. 10 000 *g* supernatant, dialysed	1 200 000	2 700	2.6	98	1.4
3. Calcium phosphate gel eluate (0.1 M-phosphate buffer, pH 7.0)	365 000	11 800	11.5	29	1.4
4. DEAE-cellulose column	94 000	39 000	38	8	1.2
5. Sephadex G-200 column	84 000	50 000	49	6	1.0

phosphate gel (0.5 vol). The inactive proteins were removed successively with 70 ml of 0.01 M- and 70 ml of 0.025 M-phosphate buffer, pH 7.0, and then carboxylase was eluted with 100 ml of 0.1 M-buffer. The eluate was concentrated by lyophilization, dialysed on Sephadex G-25 coarse and fractionated on DEAE-cellulose. The column (50 × 1 cm) was loaded with 20 - 25 mg of protein, and eluted with a linear 0.01 - 0.05 M gradient of phosphate buffer, pH 7.0, containing 5 mM-2-mercaptoethanol. The enzyme emerged at 0.02 M concentration, and was concentrated by lyophilization; 4 - 5 mg of protein was applied to Sephadex G-200 column (35 × 1 cm) and the enzyme was eluted with 0.01 M-phosphate buffer, pH 7.0. By the above procedure an about 50-fold purification of the enzyme was achieved; the preparations from the last two steps were devoid of the carboxyltransferase activity.

Carboxyltransferase was isolated from the sonicates of washed mycelium suspended in 0.05 M-Tris-HCl buffer, pH 7.4, containing 10 mM-2-mercaptoethanol. The sonicate was centrifuged and the supernatant diluted to contain 4 - 5 mg protein/ml; the fraction precipitated at 0.3 - 0.5 ammonium sulphate saturation was collected, dissolved in the same Tris buffer to contain 6 mg of protein/ml, mixed with 2 vol. of calcium phosphate gel, stirred for 15 min and centrifuged at 12 000 rev./min for 15 min. The gel with the absorbed enzyme was washed successively with 1 vol. of 0.025 M- and 1 vol of 0.05 M-Tris buffer, pH 7.0, and the enzyme was eluted with 2 vol. of 0.25 M-buffer of pH 7.0. The eluate (0.5 - 1.0 mg protein/ml) was brought to 0.5 saturation with solid $(NH_4)_2SO_4$, the precipitate was collected and dissolved in 0.01 M-phosphate buffer, pH 7.0. The solution containing 4 - 5 mg protein/ml was stirred for 30 min with 2 vol. of DEAE-Sephadex A-50 suspended in the same buffer. The fraction eluted with 1 vol. of 0.025 M-buffer was discarded and the enzyme was eluted twice with 1 vol. of 0.1 M-buffer, each time the adsorbent being stirred with the buffer for 30 min. The eluate was concentrated on Diaflo UM 10 membrane to

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contain about 5 mg protein/ml. Then 15 - 20 mg of protein was applied to DEAE--cellulose column (50 × 1 cm) and eluted with 0.01 - 0.05 M linear gradient of phosphate buffer, pH 7.0, containing 5 mM-2-mercaptoethanol. The enzyme was eluted at 0.02 M concentration. The overall purification of carboxyltransferase achieved was 45 - 50-fold (Table 2). The final preparation did not show any traces of carboxylase activity.

Table 2

Purification of methylmalonyl-CoA carboxyltransferase

For determination of the enzyme activity, propionyl-CoA was used as a substrate; the activity is expressed as nmoles of pyruvate formed per 1 h. For details see Methods.

Step	Total activity (nmoles/h)	Specific activity (nmoles/h/mg protein)	Purification factor	Yield (%)
1. Sonicated mycelium	7500	14	—	100
2. Ppt. at 0.3 - 0.5 $(NH_4)_2SO_4$ sat.	5400	26	1.8	72
3. Calcium phosphate gel eluate (0.25 M-phosphate buffer, pH 7.0)	3600	136	9.7	48
4. DEAE-Sephadex-batch (0.1 M--phosphate buffer, pH 7.0)	2050	270	19.0	27
5. DEAE-cellulose column	650	670	47.0	9

Properties of carboxylase and carboxyltransferase. The ratio of carboxylase activity towards acetyl-CoA and propionyl-CoA was practically the same at all steps of the purification procedure (Table 1), and for the final preparation it was 1.0. This indicates non-specificity of the carboxylase. For carboxyltransferase, the ratio of the activity with acetyl-CoA and propionyl-CoA was 1:1.5. The K_m values, calculated from the double reciprocal Lineweaver-Burk plots, for acetyl-CoA and propionyl-CoA were found to be virtually the same: in the case of carboxylase 1.1 and 1.6 mM, and with carboxyltransferase 2.5 and 1.25 mM, respectively. The molecular weights of the purified enzymes calculated from the elution volumes using the calibrated Sephadex G-200 column (Fig. 1) were 110 000 for carboxylase and 170 000 for carboxyltransferase.

The activities of the two carboxylating enzymes were not affected by the addition of citrate, fructose-1,6-diphosphate, glycerol-3-phosphate, 2-oxoglutarate or fumarate at concentration of 2 mM. Nystatin (5000 units/ml) was also ineffective. Citrate added to the 48 h cultures in a final concentration of 2 mM did not induce synthesis of the enzymes. The activities of carboxylase and carboxyltransferase were, however, strongly inhibited by fatty acids (Tables 3 and 4). Total inhibition by butyrate and C_{14} - C_{18} fatty acids was observed at concentration of 0.1 mM, while at the same concentration fatty acids of medium length, C_6 - C_{12}, were ineffective. Data on inhibition at various substrate concentrations showed that butyrate inhibited the carboxylase activity competitively, which may indicate that butyryl-CoA could

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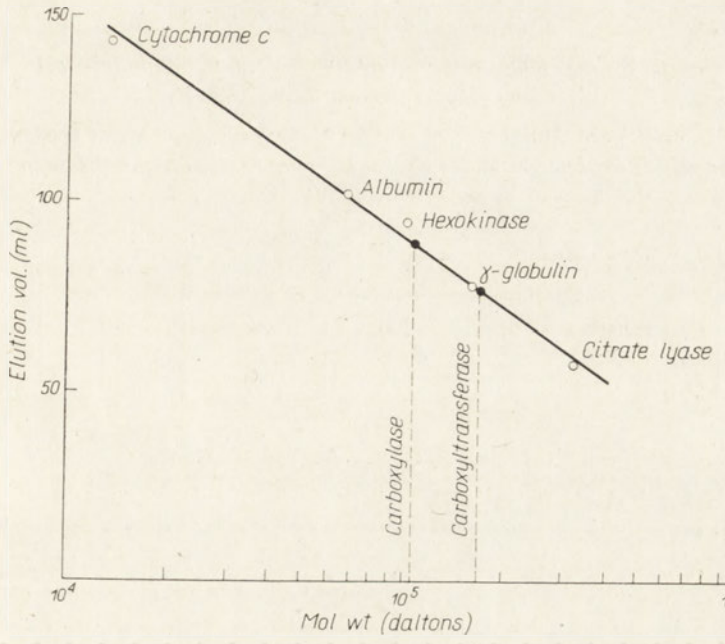

Fig. 1. Determination of molecular weight of acetyl-CoA carboxylase and methylmalonyl-CoA carboxyltransferase on the calibrated Sephadex G-200 column.

also serve as a substrate for this enzyme. Inhibition by caprylate was of the non-competitive type and that by stearate, of the mixed competitive-noncompetitive type. Free fatty acids inhibited completely the activity of carboxyltransferase at concentration by one order of magnitude higher (1 mM) than that at which carboxylase activity was inhibited.

Table 3

Effect of free fatty acids on the activity of acetyl-CoA carboxylase

The activity was determined with a 50-fold purified enzyme preparation and acetyl-CoA as a substrate, as described in Methods. The results are expresssed as percentage of inhibition in relation to the control.

Compound	Concentration (M)			
	10^{-3}	10^{-4}	10^{-5}	10^{-6}
C_4, Butyrate	—	86	57	—
C_6, Caproate	21	1	0	—
C_8, Caprylate	36	14	—	—
C_{12}, Laurate	18	5	0	—
C_{14}, Myristate	98	75	23	—
C_{18}, Stearate	—	100	67	33
C_{18}, Oleate	—	98	82	23
C_{18}, Elaidate	—	94	55	15

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Table 4

Effect of free fatty acids on the activity of methylmalonyl-CoA carboxyltransferase

The activity was determined with a 50-fold purified enzyme preparation and propionyl-CoA as a substrate, as described in Methods. The results are expressed as percentage of inhibition in relation to the control.

Compound	Concentration (M)		
	10^{-3}	10^{-4}	10^{-5}
C_4, Butyrate	100	47	20
C_8, Caproate	—	22	0
C_{18}, Stearate	83	59	0

DISCUSSION

The occurrence in *S. noursei* var. *polifungini* of the two distinct non-specific enzymes carboxylating acetyl-CoA and propionyl-CoA and showing similar substrate affinities, is of considerable interest. The Mg^{2+}- and ATP-dependent carboxylase resembles propionyl-CoA carboxylase of mammalian mitochondria in being a latent structure-bound enzyme (Scholte, 1969; Rafalski & Raczyńska-Bojanowska, 1972). However, unlike the mitochondrial enzyme, it is not specific for propionyl-CoA and carboxylates also acetyl-CoA, similarly as the soluble cytoplasmic acetyl-CoA carboxylase of mammals and acetyl-CoA carboxylases from other sources. ATP--independent carboxyltransferase of *S. noursei* var. *polifungini*, like the analogous enzyme from propionibacteria (Wood, 1972) catalyses carboxylation of both acetyl--CoA and propionyl-CoA at the expense of oxaloacetate carboxyl group; the K_m values found for the Streptomyces enzyme were, however, higher (2.5 and 1.25 mM) than those reported by Wood (1972) for the Propionibacteria enzyme (0.5 and 0.03 mM, respectively).

Similarly as in *E. coli*, the activity of the investigated carboxylase is not controlled by the intermediates of glycolysis and those of Krebs cycle (Alberts & Vagelos, 1972). Neither do these compounds affect the activity of carboxyltransferase. It seems that fatty acids may play a regulatory role in *S. noursei* var. *polifungini* both by inhibiting and repressing the activity of acetyl-CoA carboxylase. This activity is much lower in the cells grown on lipid medium (Rafalski & Raczyńska-Bojanowska, 1972), and concentration of long-chain free fatty acids found in the cells of *S. noursei* var. *polifungini* (Roszkowski *et al.*, 1972; Roszkowski, unpublished) is of the same order (0.1 mM) as that at which the enzyme is inhibited in the experiments *in vitro*. Therefore the inhibitory effect of fatty acids should be regarded as specific rather than due to the non-specific detergent action. It is also noteworthy that the effect of unsaturated fatty acids of higher detergent properties is the same as that of saturated fatty acids. Lower susceptibility of the carboxyltransferase to fatty acids may be of importance for growth and antibiotic synthesis in *S. noursei* var. *polifungini*. The physiological role of free fatty acids in regulation of fatty acids synthesis in

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mammals is still controversial (Weber *et al.*, 1967; Pande & Mead, 1968; Parvin & Dakshinamurti, 1970; Goodridge, 1972, 1973).

The molecular weights of the purified carboxylase and carboxyltransferase found by molecular sieving on Sephadex G-200 are very low as compared with those of the corresponding enzymes from other sources (Alberts & Vagelos, 1972; Wood, 1972). Although under our experimental conditions of isolation and purification both enzymes could dissociate into active subunits, none of the reported active subunits were of so low molecular weight. More experiments are therefore needed to explain these differences and the submolecular structure of the Streptomyces enzymes.

The role of the two carboxylating systems in maintaining the pool of malonyl-CoA and methylmalonyl-CoA — the precursors of poliene ring of antibiotic — remains unexplained. It may be assumed that acetyl-CoA carboxylase is associated with the fatty acids synthesis while methylmalonyl-CoA carboxyltransferase may cooperate with phosphoenolpyruvate carboxylase and pyruvate: orthophosphate dikinase (EC 2.7.9.1) catalysing direct synthesis of phosphoenolpyruvate from pyruvate in the hypothetical "oxaloacetate cycle", thus providing malonyl-CoA and methylmalonyl-CoA for biosynthesis of lactone ring of poliene antibiotics (Rafalski & Raczyńska-Bojanowska, 1972).

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NIESPECYFICZNA KARBOKSYLAZA ACETYLO-CoA I KARBOKSYLOTRANSFERAZA METYLOMALONYLO-CoA U *STREPTOMYCES NOURSEI* VAR. *POLIFUNGINI*

Streszczenie

1. Z grzybni *Streptomyces noursei* var. *polifungini* wyizolowano karboksylazę acetylo-CoA (EC 6.4.1.2.) oraz karboksylotransferazę metylomalonylo-CoA (EC 2.1.3.1). Enzymy oddzielono od siebie i oczyszczono około 50-krotnie.

2. Oba enzymy są nieswoiste, katalizują karboksylację zarówno acetylo-CoA, jak i propionylo-CoA. K_m dla obu tych substratów wynoszą: 1.1 i 1.6 mM w odniesieniu do karboksylazy acetylo-CoA oraz 2.5 i 1.25 mM dla karboksylotransferazy metylomalonylo-CoA.

3. Karboksylotransferaza metylomalonylo-CoA jest bardziej odporna niż karboksylaza acetylo-CoA na działanie wolnych kwasów tłuszczowych. Niemal całkowite hamowanie tego enzymu obserwowano przy stężeniu 0.1 mM maślanu i kwasów C_{14} - C_{18}.

4. Omówiono rolę obu enzymów karboksylujących w biosyntezie polifunginy.

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BOROHYDRIDE-REDUCIBLE COMPONENTS IN SOLUBLE COLLAGEN IRRADIATED WITH GAMMA RAYS IN SOLUTION

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1. Irradiation with 100 krad of gamma rays of neutral-salt-soluble rat skin collagen decreased the content of aldol cross-links by a factor of three, whereas it did not affect the content of allysine.

2. On reduction with tritiated sodium borohydride, five new components were detected showing different stability towards acid and alkali.

Little is known on the mechanism of collagen radiolysis. Disruption of hydrogen and peptide bonds seems to be the main radiolytic reaction accounting for the degradation of collagen (Bailey, 1968; Cooper, 1971). However, no information is available on susceptibility of cross-links in collagen to irradiation.

There are two general types of cross-linkages in collagen. The intramolecular one, derived from aldol condensation between the two residues of the adipic acid semialdehyde derivatives, and the intermolecular one, resulting from the Schiff base formation between intrinsic collagen aldehydes and ε-amino group of a lysyl or hydroxylysyl residue (Bailey *et al.*, 1974; Tanzer, 1973). Selective reduction of collagen with tritiated borohydride stabilizes these cross-links and by introduction of a tritium label allows the detection and isolation of the reduction products. Hence, the application of borohydride reduction technique to the irradiated collagen can supply information on changes in cross-linkages due to irradiation.

The results presented in this paper indicate that irradiation decreases the number of aldol cross-links in collagen and induces formation of new reducible components of an unknown structure.

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MATERIALS AND METHODS

Collagen. Collagen soluble in neutral salt was prepared from skins of young Wistar rats (30 - 40 g) essentially by the method of Bornstein & Piez (1964). Two additional reprecipitations with sodium chloride were introduced to the procedure to increase homogeneity of the preparation. The final preparation was stored as a 0.15% solution in 0.15 M-NaCl at −20°C.

Irradiation. Collagen solution adjusted to pH 5.0 was deoxygenated by bubbling the oxygen-free nitrogen for 30 min, and was sealed in a test-tube. The ampoule was placed in an ice-bath and irradiated with a 900 Ci cobalt-60 gamma source at a dose rate of 0.1 Mrad per hour.

Reduction with sodium borohydride. Reduction of collagen was carried out with a 500-fold molar excess of tritiated sodium borohydride as described by Paz *et al.* (1969). The excess of reagent was removed by 48 h dialysis against water and the dialysed samples were lyophilized.

Hydrolysis. The freeze-dried samples of collagen were hydrolysed alternatively in 2 M-NaOH, in 6 M-HCl or in 3 M-*p*-toluenesulphonic acid at 110°C for 22 - 24 h (Liu & Chang, 1971).

Analytical methods. The samples of hydrolysed collagen were analysed with the Unichrom automatic amino acid analyser (Beckman Instruments Inc.) according to the procedure of Spackman *et al.* (1958). In the effluents, the distribution pattern of amino acids and that of radioactivity were examined. For radioactivity measurements the effluents were collected in 1 ml fractions. To relate the positions of the radioactivity with the elution peaks of amino acid fractions, the two elution profiles were superimposed. The tritium activity of the collected effluents was measured by mixing aliquots of the fractions (0.2 ml) with Bray's solution (5 ml), and the radioactivity determined in a liquid scintillation counter, type SL-30 (Intertechnique, Paris). Protein concentration was estimated by the Kjeldahl method.

RESULTS AND DISCUSSION

Incorporation of tritium into collagen during its reduction with tritiated sodium borohydride was higher in the collagen irradiated under nitrogen than in corresponding non-irradiated samples (Table 1). This indicates formation on irradiation of new reducible components. Since under the experimental conditions used the aldehyde

Table 1

Relative specific radioactivity of collagen reduced with tritiated sodium borohydride

Collagen	Radioactivity
Non-irradiated	1.0
Irradiated (30 krad)	1.8
Irradiated (100 krad)	4.1

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group is reducible, one may assume that the increase in aldehyde content in irradiated collagen (Jeleńska & Dancewicz, 1969) could account for the increase in the reducible components. Structure of these aldehydes is unknown. Our preliminary results suggest that the radiation-induced aldehydes are of the "saturated" type (Jeleńska *et al.*, 1975).

Analysis of the alkaline hydrolysates of the reduced collagen (Fig. 1) showed that, in addition to aldol and allysine[1] — the two reducible structures present in native collagen — three new radioactive components (I, II, III) appeared in irradiated collagen. The content of all these three new components increased with the irradiation dose. In the literature there is no available information which would allow any reasonable assumption to be made as to the nature of these components.

Table 2

Total radioactivity in ε-hydroxynorleucine, reduced aldol condensation product (Aldol) and in the product eluted at the position of "degraded aldol"

Separation of the products from the alkaline hydrolysates (ε-hydroxynorleucine, Aldol) and acid hydrolysates ("degraded aldol") of collagen, reduced with tritiated sodium borohydride was carried out on the automatic amino acid analyser.

Collagen	Radioactivity (c.p.m. $\times 10^{-3}$/mg of protein)		
	ε-hydroxynor-leucine	Aldol	"degraded aldol"
Non-irradiated	4.68	4.50	3.50
Irradiated (30 krad)	4.94	2.40	5.30
Irradiated (100 krad)	4.40	1.44	7.50

Data given in Fig. 1 and Table 2 show also that the content of aldol cross-links was decreased threefold on irradiation of collagen with 100 krad. The content of allysine remained unchanged. This implies that the saturated type of aldehyde, such as allysine, is more radioresistant than the α,β-unsaturated aldehyde grouping in the aldol cross-link. The precise nature of the aldol radiolytic product is unknown.

Some information on the reducible components in irradiated collagen was obtained from the distribution of radioactivity in the reduced collagen subjected to acid hydrolysis. Under these conditions, aldol is destroyed and eluted as "degraded aldol", while the reduction product of allysine, hydroxynorleucine, is converted partly to chloronorleucine (Lent *et al.*, 1969). The elution profile of the acid hydrolysate (Fig. 2) shows seven peaks of radioactivity. Three of them, I, II and III, were eluted at the same positions as those of the alkaline hydrolysates. However, components of peaks II and III were less stable to acid than to alkaline hydrolysis, while the product eluted in peak I was more stable to acid hydrolysis.

In the acid hydrolysate, in addition to these three components, a fourth peak of radioactivity (IV) was eluted after histidine. Since this peak was not found in

[1] Abbreviation used: allysine, α-aminoadypic-δ-semialdehyde.

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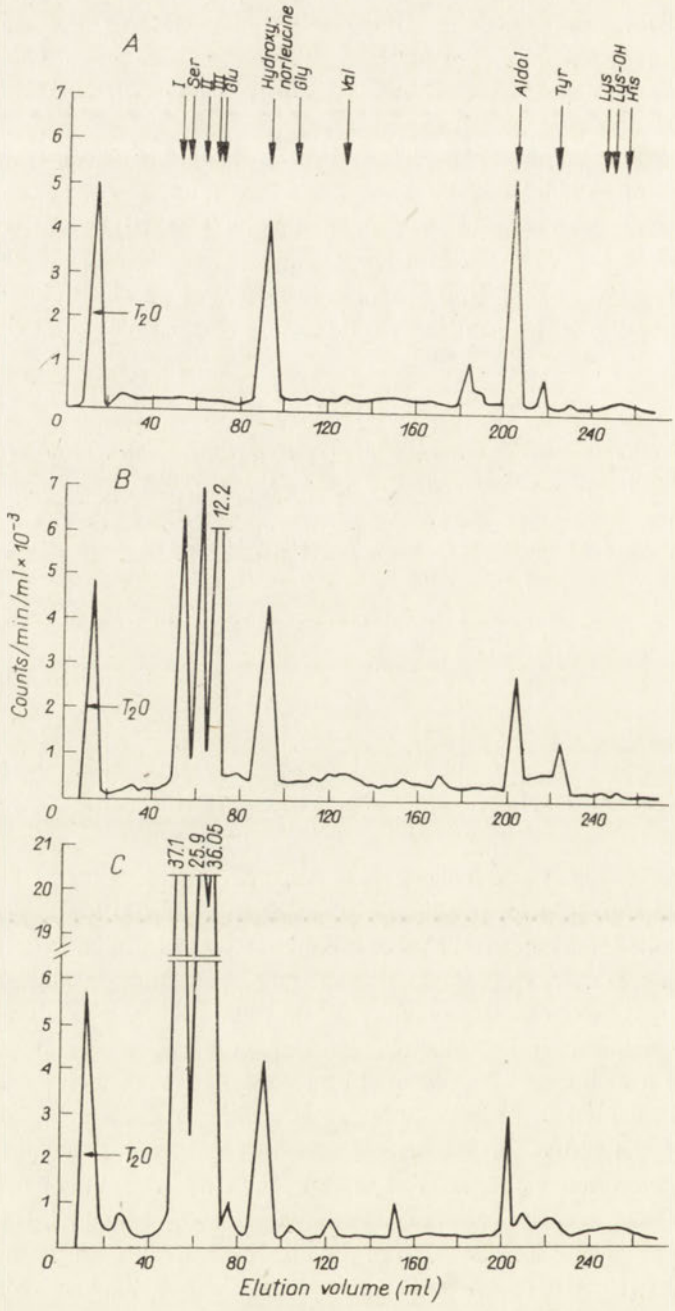

Fig. 1. Distribution of radioactivity in the amino acid fractions separated from alkaline hydrolysates of collagen (5 mg) reduced with tritiated sodium borohydride. Elution positions of some amino acids are indicated. Collagen: *A*, non-irradiated; *B*, irradiated with 30 krad; *C*, irradiated with 100 krad. I, II, III, unknown reducible components appearing in the irradiated collagen.

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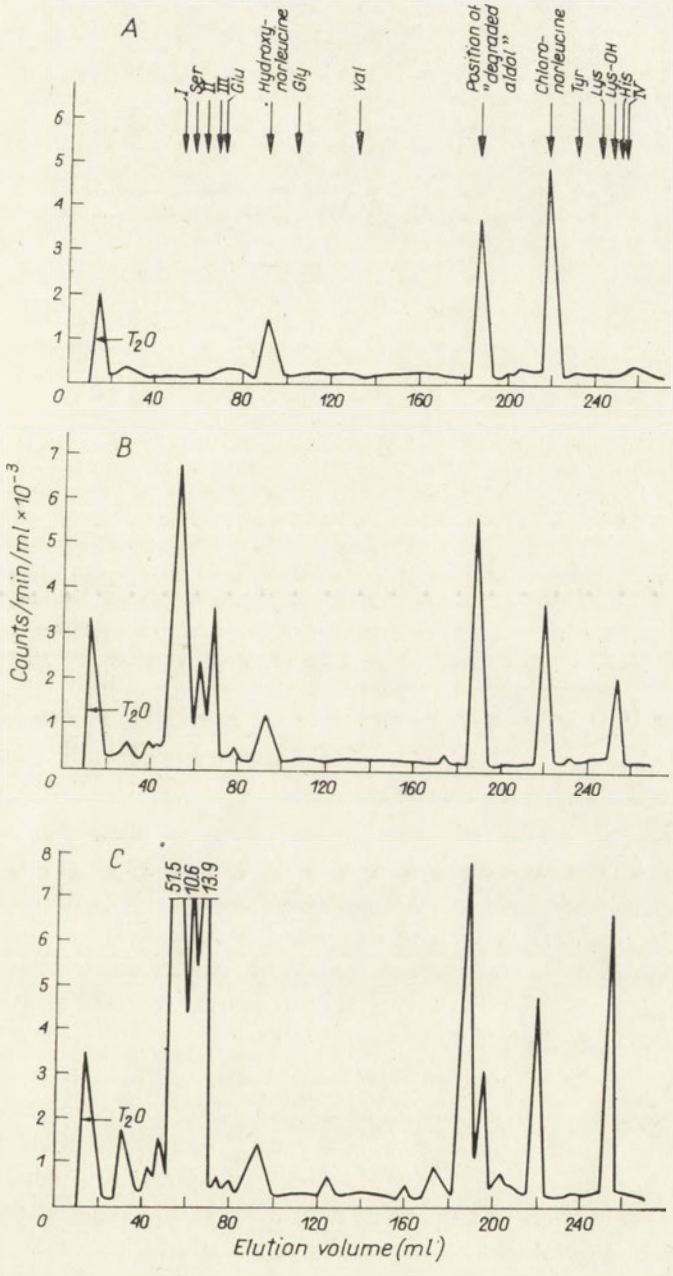

Fig. 2. Distribution of radioactivity in the amino acid fractions separated from acid hydrolysates of collagen (5 mg) reduced with tritiated sodium borohydride. Elution positions of some amino acids are indicated. *A*, Non-irradiated collagen; *B*, collagen irradiated with 30 krad; *C*, collagen irradiated with 100 krad. I, II, III and IV, unknown reducible components from the irradiated collagen.

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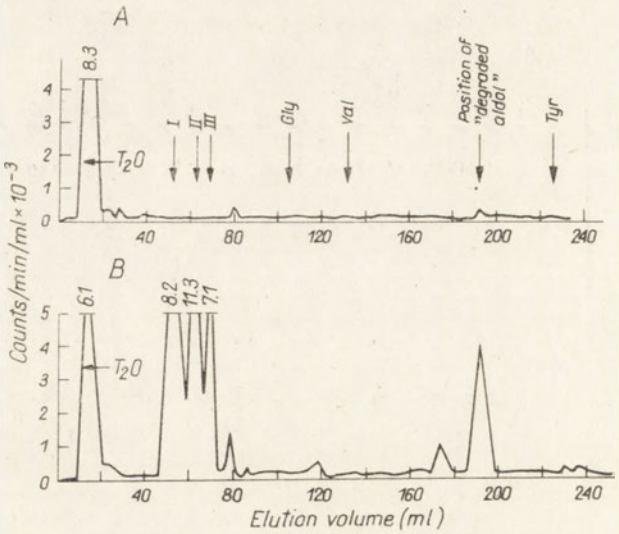

Fig. 3. Distribution of radioactivity in the amino acid fractions separated from acid hydrolysates of collagen (5 mg) reduced before and after irradiation with, respectively, non-radioactive and radioactive sodium borohydride. Elution positions of some amino acids are indicated. *A*, Non-irradiated collagen; *B*, collagen irradiated with 100 krad. I, II and III, unknown reducible components appearing after irradiation of collagen devoid of aldol and allysine.

alkaline hydrolysates, the possibility exists that it is a product of acid degradation of the components eluted in peaks II and/or III.

The elution positions of the remaining three out of seven radioactivity peaks were found to be the same as those established by Lent *et al.* (1969) for ε-hydroxynorleucine, "degraded aldol" and chloronorleucine. The additional proof for the presence of chloronorleucine was obtained with hydrolysates performed with *p*-toluenesulphonic acid. Under these conditions, chloronorleucine is not formed, and in our experiments no radioactivity appeared at the expected position of chloronorleucine in the elution profile.

The amount of the "degraded aldol" found in the acid hydrolysates of irradiated collagen increased with the irradiation dose while the corresponding amount of aldol in alkaline hydrolysates decreased. This apparent discrepancy could be explained by the results of the experiment in which collagen previously reduced with non-radioactive borohydride was exposed to irradiation and reduced for the second time with tritiated borohydride. The results presented in Fig. 3 show that the non-irradiated collagen contained neither aldol nor its precursor, allysine. In irradiated collagen, however, a new reducible component appeared which had the R_T the same as that of the "degraded aldol". Identification of this, as well as four other newly detected reducible components induced in collagen on irradiation, awaits further investigation.

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ZWIĄZKI REDUKOWANE BOROWODORKIEM W ROZPUSZCZALNYM KOLAGENIE NAPROMIENIONYM PROMIENIAMI GAMMA W ROZTWORZE

Streszczenie

1. Napromienienie kolagenu ze skóry szczurzej, rozpuszczalnego w solach obojętnych, dawką 100 krad promieniowania gamma zmniejszało trzykrotnie zawartość aldolowych wiązań krzyżowych i nie wpływało na zawartość allizyny.

2. Po redukcji trytowanym borowodorkiem sodu stwierdzono obecność pięciu nowych związków charakteryzujących się różną trwałością wobec kwasów i zasad.

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THE ACTIVATION BY STAPHYLOKINASE OF HUMAN PLASMINOGEN

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The activation of human plasminogen by a highly purified staphylokinase was investigated using casein or an active site titrant (*p*-nitrophenyl-*p'*-guanidinobenzoate, NPGB) as a substrate. The reaction rate was time dependent, showing a pronounced lag period with either substrate. Saturation curve estimated from the caseinolytic assay was sigmoid, but changed to quasi-hyperbolic in the presence of pre-formed human plasmin. With NPGB, the extent of plasminogen conversion into esterolytic plasmin was directly proportional to staphylokinase concentration, and the saturation point was reached when the molar concentration of staphylokinase equaled that of plasminogen. It is concluded that staphylokinase acts stoichiometrically, forms an equimolar complex with plasminogen, and thus is not an enzyme but a modifier. Staphylokinase-activated plasminogen exhibits properties of a hysteretic enzyme.

Staphylokinase is an extracellular protein produced by many strains of *Staphylococcus aureus* which can activate the fibrinolytic system of human blood. It has the capacity to convert the proenzyme, plasminogen, into a trypsin-like proteolytic enzyme, plasmin. In that respect, staphylokinase resembles another plasminogen activator of bacterial origin, streptokinase, but it has been much less extensively studied than streptokinase.

Several attempts were made to purify staphylokinase. Various salting-out and/or chromatographic procedures yielded a protein fraction of molecular weight estimated by gel filtration to be 22 500 (for a recent review, see Lack & Glanville, 1970), and containing at least three proteins of similar plasminogen-activating properties but of different isoelectric points (Vesterberg & Vesterberg, 1972). While some extraneous proteins were present in the purified preparations, staphylokinase was shown, by *in vitro* assays, to be a protein entity distinct from typical staphylococcal toxins, and free of protease, DNase, and hyaluronidase activities (Glanville, 1963).

The only biological activity of staphylokinase known so far is the activation of plasminogen of several, but not all, animal species, including human, rabbit, guinea pig, but excluding ox plasminogen. The rate of the activation, and the yield of

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plasmin depend on the concentration of staphylokinase, but the details of staphylokinase interaction with plasminogen have not yet been elucidated. In contrast to streptokinase, staphylokinase does not seem to be consumed during the activation, and to require a "proactivator" (Davidson, 1960). On that basis it has been suggested that staphylokinase is an enzyme, even though no other enzymic activity has been detected in it.

This report is concerned with the kinetics of the activation of human plasminogen by a highly purified preparation of staphylokinase. Using casein or *p*-nitrophenyl-*p'*--guanidinobenzoate as a substrate, the results were obtained which indicated that staphylokinase reacted stoichiometrically with plasminogen to produce an enzyme which shows a hysteretic response.

MATERIALS AND METHODS

Staphylokinase. Staphylokinase was extracted from supernatants of submerged culture of *Staphylococcus aureus*, strain 502A using a column of CM-Sephadex C-50 in a fully protonated form. The active protein was eluted with 0.02 M-phosphate buffer, pH 7.2, and chromatographed on CM-Sephadex C-50 equilibrated in 0.05 M--acetate buffer, pH 5.0. Staphylokinase-containing fraction was eluted with the same acetate buffer supplemented with 0.3 M-sodium chloride. The fraction containing staphylokinase activity was used for most caseinolytic assays; its activity was about 20×10^3 uSF per E_{275} unit (for the methods of assay, and the definition of staphylokinase units, see below). For other experiments, staphylokinase was further purified by isoelectric focusing in sucrose density columns. Three fractions were recovered from the column, with pI 5.50, 5.90 and 6.65. The specific activity of pI 6.65 staphylokinase was 35×10^3 uSF/mg protein. When this preparation was freed of ampholines and concentrated by ultrafiltration, the value of $E^{1\%}_{280\,nm} = 12.6$ was determined from the microbiuret method using a purified preparation of human serum albumin as a reference preparation. The molecular weight of staphylokinase determined from gel filtration on Sephadex G-50 was $11\,000 \pm 1000$, and from SDS-polyacrylamide gel electrophoresis $14\,000 \pm 3\,000$; the value of 12 000 was assumed for stoichiometric calculations.

Human plasminogen. Various preparations of human plasminogen were used in this work. For routine caseinolytic assay of staphylokinase activity (see below), a commercial preparation was employed which contained 30 to 40 caseinolytic units/mg of protein, and a variable amount of pre-formed plasmin; active site titration (see below) showed these preparations to be about 45% pure. For most of the experiments, plasminogen was extracted from placental serum, or from Cohn Fraction III obtained from that serum, using the lysine-substituted Sepharose as described by Deutsch & Mertz (1970). This preparation contained about 2% of pre-formed plasmin, and was about 80% pure, by active site titration. To remove the residual plasmin activity, the preparation was passed through a column of soybean trypsin inhibitor which had been covalently coupled with Sepharose 4B, following the procedure of Cuatrecasas *et al.* (1970). Plasmin-free plasminogen

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preparations are explicitly referred to as such, to distinguish them from the routine plasminogen preparations which were contaminated with plasmin. The values of $E^{1\%}_{280\,mn}=17.0$, and 85 000 for molecular weight of plasminogen were assumed for stoichiometric calculations. All plasminogen preparations were stored frozen until used.

Human plasmin. Plasmin was extracted from the lysine-Sepharose extracted plasminogen which was activated with catalytic quantities of streptokinase in 25% glycerol at 37°C for 30 min. The reaction mixture diluted to 12.5% in glycerol with pH 7.4 phosphate buffer was passed through a column of immobilized soybean trypsin inhibitor, described above, and the enzyme was dissociated from the sorbent with 6 M-urea adjusted to pH 1.6 with hydrochloric acid. The eluted protein was brought to pH 7.4 and to 50% in glycerol, in which it has been stored in the cold. The yield of plasmin was about 80%.

Other materials. Streptokinase (intravenous) was purchased from Biomed, Warsaw, and was purified as described previously (Biliński *et al.*, 1968). International Reference Preparation of Streptokinase-Streptodornase was obtained through the courtesy of The National Institute for Biological Standards, Mill Hill, London. Casein, Hammarsten-type, was a Merck (Darmstadt, G.F.R.) product. Sephadex and Sepharose were Pharmacia (Uppsala, Sweden) products. Other reagents were purchased from local outlets, and were used without further purification.

p-Nitrophenyl-p'-guanidinobenzoate (NPGB) was synthesized as described by Chase & Shaw (1967). The product was virtually pure as determined from the amount of *p*-nitrophenolate liberated by alkaline hydrolysis. The values of ε_{410nm} of *p*-nitrophenolate were assumed: 17 500 in 0.1 M-sodium hydroxide, and 11 600 in pH 7.4 phosphate buffer. Stock solutions of NPGB, approximately 6 mM, were prepared in anhydrous dimethylformamide, and were used within 4 days.

Caseinolytic assay. The procedure described by Johnson *et al.* (1969) was followed with minor modifications: the reactants were kept in crushed ice until placed in the 37°C water bath; aliquots were withdrawn, unless otherwise stated, just prior to the incubation, and after 30 minutes at 37°C; casein concentration was 7 mg/ml in the reaction mixture. The assay was not standardized with CTA reagents; one caseinolytic unit (c.u.) of plasmin was defined as that amount of the enzyme which generated an increase in E_{275nm} of 0.300 after 30 minutes.

To determine staphylokinase activity, various amounts of it were added to the assay system containing between 0.6 and 0.7 c.u. of plasminogen per ml. Under these conditions, one international unit of streptokinase yielded an increase in the concentration of perchloric acid soluble casein digestion products equivalent to $\Delta E_{275nm}=0.012$ after a 30-min incubation. For streptokinase, the calibration plot was linear over the range of 1 to 6 units per ml of the reaction mixture. For staphylokinase, the same 0.012 value of ΔE was taken as one unit (uSF), but the calibration plot was sigmoid with a narrow portion of quasi-linearity. The solutions of the assayed staphylokinase were diluted, on the basis of preliminary titrations, so as to yield the ΔE_{275nm} between 0.150 and 0.350. The calibration of the assay system was included in each titration.

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Active site titration. The procedure based on that described by Chase & Shaw (1969) was followed. Stock solution of NPGB was diluted with 0.1 M-phosphate buffer, pH 7.4, to 0.2 mM, and the actual concentration of NPGB was determined from alkaline hydrolysis. Other reactants employed in the active site titration were also made up in the same phosphate buffer. Dilute NPGB solution, 0.2 ml, was pipetted into a semimicrospectrophotometer cell, and was followed by 0.6 ml of plasminogen solution. After the extinction at 410 nm reached a steady-state, various amounts of staphylokinase in 0.4 ml volume were added. The extinction was recorded at frequent time intervals until a steady-state was established. The measurements were carried out in thermostated cell compartment of SP 500 Unicam spectrophotometer at 20°C against a blank consisting of NPGB in the same pH phosphate buffer.

RESULTS

A family of progress curves in Fig. 1 illustrates the activation of plasminogen (containing about 5% of pre-formed plasmin), 0.6 c.u./ml, with various amounts of staphylokinase under the conditions of caseinolytic assay. All the curves appear sigmoid: a pronounced lag period is followed by an accelerated rate of casein digestion which levels off later on. The duration of the lag extends over some 40 minutes at the lowest staphylokinase concentration, 0.5 uSF/ml, becomes shorter with the increasing staphylokinase concentrations, but persists also even with 48 uSF/ml (cf. curve *d*, Fig. 1), which is equivalent, with the employed plasminogen concentration of 4×10^{-7} M in active site, to the equimolar staphylokinase:plasminogen mixture. The steepness of the intermediate portion of the caseinolytic progress curves is

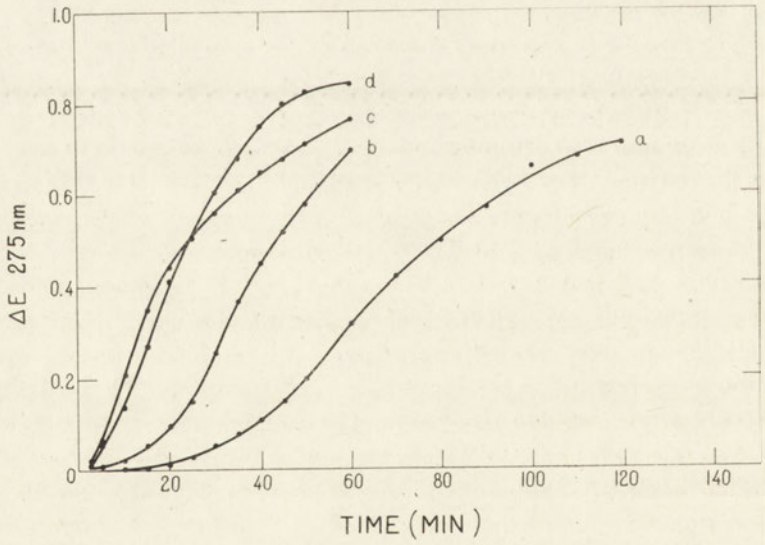

Fig. 1. Activation of human plasminogen by staphylokinase. Caseinolytic assay: plasminogen, 0.6 c.u./ml; pH 7.4 phosphate buffer; temp. 37°C. Staphylokinase: (*a*) 0.5 uSF/ml; (*b*) 1.6 uSF/ml; (*c*) 24 uSF/ml; (*d*) 48 uSF/ml.

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inversely related to the duration of lag period, and increases with the increasing staphylokinase - plasminogen ratios. Since all the curves tend to converge at a similar maximum level, and the experiments were performed with excess casein, it is evident that the final extent of casein digestion does not depend on staphylokinase concentration. On the other hand, within a limited time of digestion, the amount of casein digestion products was different for various staphylokinase concentrations. A 30-min incubation time was chosen for staphylokinase assay as it appeared to include a relatively wide range of staphylokinase concentrations. However, the relationship between the amount of staphylokinase in the reaction mixture and the amount of perchloric acid soluble products liberated from casein was not linear, necessitating the adjustment of staphylokinase concentration to a fixed value in the assay system, and its careful standardization (cf. Materials and Methods).

In the preliminary studies on the relationship between the concentration of staphylokinase and the yield of plasmin, it has been observed that the results varied with different batches of plasminogen. However, with the plasmin-free plasminogen the saturation curve shown in Fig. 2 (curve *a*) was reproducibly obtained. This curve is markedly different from what could be expected on the basis of progress curves shown in Fig. 1, despite an almost insignificant difference in plasminogen concentration, which was 0.60 c.u./ml in the experiment illustrated on Fig. 1, and 0.67 c.u./ml in that on Fig. 2. For instance, after 30-min incubation with routine plasminogen solution, 0.5 uSF/ml yielded 0.05, and 1.6 uSF 0.26 E_{275nm} unit (cf. curve *a* and curve *b*, Fig. 1), while with the plasmin-free plasminogen only a trace of digestion products was detected with 0.5 uSF/ml, and the $E_{275nm}=0.04$ was found with 1.5 uSF/ml (cf. curve *a*, Fig. 2).

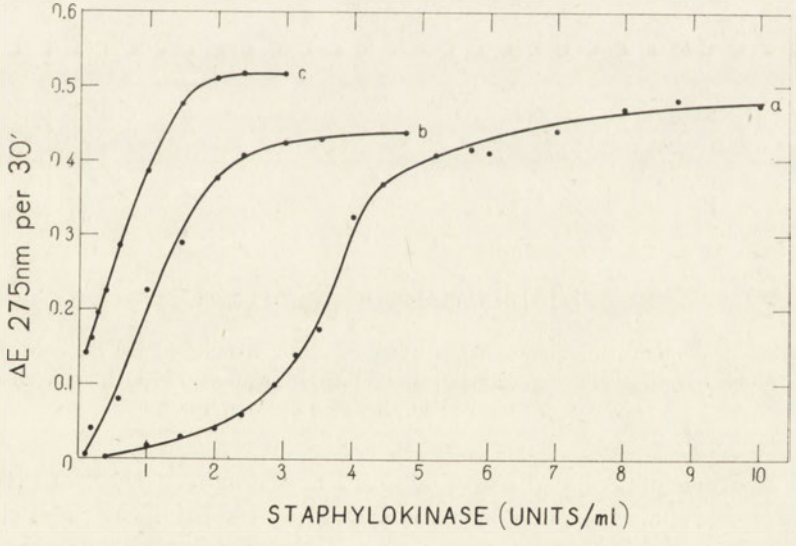

Fig. 2. The effect of pre-formed plasmin on the activation of human plasminogen by staphylokinase. Caseinolytic assay: plasmin-free plasminogen, 0.67 c.u./ml; pH 7.4 phosphate buffer; 30-min incubation time at 37°C. Human plasmin added: (*a*) none; (*b*) 2%; (*c*) 10%.

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The effect of pre-formed plasmin was investigated on the activation kinetics using plasmin-free plasminogen to which various amounts of purified human plasmin (cf. Materials and Methods) were added. The addition of plasmin resulted in a striking change of the rate of casein digestion. The saturation curve for plasmin-free plasminogen (curve *a*, Fig. 2) was sigmoid: the yield of plasmin was increasing slowly up to about 2 uSF/ml, then rapidly up to about 5 uSF/ml, and again very slowly at higher staphylokinase:plasminogen ratios. In the presence of a low amount of pre-formed plasmin (2% of total potential plasmin activity) the initial slowly rising portion of the saturation curve disappeared; at the higher (10%) plasmin content, the saturation curve was shifted towards lower staphylokinase concentrations but was otherwise identical, if corrected for the activity of the added plasmin, with the curve obtained with a lower plasmin content (cf. curves *b* and *c*, Fig. 2). In the presence of pre-formed plasmin, then, the sigmoid kinetics apparent with plasmin-free plasminogen is changed into what appears a hyperbolic kinetics. This is illustrated by the linearity of double reciprocal plot of the data obtained when plasmin-free plasminogen was enriched with 10% of plasmin prior to the addition of staphylokinase, shown in Fig. 3.

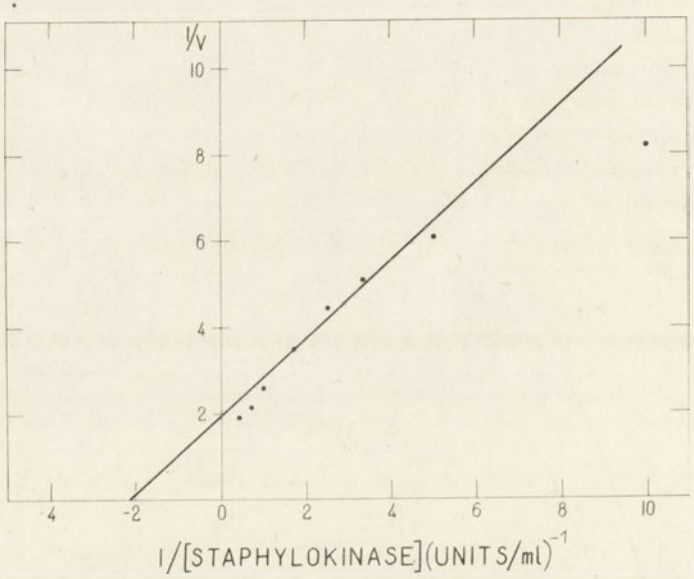

Fig. 3. Kinetics of human plasminogen activation by staphylokinase in the presence of human plasmin. Double reciprocal plot. Caseinolytic assay, 30-min incubation at 37°C; human plasminogen, 0.67 c.u./ml; purified human plasmin, 0.07 c.u./ml.

The experiments presented above indicated a predominant effect of pre-formed plasmin on the course of plasminogen activation by staphylokinase, and suggested that an initial stage in the activation might consist of the interaction between staphylokinase and plasmin rather than with plasminogen. To examine this question, the activation was investigated using NPGB instead of casein as a substrate. NPGB

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is an active site titrant for serine proteases including plasmin (Chase & Shaw, 1969). The catalytic disruption of the ester bond in NPGB liberates the alcohol moiety, *p*-nitrophenol, which dissociates rapidly from the enzyme; while the acid moiety, guanidinobenzoate, remains attached to the enzyme. As the rate of the dissociation of guanidinobenzoate from the enzyme is extremely low, plasmin remains effectively inactivated, making it possible to study the activation of plasminogen without the participation of pre-formed or generated plasmin (McClintock & Bell, 1971).

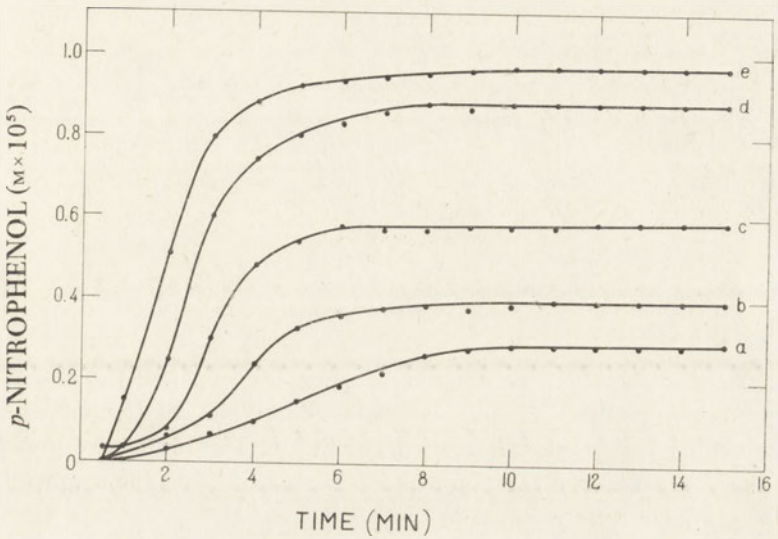

Fig. 4. Time-course of the activation of human plasminogen by staphylokinase in the presence of *p*-nitrophenyl-*p'*-guanidinobenzoate (NPGB). Spectrophotometre cell contained: 3.5×10^{-5} M NPGB, 0.2 ml; 1.08×10^{-5} M (in active site) human plasminogen, 0.6 ml; and staphylokinase, 0.4 ml: (*a*) 0.19×10^{-5} M; (*b*) 0.36×10^{-5} M; (*c*) 0.59×10^{-5} M; (*d*) 0.83×10^{-5} M; (*e*) 0.89×10^{-5} M. All reactants in pH 7.4 phosphate buffer; temp. 20°C.

For the NPGB experiments, staphylokinase fraction isolated from isoelectric focusing as pI 6.65 protein (cf. Materials and Methods) was used. Its specific activity was 36×10^3 uSF/mg of protein. A family of progress curves shown in Fig. 4 was obtained when increasing amounts of staphylokinase were added to a constant amount of plasminogen with an approximately threefold excess of NPGB. All the curves exhibit a definite lag period, resembling in that respect the caseinolytic progress curves (cf. Fig. 1). In both, the duration of the lag and the steepness of the intermediate portion of the curve were related to the concentration of staphylokinase. However, with NPGB the amount of *p*-nitrophenolate liberated eventually reached a maximum value, which remained constant throughout the experiment (if corrected for a small steady-state, post-burst, production of *p*-nitrophenolate). Thus with NPGB as a substrate, the concentration of staphylokinase was a limiting factor for the extent of product formation, in contrast to what has been observed with casein as a substrate.

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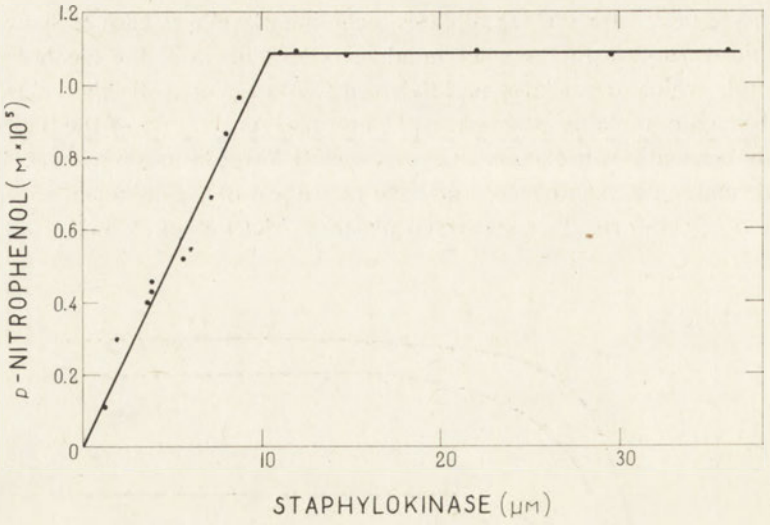

Fig. 5. Saturation of human plasminogen by staphylokinase determined with NPGB as a substrate. Conditions as in Fig. 4.

The relationship between the amount of staphylokinase and the magnitude of plasminogen conversion into plasmin was estimated by plotting the maximum yield of *p*-nitrophenol *versus* staphylokinase concentration. The results are presented in Fig. 5. It is evident that the saturation point is reached when the staphylokinase : : plasminogen molar ratio is equal to unity.

DISCUSSION

Very few studies have yet been reported on the kinetics of the activation by staphylokinase of human plasminogen, and the purity of the employed reactants was often questionable. Staphylokinase used in this work was freed of most, if not all, extraneous proteins, and was inactive towards the substrates employed, casein and NPGB. The molecular weight of our staphylokinase, 12 000, was considerably below 22 500 reported by Lack & Glanville (1970). The cause of this discrepancy remains obscure. While it cannot be excluded that staphylokinase forms a dimer (or is split) under some experimental conditions, a more likely explanation is suggested by several findings concerning its heterogeneity. We have observed a considerable spread (but no evidence of discontinuity) in the pattern given by exhaustively reduced staphylokinase in SDS-polyacrylamide gel electrophoresis (unpublished); Arvidson *et al.* (1973) reported that the molecular weight of a highly purified staphylokinase ranged from 16 000 to 22 000; the charge microheterogeneity of staphylokinase in culture medium was documented by Vesterberg & Vesterberg (1972), and has been found by ourselves in the CM-Sephadex purified staphylokinase by isoelectric focusing (cf. Materials and Methods). It may well be that some bacterial strains produce staphylokinase in which a particular protein species predominates. The matter deserves further studies for more than one reason.

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The activity of staphylokinase in this work was determined by a simultaneous incubation of all the components of the activation mixture. Other authors separated the activation system from the proteolytic system (Davidson, 1960; Vesterberg *et al.*, 1967; Lack & Glanville, 1970). In these latter methods, higher values are obtained for the proteolytic activity, possibly because the inhibition is obviated of the activation by casein substrate (Davidson, 1960). However, we have found the simultaneous incubation procedure more reproducible, and ascribe it to the fact that staphylokinase-induced plasmin activity is relatively rapidly destroyed in the absence of casein substrate. The disadvantage of the simultaneous procedure consists in a more limited linearity of the calibration plot, but a judicious use of a fixed dilution of the assayed preparation together with the introduction of standard streptokinase as a reference preparation reduced the uncertainty of the assay (inherent in all methods for staphylokinase titration), and may eventually help in interlaboratory comparisons.

Sigmoid saturation curve, such as has been obtained when a plasmin-free plasminogen was used in caseinolytic assay, suggests that a cooperative effect is involved in the activation. This seems to be abolished if plasmin is added prior to the initiation of the activation by staphylokinase. While the saturation profile alone is not a sufficient evidence for cooperativity (or otherwise), the effect may be due to the formation of a ternary complex composed of staphylokinase, plasminogen, and plasmin. If this is so, the presence of pre-formed plasmin could accelerate the complexing, and shift the saturation curve towards lower staphylokinase concentrations. However, it cannot be established with the present data whether the quasi-hyperbolic kinetics of plasmin-cortaining plasminogen activation by staphylokinase really reflects the absence of cooperativity, as it would require a much more sensitive method for the observation of activation process in the presence of low concentrations of staphylokinase. Whatever the mechanism(s) involved, the caseinolytic calibration curve for staphylokinase is bound to be highly sensitive to the concentration of pre-formed plasmin, and — since that factor is exceedingly difficult to control — the assay requires a careful standardization.

The main findings in the present work concern the interaction between staphylokinase, plasminogen, and NPGB. Esterolytic plasmin activity is evidently generated by staphylokinase in the solution of plasminogen which has been pre-treated with NPGB. It is thus clear that pre-formed plasmin is not an obligatory factor in the activation of human plasminogen by staphylokinase. Rising amounts of staphylokinase produce increasing esterolytic activity. The incremental responses of plasminogen were a linear function of staphylokinase concentration until a distinct saturation point was reached at 1:1 molar ratio of staphylokinase to plasminogen. This shows that staphylokinase functions stoichiometrically, and argues against the supposition that staphylokinase is an enzyme (Lack & Glanville, 1970). It can be concluded that staphylokinase is a modifier which induces the active site in plasminogen molecule, thus resembling streptokinase for which a similar effect has been well documented by McClintock & Bell (1971) and Reddy & Markus (1972). Even though a technique has not yet been elaborated to directly evidence the integrity

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of peptide chain in staphylokinase-activated but NPGB-inhibited plasminogen, it may reasonably be assumed that staphylokinase shares with streptokinase the property of inducing the active site in human plasminogen prior to, and independent of, the irreversible cleavage of the sensitive arginyl-valine bond in plasminogen.

In the presence of excess plasminogen and NPGB, a stable maximum level of the activated plasminogen was generated, as it has been found also in the case of streptokinase by McClintock & Bell (1971) and Reddy & Markus (1972). Thus, staphylokinase, like streptokinase, is not recycled after the initial interaction with plasminogen. This similarity, however, is restricted to experimental conditions which ensure that the generated plasmin is immediately trapped by NPGB cleavage product. Without the inhibitor, as in the caseinolytic assay, functional staphylokinase remains in the plasmin-containing solution, and is available for further portions of plasminogen long after the initial activation had been completed (Davidson, 1960); we have confirmed that by isolating an apparently intact staphylokinase from its mixture with plasminogen using Sephadex G-100 chromatography in pH 7.4 phosphate buffer free of dissociating agents (unpublished). Contrasting with staphylokinase, streptokinase disappears rapidly from the activation mixture, being recoverable only in the complex with plasmin, at first as an altered but active, and later as a fragmented and inactivated protein (Summaria *et al.*, 1971; Brockway & Castellino, 1974; Summaria *et al.*, 1974). Reddy & Markus (1972) have shown that streptokinase is more strongly bound with plasminogen, a single-chain protein, than with the two-chain plasmin. It seems likely that staphylokinase exhibits a similar binding preference but — being evidently resistant to the plasmin proteolytic attack — remains active in the caseinolytic reaction mixture.

The examination of NPGB progress curves (cf. Fig. 4) reveals an important difference between the kinetics of staphylokinase and streptokinase action. With streptokinase a typical "burst" of *p*-nitrophenol is obtained (Chase & Shaw, 1969), the rate of which was so rapid that it necessitated the addition of inhibitors to make the pre-burst rate observable under the conditions of normal spectrophotometry (Sodetz & Castellino, 1972). On the other hand, the liberation of *p*-nitrophenol with the staphylokinase-activated plasminogen was slow, even though the maximum extent of plasminogen conversion was attained with staphylokinase, as with streptokinase, when an equimolar complex was formed. The distinct lag period in the reaction course suggested that staphylokinase-plasminogen system may belong to the class of enzymes which respond slowly to a rapid change in ligand (modifier or substrate) concentration, and for which Frieden (1970) introduced the term "hysteretic enzymes". The time-dependence of the velocity of reactions catalysed by hysteretic enzymes can be described by the equation developed by Frieden (1970): $V_t = V_f + (V_0 - V_f)\, e^{-k't}$. This equation yields a straight line when $\ln(V_f - V_t)$ is plotted *versus* t, the reaction time. To evaluate V_t, the velocity at t, the procedure of Kim & Graves (1973) was followed: tangents were constructed to the consecutive time-points, as illustrated in Fig. 6. These slope values were used to plot the logarithmic form of the Frieden equation, which is shown in Fig 6 (cf. Insert). The linearity of the plot, extending over the initial 3 minutes of the reaction, indicates that the

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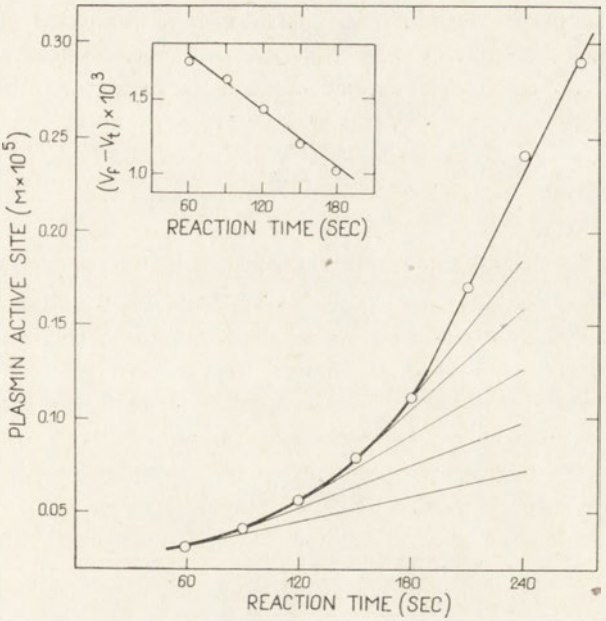

Fig. 6. Estimation of the initial velocity of NPGB cleavage by staphylokinase-activated human plasminogen. Plasminogen, 1.1×10^{-5} M (in active site); staphylokinase (36×10^3 uSF/mg), $0.6 \times \times 10^{-5}$ M; NPGB, 3.5×10^{-5} M; pH 7.4 phosphate buffer; temp. 20°C. Final velocity, V_f, and velocities at time t, V_t, determined as indicated by thin lines. The insert: semi-logarithmic plot of Frieden's equation (see Text).

esterolytic activity induced in plasminogen by staphylokinase develops in accordance with the theory of hysteretic enzymes. In that respect, Mechanism III (single substrate — single modifier case) elaborated by Frieden (1970) may be of particular interest. The mechanism underlying the lag period can consist of a dissociation--association process and/or a conformational change. This could not be evaluated from the available results, but some preliminary calculations showed that V_0 (which can be evaluated graphically from the logarithmic form of the Frieden equation, cf. the insert, Fig. 6) was sharply dependent on the concentration of staphylokinase, pointing to the possibility that it is the rate of staphylokinase dissociation or association which is an important factor in the kinetics of plasminogen activation.

Of the few identified, and several presumed, plasminogen activators (Astrup & Kok, 1970) streptokinase was until now unique in being conclusively shown to act through a non-enzymatic mechanism. The results presented in this work afford evidence for a non-enzymatic activation of human plasminogen also by staphylokinase. It may be envisaged that these two modifiers appertain to a wider family of specific plasminogen activators, possibly not limited to microorganisms, which can induce the active site without affecting the integrity of plasminogen peptide chain. In that respect it is of particular interest that the kinetics of staphylokinase-plasminogen system differs from that of the streptokinase-plasminogen system. It may be that some properties of modifier-activated plasminogen are

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defined by the nature of the modifier. Considered in the terms of the theory of hysteretic enzyme reactions, staphylokinase-plasminogen system might exemplify the adaptation of plasminogen-derived enzyme for regulatory functions.

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AKTYWACJA LUDZKIEGO PLAZMINOGENU PRZEZSTAFILOKINAZĘ

Streszczenie

Aktywację ludzkiego plazminogenu przez wysoko oczyszczoną stafilokinazę badano stosując jako substrat kazeinę lub odczynnik miejsca aktywnego (*p*-nitrofenylo-*p'*-guanidynobenzoesan, NPGB). Szybkość reakcji była zmienna w czasie, wykazując znaczny okres początkowej zwłoki z każdym z substratów. Krzywa wysyceniowa, oznaczona z pomiarów kazeinolitycznych, była sigmoidalna lecz zmieniała się w quasi-hiperboliczną w obecności niewielkich ilości preformowanej ludzkiej plazminy. Stosując NPGB stwierdzono, że przekształcenie plazminogenu w esterolityczną plazminę jest proporcjonalne do stężenia stafilokinazy i że punkt wysycenia osiąga się, gdy stężenie molarne stafilokinazy jest równe stężeniu plazminogenu. Z doświadczeń tych wyprowadzono wniosek, że stafilokinaza działa stechiometrycznie, tworzy równomolarny kompleks z plazminogenem będąc, wobec tego, nie enzymem ale modyfikatorem. Plazminogen aktywowany przez stafilokinazę posiada cechy enzymu histeretycznego.

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