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ACTA BIOCHIMICA POLONICA

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MIROSŁAWA WEBER, ZDZISŁAW ŻAK and JANUSZ STECZKO

EFFECT OF ANTIBODY ON INTERACTION BETWEEN VITAMIN B_2 AND RIBOFLAVIN APOPROTEIN

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1. Dissociation of riboflavin from flavoprotein and from the flavoprotein-antibody complex occurs under the same conditions.

2. The precipitated apoprotein-antibody complex retains 15% of the apoprotein capacity to bind riboflavin. After solubilization of the complex in 0.3 M-KCl or 1 M-urea, the binding of riboflavin amounts to 80 - 90% of its capacity.

3. The apoprotein modified by oxidation of 50% of tryptophan residues loses the ability to bind riboflavin but its immunological reactivity with the anti-flavoprotein antibody is similar to that of native apoprotein. The apoprotein with all tryptophan residues oxidized shows much lower immunoreactivity.

4. The obtained results suggest that in riboflavin flavoprotein the region around the riboflavin-binding site does not show the properties of an antigenic determinant.

Riboflavin flavoprotein from chicken egg-yolk (Ostrowski *et al.*, 1962; Żak & Ostrowski, 1963) belongs to proteins that store and transport vitamin B_2, and it has antigenic properties (Weber *et al.*, 1966; Żak *et al.*, 1968). The formed antibody reacts also, although in a somewhat different way, with the apoprotein obtained by dissociation of riboflavin. This is due to different conformation of these proteins. As reported by Żak *et al.* (1972), the apoprotein on binding riboflavin undergoes changes, probably in a part of polypeptide chain which contains binding site for riboflavin. Participation of tryptophan residues in binding of riboflavin (Żak *et al.*, 1972) and hydrophobic nature of this interaction have been demonstrated by Steczko & Ostrowski (1975).

The aim of the present work was to elucidate whether the region in proximity of the binding site for riboflavin in the protein molecule shows properties of an antigenic determinant; an attempt has been made to establish whether the reaction of riboflavin flavoprotein with the specific antibody impairs dissociation of riboflavin, and whether the complex of apoprotein with the anti-flavoprotein antibody is able

to bind riboflavin. Moreover, immunological reactivity of native apoprotein has been compared with that of the apoprotein modified by oxidation of tryptophan residues.

MATERIALS AND METHODS

Riboflavin flavoprotein was prepared from chicken egg-yolk as described by Ostrowski *et al.* (1962) and Żak & Ostrowski (1963); its molecular weight is 36 000 daltons. The apoprotein was obtained by dissociation of riboflavin in citrate buffer at pH 3.0 (Żak & Ostrowski, 1963). Tryptophan residues in the apoprotein were modified by oxidation with *N*-bromosuccinimide[1] as described by Steczko & Ostrowski (1975).

Anti-flavoprotein serum was obtained by immunization of rabbits, and its activity was tested by the methods published previously (Weber *et al.*, 1966; Żak *et al.*, 1968). Immunochemical assays included: immunodiffusion according to Ouchterlony (1948), immunoelectrophoresis by the method of Grabar & Williams in the modification of Scheidegger (1955), and complement fixation as described by Levine (1967).

Protein concentration was determined according to the method of Lowry *et al.* (1951) with bovine albumin as a standard, or by measuring absorbance at 280 nm using Unicam SP-800 spectrophotometer.

Equilibrium dialysis. Samples, 1 ml, of 0.1 mM-flavoprotein and flavoprotein-antibody complex in 0.01 M-sodium phosphate buffer, pH 7.5, containing 0.9% NaCl, were dialysed against 3 ml of the above buffered saline solution. The outer buffer was kept at a temperature of 22°C with stirring, and the concentration of liberated riboflavin was determined fluorimetrically with SF-1 spectrofluorimeter (Beard Atomic, Cambridge, Mass., U.S.A.). The fluorescence intensity was measured at 530 nm after excitation at 450 nm.

On the basis of changes in molar concentration of the liberated and bound riboflavin, the Scatchard plot was constructed (Scatchard, 1949; Kahn *et al.*, 1973).

Chemicals: Riboflavin (B.D.H.Ltd, London, England), *N*-bromosuccinimide (VEB Laborchemie, Apolda, G.D.R.), Sephadex gels (Pharmacia, Uppsala, Sweden); bovine albumin (Sigma Biochem. Corp., St. Louis, Mo., U.S.A.). Other reagents were analytical grade products from POCh (Gliwice, Poland).

RESULTS

Dissociation of flavoprotein-antibody complex

As reported by Żak & Ostrowski (1963), in buffer solution of pH 3.0 riboflavin dissociated from the flavoprotein. To find out whether there are differences in the riboflavin binding in flavoprotein and in flavoprotein-antibody complex, the effect

[1]Abbreviation: NBS, *N*-bromosuccinimide.

of pH on riboflavin dissociation was examined. At pH 3.0, the flavoprotein-antibody complex dissociated with simultaneous liberation of riboflavin.

Kinetics of riboflavin dissociation from flavoprotein and flavoprotein-antibody complex was studied by equilibrium dialysis in 0.01 M-sodium phosphate buffer, pH 7.5. After 2 h, in either case the equilibrium was attained (Fig. 1). The obtained results permitted to establish the ratio of unbound to bound riboflavin at varying amounts of the dissociated riboflavin, and to construct the Scatchard plot (Fig. 2). The dissociation plot for the riboflavin flavoprotein-antibody complex was parallel to that for flavoprotein. The dissociation constants for riboflavin were 2.5 and 2.91 nM, respectively. The dissociation constant for riboflavin flavoprotein determined by Ostrowski & Krawczyk (1963) was practically the same (2.6 nM).

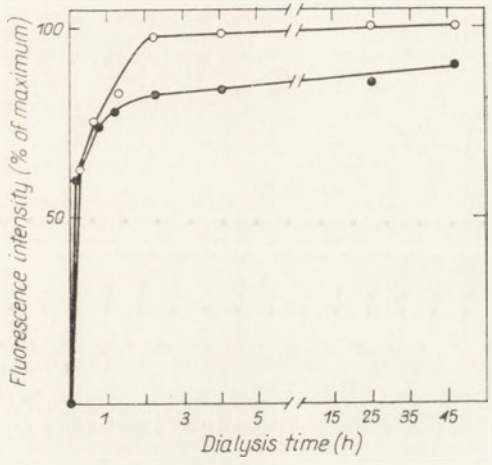

Fig. 1

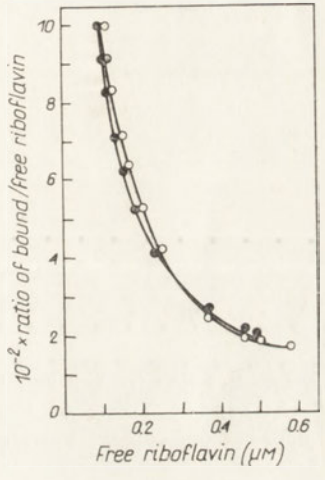

Fig. 2

Fig. 1. Dissociation of riboflavin flavoprotein (○) and flavoprotein-antibody complex (●) during dialysis. The proteins were at 0.1 mM concentration, and were dialysed against 0.01 M-sodium phosphate buffered saline, pH 7.5. For details see Methods.

Fig. 2. Scatchard plot of the data obtained from dialysis. Riboflavin flavoprotein (○) and flavoprotein-antibody complex (●) were at 0.1 mM concentration. For details see legend to Fig. 1 and Methods.

Binding of riboflavin by the apoprotein-antibody complex

The complex was obtained by treatment of apoprotein with anti-flavoprotein serum in 0.01 M-sodium phosphate buffer, pH 7.5, containing 0.9% NaCl, and the obtained sediment was incubated with riboflavin in phosphate buffer, with vigorous stirring, at 37°C. At appropriate time intervals, the decrease of riboflavin in the supernatant was determined fluorimetrically. Even after 90 min of incubation, the amount of bound riboflavin was not higher than about 15% of the binding capacity of the apoprotein. This showed that apoprotein complexed with antibody did not lose the ability to bind riboflavin. The low efficiency of binding may indicate

that aggregation of the sedimented apoprotein-antibody complex limits the access of riboflavin to the binding sites.

For further experiments, the sedimented apoprotein-antibody complex was solubilized at different KCl concentrations. Samples of the sediment were incubated at room temperature in KCl solutions with riboflavin, and the amount of the complex dissolved and of riboflavin bound were determined (Fig. 3A). At 0.05 M-KCl, the sediment began to pass into solution, and at 0.15 M-KCl it was practically solubilized. The binding of riboflavin increased rapidly above 0.1 M-KCl, and at 0.3 M-KCl reached 80% of the binding capacity of free apoprotein. At higher KCl concentrations, binding of riboflavin did not exceed 90%.

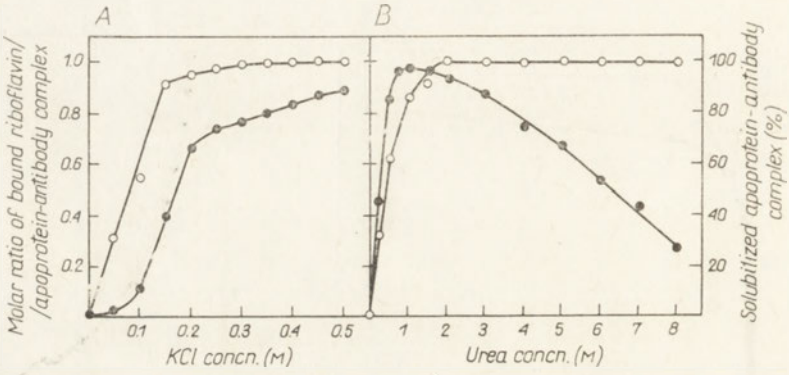

Fig. 3. Effect of KCl (*A*) and urea (*B*) concentration on solubility of the apoprotein-antibody complex (○) and its capacity for binding riboflavin (●). The maximum concentration of soluble apoprotein-antibody complex was 2 μM, as measured on the basis of riboflavin-binding capacity.

The observed binding of riboflavin may be accounted for by binding either by the apoprotein-antibody complex, or by the apoprotein dissociated from the complex. It was found by thin-layer chromatography on Sephadex G-100 that the apoprotein-antibody complex did not dissociate in solutions of KCl concentration up to 0.5 M. The complex showed very low mobility and remained near the point of application. At KCl concentration exceeding 0.5 M, the complex dissociated, the products showing greater mobility, corresponding to that of free apoprotein and γ-globulin.

The binding of riboflavin by apoprotein-antibody complex was confirmed using urea solution as solubilizer. Urea was found to be adequate as only at its concentration as high as 8 M, apoprotein lost the riboflavin-binding ability, and up to 1.5 M-urea the apoprotein-antibody complex was practically stable, as demonstrated by thin-layer chromatography. Complete solubilization of the sedimented complex was obtained with urea solutions of concentration exceeding 1 M, and simultaneously the highest binding of riboflavin by the complex was observed at 0.75 - 1.5 M-urea (Fig. 3B). A further increase in urea concentration lowered riboflavin binding.

Immunochemical reactivity of the oxidized apoprotein

Żak *et al.* (1972) demonstrated differences in immunological properties toward the anti-flavoprotein antibody between riboflavin flavoprotein and the apoprotein possessing full capacity to bind riboflavin. The apoprotein contains eight tryptophan residues (Żak & Ostrowski, 1963) and, as reported by Steczko & Ostrowski (1975), by treatment with hydrogen peroxide, 2-hydroxy-5-nitrobenzylbromide or NBS, maximally six residues could be oxidized; after oxidation of 3 - 4 tryptophans, the apoprotein completely loses the capacity for binding riboflavin.

The immunological reactivity of native apoprotein and riboflavin flavoprotein were compared with that of the apoprotein oxidized with NBS. Using different concentrations of NBS, two forms of the modified apoprotein were obtained: Apo-50, oxidized in 50% (2 - 3 indole residues), and Apo-100, with all exposed residues oxidized. In the immunodiffusion test of Ouchterlony (Fig. 4A) and in immunoelectrophoresis (Fig. 4B), riboflavin flavoprotein, native apoprotein and Apo-50 gave precipitation bands with the anti-flavoprotein serum.

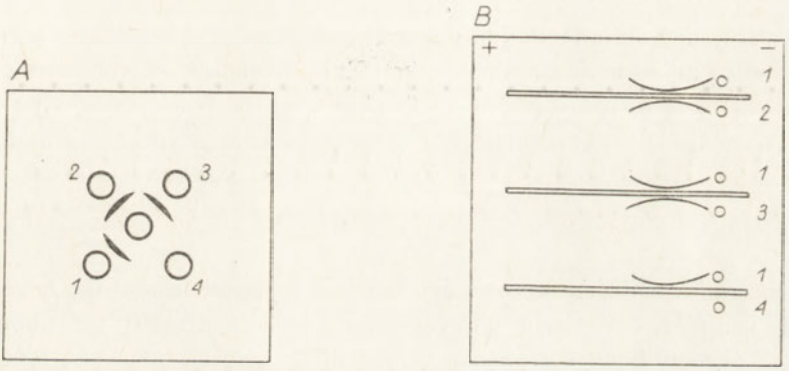

Fig. 4. The immunodiffusion test (*A*) and immunoelectrophoresis (*B*) of: *1*, riboflavin flavoprotein; *2*, native apoprotein; and *3*, apoprotein modified by oxidation of 50% of tryptophan residues (Apo-50) or *4*, of all exposed tryptophans (Apo-100). In both assays, 0.5% protein solution was used and undiluted anti-serum (1 ml having the capacity to bind 0.35 mg of flavoprotein). Electrophoresis was run in veronal buffer of pH 8.2, $I=0.025$, at 6 - 8 V/cm.

Immunological reactivity of the modified apoprotein was also tested by the complement fixation assay. The anti-serum was diluted from 1:32 to 1:2048; the dilutions 1:128 and 1:512 were found to be optimal. At the 1:128 dilution of anti-serum (Fig. 5A), the plot for complement fixation as a function of antigen concentration was similar for Apo-50 and native apoprotein. The maximum complement fixation was obtained with 15 μg of antigen. On the other hand, the plot for Apo-100 was shifted in the direction of higher antigen concentrations and had no distinct maximum. At the 1:512 dilution (Fig. 5B), distinct maxima of complement fixation were observed for all three proteins studied. The plots for native apoprotein and Apo-50 were closely similar, whereas that for Apo-100 showed a lower maximum at higher antigen concentration.

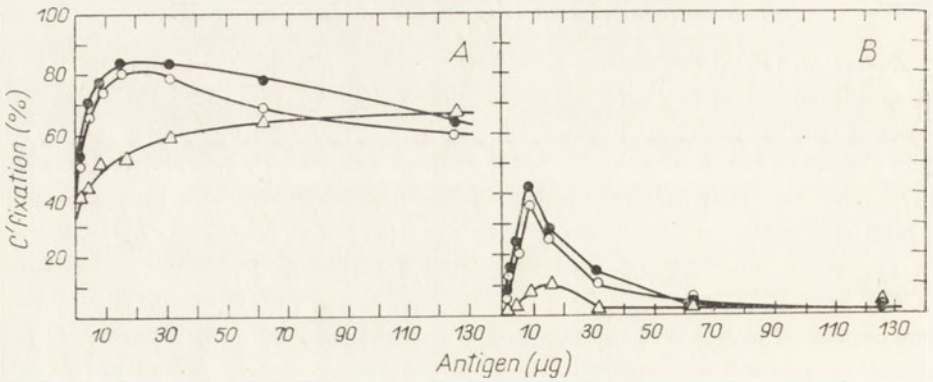

Fig. 5. Complement fixation by native apoprotein (●) and the apoprotein modified by oxidation of 50% of tryptophan residues (○) or of all accessible tryptophan residues (△), i.e. Apo-50 and Apo-100, respectively. Anti-flavoprotein serum was diluted at the following ratios: *A*, 1:128; *B*, 1:512.

Thus, although on immunodiffusion and immunoelectrophoresis the preparation Apo-100 gave no precipitation bands, its affinity to the anti-flavoprotein antibody was demonstrated in the complement fixation test; the reactivity of Apo-100 was low and showed different properties as compared with native apoprotein and Apo-50.

DISCUSSION

Kinetics of equilibrium dialysis of riboflavin flavoprotein and the flavoprotein-antibody complex, and the results concerning binding capacity for riboflavin of the apoprotein-antibody complex, provided direct evidence that the binding site for riboflavin was not blocked on complexing with antibody. The binding of riboflavin by the apoprotein-antibody precipitate was low; full binding capacity was obtained on solubilization of the precipitated complex with low KCl or urea concentration under conditions where no dissociation of the complex occurred. This indicates that the part of polypeptide surrounding the binding site for riboflavin is not an antigenic determinant.

Participation of tryptophan residues in the antigenic determinant of protein has been extensively studied. Several proteins obtained from snake venom have one tryptophan residue. Chang & Yang (1973) modified the tryptophan residue of cobrotoxin, causing almost complete loss of toxicity without affecting the antigenic specificity. One tryptophan residue was demonstrated to participate in the antigenic determinant of lysozyme (Strosberg & Kanarek, 1969).

Sperm-whale myoglobin has two tryptophan residues at positions 7 and 14 (Atassi & Caruso, 1968; Atassi, 1975). It has been demonstrated that modification of tryptophan at position 7 does not affect the antigenic specificity. On the other hand, modification of both tryptophan residues causes a discernible change in myoglobin structure and a lowering of antigenicity (Atassi & Caruso, 1968).

In the egg-yolk riboflavin flavoprotein two kinds of exposed tryptophan residues may be discerned.The first, consisting of 2 - 3 residues, is located at the riboflavin--binding site, and probably participates in the conformational changes of apoprotein occurring during binding of riboflavin (Żak *et al.*, 1972; Żak, 1973; Steczko & Ostrowski, 1975). Oxidation of these residues by NBS results in the loss of riboflavin-binding capacity without affecting the immunological reactivity.

The second kind consists of 3 - 4 tryptophan residues. They are less susceptible to modification by NBS and are probably located in the hydrophobic part of the apoprotein molecule, distant from the riboflavin binding site. Modification of these residues lowers the immunological reactivity. Probably, their oxidation affects the conformation of the apoprotein in the hydrophobic part of the molecule, which leads to changes in localization of polar amino acid residues in antigenic determinants. Such changes (Atassi & Caruso, 1968; Atassi, 1975) in conformation of antigenic determinants lower the immunochemical reactivity of the protein.

The authors are thankful to Professor Włodzimierz Ostrowski for critical reading of the manuscript; to Doctor Bogumiła Panczenko for fluorimetric measurements and to Miss Alina Bulsiewicz, B. Sc., for her help in complement fixation experiments.

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WPŁYW PRZECIWCIAŁA NA INTERAKCJĘ WITAMINY B_2 Z RYBOFLAWINOWYM APOPROTEIDEM

Streszczenie

1. Porównano dysocjację ryboflawinowego flawoproteidu z kompleksem flawoproteid-przeciwciało i wykazano, że odszczepianie ryboflawiny zachodzi w tych samych warunkach dla obu kompleksów.

2. Apoproteid w połączeniu z przeciwciałem w postaci osadu wytrąconego w obojętnym buforze fosforanowym wykazuje zdolność do wiązania ryboflawiny w 15%. Natomiast w 80 - 90% wiąże ryboflawinę kompleks apoproteid-przeciwciało w 0,3 M roztworze chlorku potasu i w 1 M moczniku.

3. Zmodyfikowany apoproteid po utlenieniu 50% reszt tryptofanu traci zdolność do wiązania ryboflawiny ale ma podobne własności antygenowe do natywnego apoproteidu. Apoproteid z utlenionymi całkowicie resztami tryptofanu (Apo-100) ma znacznie obniżoną antygenowość.

4. Przeprowadzone badania sugerują, że otoczenie miejsca wiązania ryboflawiny w ryboflawinowym flawoproteidzie nie ma własności determinanty antygenowej.

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MARIA GUMIŃSKA and JAN STERKOWICZ

EFFECT OF SODIUM FLUORIDE ON GLYCOLYSIS IN HUMAN ERYTHROCYTES AND EHRLICH ASCITES TUMOUR CELLS *IN VITRO* *

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In human erythrocytes and Ehrlich ascites tumour cells, NaF inhibited aerobic glucose utilization and lactate formation. The inhibition of glycolysis was accompanied by a decrease in cellular pyruvate and ATP, and by accumulation of 2-phosphoenolpyruvate. These results and direct enzymatic determinations showed that fluoride inhibits, in addition to enolase (phosphopyruvate hydratase, EC 4.2.1.11), also pyruvate kinase (EC 2.7.1.40).

Fluoride was one of the first compounds known to inhibit the glycolytic pathway (Lohmann & Meyerhof, 1934) and has long been used as an inhibitor of unnecessary fermentation. The target enzyme of fluoride action was found to be enolase (Warburg & Christian, 1941) which is known to require for its activity Mg^{2+} ion, and to form, in the presence of phosphate, inactive magnesium - fluoride - phosphate complex. Later, the inhibitory effect of fluoride was demonstrated with respect to other glycolytic enzymes activated by magnesium (see Najjar, 1962; Pizer, 1962; Malmström *et al.*, 1962) and glucose-6-phosphatase (Stetten, 1964). Thus, although fluoride may affect different enzymes, it seems that its predominant effect in the intoxication is on the cell energy metabolism.

Due to the increasing environment pollution in some industries, we have studied the erythrocytes of workers exposed to prolonged contact with fluoride, and found that, along with a decrease in ATP content, there appeared an adaptive increase in the activity of magnesium-dependent enzymes, namely enolase, pyruvate kinase and ATPase (Gumińska & Sterkowicz, 1975).

To gain more insight into this paradoxical effect of fluoride, enzymatic and metabolic studies were performed *in vitro* on human erythrocytes and Ehrlich ascites tumour cells.

* This work was supported partly by the Committee of Medical Sciences of the Polish Academy of Sciences (Division in Kraków).

MATERIAL AND METHODS

Erythrocytes. Blood of healthy persons was collected into tubes containing disodium versenate (2 mg/ml of blood) and centrifuged for 10 min at 1000 *g*. Plasma and the buffy coat were discarded, and the erythrocytes were washed twice with physiological saline solution.

Ehrlich ascites tumour cells, passaged on CBA mice, were taken for experiments 10 days after inoculation, centrifuged for 10 min at 1000 *g* and washed twice with physiological saline solution.

Incubations. The washed cells were resuspended in Krebs-Ringer phosphate buffer, pH 7.4 (Umbreit *et al.*, 1951) containing 10 mM-glucose, and incubated at 37°C in the absence (control) or in the presence of 0.24 or 1.2 mM-NaF. At zero time and at intervals of 15 - 30 min, portions were withdrawn and added to 0.6 M-perchloric acid for determination of glucose by the method of Hugget & Nixon (1957) and lactate according to Horn & Bruns (1956). Up to 90 min incubation, lactate formation and glucose utilization were linear; the results are expressed as nanomoles per 60 min per mg protein. After 60 or 90 min of incubation, the remaining cell suspensions were centrifuged, the sedimented cells homogenized with cold 0.6 M-perchloric acid, the supernatant was neutralized with $KHCO_3$ (*in substantia*) to remove the excess of perchloric acid, then ATP was determined according to Adam (1965), pyruvate by the method of Horn & Amelung (1957) and, following pyruvate determination, in the same samples 2-phosphoenolpyruvate[1] was assayed by the method of Czok & Eckert (1965). Concentration of metabolites was expressed as nmoles per milligram of protein.

Protein was determined according to Lowry *et al.* (1951).

Enzyme assays. Erythrocytes were haemolysed by freezing and addition of an equal volume of water, then 40-fold diluted with 200 mM-Tris-HCl buffer, pH 7.4, containing 115 mM-KCl, 10 mM-$MgCl_2$ and 2 mM-EDTA, and the activity of enolase and pyruvate kinase was estimated spectrophotometrically at 340 nm as described by Gumińska & Sterkowicz (1975) and Gumińska *et al.* (1975).

Special reagents used, including preparations of enolase and pyruvate kinase from rabbit muscle, were products of Boehringer GmbH (Mannheim, G.F.R.).

RESULTS

The effect of two different concentrations of NaF on glycolysis and the content of metabolites in human erythrocytes *in vitro*, is presented in Table 1. Lactate formation at 1.2 mM-NaF was inhibited by more than 50%, whereas glucose utilization was somewhat less affected, the inhibition not exceeding 25%. The decrease in lactate formation was accompanied by a decrease in pyruvate content which, depending on NaF concentration, dropped even to about 25% of the control value. Inhibition of glycolysis resulted in a decrease by a half of ATP, connected with accumulation

[1] Abbreviation: PEP, 2-phosphoenolpyruvate.

of PEP. The greater was the decrease of lactate formation and pyruvate and ATP contents, the higher was the accumulation of PEP, reaching values even twice as high as in the control. Thus, at the stage of PEP to pyruvate conversion there ap-

Table 1

Effect of sodium fluoride on aerobic glycolysis and concentration of some metabolites in human erythrocytes

For incubation, Krebs-Ringer phosphate buffer, pH 7.4, containing 10 mM-glucose and 50 - 60 mg of erythrocyte protein/ml was used. Temperature 37°C. Lactate formation and glucose uptake are expressed as nmoles/mg protein/hour. Concentration of pyruvate, PEP and ATP were determined in cell sediment after 90 min of incubation and expressed as nmoles/mg protein. The results are mean values from 6 independent experiments (except for lactate — 12 experiments).

Estimated compound	Control (nmoles/mg protein)	0.24 mM-NaF		1.2 mM-NaF	
		nmoles/mg protein	% of control	nmoles/mg protein	% of control
Lactate formation (per hour)	43±17	26±12[b]	60.4	20±9[b]	46.5
Glucose uptake (per hour)	49±20	37±15	75.5	39±15	79.5
Pyruvate concn.	22.9±3.4	12.8±7.7[b]	55.8	6.0±3.9[a]	26.2
ATP concn.	4.8±1.5	4.5±1.5	93.7	2.4±2.1[c]	50.0
2-Phosphoenol-pyruvate concn.	10.6±7.3	18.7±8.6[b]	176.4	23.6±19.8[b]	222.6

[a] $P=0.001$; [b] $P=0.01$; [c] $0.05<P<0.1$.

Table 2

Effect of sodium fluoride on aerobic glycolysis and concentration of some metabolites in Ehrlich ascites tumour cells

For incubation, Krebs-Ringer phosphate buffer, pH 7.4, containing 10 mM-glucose and 3 mg of cell protein/ml, was used. Temperature 37°C. Lactate formation and glucose uptake are expressed as nmoles/mg protein/hour, and are mean values ± S.D. from 18 independent experiments. Concentration of pyruvate, PEP and ATP were determined in cell sediment after 60 min incubation and are expressed as nmoles/mg protein; the results of two separate experiments are given.

Estimated compound	Control (nmoles/mg protein)	0.24 mM-NaF		1.2 mM-NaF	
		nmoles/mg protein	% of control	nmoles/mg protein	% of control
Lactate formation (per hour)	600±160	510±160	85.0	410±190[a]	68.3
Glucose uptake (per hour)	370±120	310±70[c]	83.7	270±70[b]	72.9
Pyruvate concn.	2.33; 2.00	0.86; 1.33	36.9; 66.5	0.48; 0.81	20.6; 40.5
ATP concn.	7.38; 5.40	3.60; 2.15	48.7; 39.8	2.00; 1.07	27.1; 19.8
2-Phosphoenol-pyruvate concn.	2.70; 2.25	7.24; 8.00	268.1; 355.5	8.72; 10.00	322.9; 444.4

[a] $0.001<P<0.005$; [b] $P=0.01$; [c] $0.01<P<0.05$.

peared an unexpected cross-over in the concentration of these compounds, which pointed to the inhibition of pyruvate kinase.

Since erythrocytes have low glycolytic activity, the possible occurrence of an analogous effect was studied in Ehrlich ascites tumour cells which show high aerobic glycolysis (Table 2). The aerobic lactate formation and glucose utilization in these cells exceeded tenfold the values found for erythrocytes, and inhibition by NaF did not exceed 30%.

Inhibition of glycolysis in tumour cells was accompanied by similar changes in the metabolite content. The decrease in lactate formation was concomitant with a decrease in pyruvate to about 30%, and in ATP to 25% of the control values, and by a threefold increase in PEP concentration. This shows that in tumour cells, similarly as in erythrocytes, the inhibition by NaF affects, in addition to enolase, also pyruvate kinase.

In Ehrlich tumour cells the sum of ATP and PEP, the two high-energy metabolites, was the same after incubation with and without NaF, whereas in erythrocytes in the presence of NaF it was even somewhat higher (Fig. 1). The shift in ATP concentration in favour of PEP, effected by NaF, points to the importance of pyruvate kinase in maintaining cellular energy equilibrium.

The effect of fluoride on pyruvate kinase activity was tested on erythrocyte haemolysate. Similarly to enolase, pyruvate kinase was inhibited, although to a somewhat smaller extent (Fig. 2). The same results were obtained with commercial enzyme preparations from rabbit muscle.

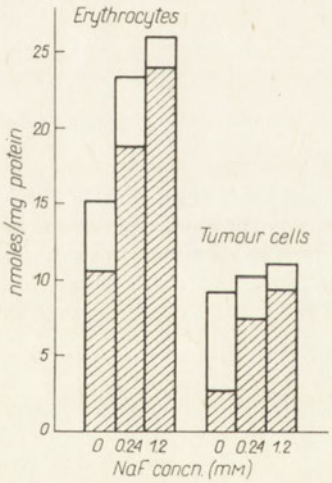

Fig. 1

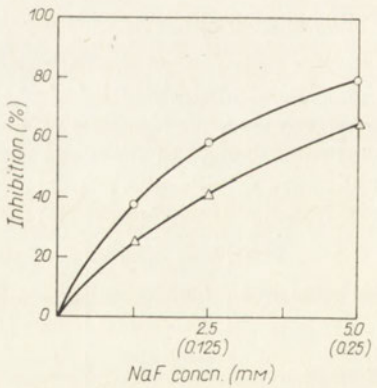

Fig. 2

Fig. 1. Effect of sodium fluoride on the content of 2-phosphoenolpyruvate (hatched columns) and ATP (outlined columns) in human erythrocytes and Ehrlich ascites tumur cells after, respectively, 90 and 60 min incubation in Krebs-Ringer phosphate buffer, pH 7.4, containing 10 mM-glucose.

Fig. 2. Inhibition of enolase (○) and pyruvate kinase (△) activity in erythrocyte haemolysate by sodium fluoride. The indicated NaF concentrations are the initial ones (after addition of inhibitor to the enzyme); final NaF concentrations in the incubation mixture are given in parentheses.

DISCUSSION

Shearer & Suttie (1970) demonstrated that in rats NaF, by inhibiting enolase activity, lowered liver lactate and pyruvate concentrations. In the present work, the inhibitory effect of NaF was confirmed *in vitro* with respect to glucose utilization and lactate formation, both in erythrocytes which have no mitochondria and in Ehrlich ascites tumour cells which show a high rate of aerobic glycolysis. In the Ehrlich tumour cells, lactate formation and pyruvate concentration may be dependent not only on glycolysis but also on the rate of their final oxidation at the stage catalysed by succinate dehydrogenase (Lovelace & Miller, 1967).

As demonstrated in the present work, fluoride inhibits not only enolase but also pyruvate kinase. This was testified to by the accumulation of PEP connected with a decrease of pyruvate in both studied types of cells, and by the direct inhibition of the erythrocyte pyruvate kinase activity. There are also some data pointing to formation of pyruvate kinase - metal - fluorine - phosphoryl complex (Mildvan, 1970). The inhibition of pyruvate kinase seems to be of greater importance for the over-all cell energy metabolism than the inhibition of enolase. Pyruvate kinase is a key and regulatory enzyme of glycolysis (Weber, 1969) and its activity in normal cell is lower than that of enolase (Gumińska, 1971). Pyruvate kinase, being an allosteric enzyme (Haeckel *et al.*, 1968) is also affected by various physiological conditions (Tanaka *et al.*, 1965; Weber, 1969; Tanaka & Pagilia, 1971).

Shearer & Suttie (1970), who also reported on the cross-over between PEP and pyruvate concentration in liver of animals exposed to intoxication with fluoride, related this effect to starvation caused by decreased ingestion of carbohydrates, which induce pyruvate kinase in liver. As it is known, in starvation or diabetes, liver pyruvate kinase activity is lowered (Krebs & Eggleston, 1965). This cannot, however, account for the effect of NaF on isolated cells, and for the inhibition of enzyme activity *in vitro*.

Inhibition of pyruvate kinase activity results in changes not only in concentration of final glycolysis products but, first of all, in cell ATP concentration. The decrease of ATP under the influence of fluoride in HeLa cells was observed by Berny (1969) and in erythrocytes by Feig *et al.* (1971). Erythrocytes, for which the glycolytic pathway provides practically all the energy, have a lower content of ATP than Ehrlich ascites tumour cells. Tumour cell ATP may be derived, however, not only from respiration but be related to much lower accumulation of PEP, due to the high pyruvate kinase activity (Gumińska, 1969, 1971).

The effect of fluoride on the energy metabolism may explain the inhibition of tissue explants development *in vitro* (Myers, 1973), which could be overcome by pyruvate entering into energy metabolism below the step blocked. In chronic NaF intoxication, inhibition of animal growth and lowering of muscular tension (Gardiner, 1970) could also be explained by the decreased ATP supply. So far, however, these effects were related to starvation of the intoxicated animals.

It seems worth noting that the sum of molar ATP and PEP concentrations in erythrocytes was even raised, probably due to inhibition of some energy-requiring

processes. However, the relative stability of the sum of these high-energy compounds, and the shifts among them caused by NaF, stress the importance of pyruvate kinase for the cellular energy metabolism.

The presented experiments performed on isolated erythrocytes and tumour cells suggest that the effect of NaF on energy metabolism may have a more general character, especially for these tissues which accumulate fluoride. In workers chronically exposed to fluoride a decrease in ATP was found in erythrocytes although they do not accumulate fluoride. At the same time, pyruvate kinase activity was elevated (Gumińska & Sterkowicz, 1975). This discrepancy with the present results becomes understandable when the enzyme activity inhibited by fluoride *in vivo* and *in vitro* is distinguished from concentration of the enzymatic protein, which might be induced in response to prolonged action of fluoride.

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WPŁYW FLUORKU SODOWEGO *IN VITRO* NA METABOLIZM GLIKOLITYCZNY ERYTROCYTÓW LUDZKICH I KOMÓREK RAKA WYSIĘKOWEGO EHRLICHA

Streszczenie

Fluorek sodu hamował tlenowe zużycie glukozy i produkcję mleczanu w erytrocytach ludzkich i w komórkach raka wysiękowego Ehrlicha. Zahamowanie glikolizy było połączone ze spadkiem zawartości pirogronianu i ATP w komórkach oraz z akumulacją 2-fosfoenolopirogronianu. Efekt ten oraz bezpośrednie oznaczenia aktywności enzymatycznej wskazują, że fluorki obok enolazy (hydrataza fosfopirogronianowa, EC 4.2.1.11) hamują także kinazę pirogronianową (EC 2.7.1.40).

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GLYCOPROTEIN NATURE OF TANNASE IN *ASPERGILLUS NIGER*

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1. Tannase (tannin acyl-hydrolase, EC 3.1.1.20) was isolated from the culture medium of *Aspergillus niger* and purified about 200-fold. On polyacrylamide-gel electrophoresis it gave a single band.

2. The molecular weight of the enzyme was of the order of 55 000 as determined by gel filtration. The enzyme contains 21.5% of carbohydrates (mannose and glucose).

3. Treatment of tannase with alkaline borohydride decreased the content of threonine, serine and mannose, suggesting that the carbohydrate - peptide linkage is of the *O*-glycoside type, involving mannose linked to threonine and serine.

Although previous publications (Haworth *et al.*, 1958; Haslam *et al.*, 1961; Armitage *et al.*, 1961; Dhar & Bose, 1964) dealt with partial purification of tannase from *Aspergillus niger*, little is known about its chemical nature. In this paper, the purified tannase from *A. niger* was characterized as a glycoprotein, and the nature of its carbohydrate - peptide linkage has been studied.

MATERIALS AND METHODS

Assay of tannase activity. For the estimation of tannase activity, the method of Dhar & Bose (1964) was adopted using methyl gallate as the substrate. One mg of gallic acid liberated from digestion mixture (11 ml) containing tannase preparation (1 ml) and 10 ml of methyl gallate (10 mg) solution was designated as one unit of tannase activity.

The protein content was estimated by the method of Lowry *et al.* (1951) using twice crystallized trypsin (Nutritional Biochemical Corporation, U.S.A.) as the reference protein (Dhar & Bose, 1961a).

Purification procedure. The enzyme extract was prepared from the pads of *A. niger* grown on a synthetic medium containing tannic acid, and was purified sequentially by protamine sulphate treatment, ammonium sulphate precipitation at pH 3.6 and reprecipitation by acetone as previously described (Dhar & Bose, 1964). For further

purification, the lyophilized enzyme was dissolved in 5 ml of 0.05 M-Tris·HCl buffer (pH 7.0) and applied to Sephadex G-200 (Pharmacia Fine Chemicals, Sweden) column (0.9 × 108 cm) which was equilibrated and eluted with the same buffer. The flow rate was maintained at 10 ml/h and 5 ml fractions were collected. The active fractions (11 - 15) were pooled, dialysed against water and then concentrated to dryness *in vacuo*. The dry enzyme obtained after Sephadex G-200 chromatography was dissolved in 10 ml of 0.1 M-acetate buffer (pH 5.8) and subjected to DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Sweden) chromatography. The column (36 × × 2.5 cm) was equilibrated and eluted first with the same buffer, then with a linear gradient of 0.1 - 0.6 M-acetate buffer (pH 5.8). The flow rate was maintained at 25 ml/h and 5 ml fractions were collected. The active fractions (21 - 26) were pooled, dialysed against water and then concentrated to dryness *in vacuo*.

The purification was carried out with several batches of the enzyme extract from *Aspergillus niger* to obtain an amount (about 20 mg) of the purified tannase sufficient for its characterization.

Polyacrylamide-gel electrophoresis. The purified tannase was subjected to polyacrylamide-gel disc electrophoresis as described by Davis (1964) using 7.5% gel in Tris-glycine buffer (pH 8.3).

Molecular weight determination. The approximate molecular weight of tannase was determined by the gel filtration procedure of Andrews (1964). The protein standards, viz. α-chymotrypsin of mol.wt. 22 500, and trypsin, mol.wt. 23 800 (both from Nutr. Biochem. Corp., U.S.A.), pepsin, mol.wt. 35 500 (Worthington Biochem.Corp., U.S.A.), ovalbumin (45 000), and serum albumin (68 000) were applied to Sephadex G-100 column (2.5 × 116 cm) together with tannase (2 mg); 0.05 M-Tris·HCl buffer containing 0.1 M-KCl (pH 7.3) was used for elution. The flow rate was maintained at 30 ml/h; 10 ml fractions were collected and protein was monitored by measuring the absorbance at 280 nm.

Carbohydrate analysis. The content of neutral and amino sugars in tannase (2 mg) was determined after resin hydrolysis (Bose, 1963) which released sugars without any appreciable destruction. The phenol-sulphuric acid method of Dubois *et al.* (1956) was adopted for quantitative estimation of total neutral sugars. The descending paper (Whatman no. 1) chromatography (Gebhardt, 1960) and the quantitative method of Joseph & Bose (1959) were used for identification and the assay of the individual neutral sugars. Amino sugars were estimated by the method of Elson & Morgan (1933) as modified by Rimington (1940).

Treatment of tannase with alkaline borohydride. Purified tannase (5 mg) made 1 M with respect to sodium borohydride was incubated at 25°C for 20 min. The required quantity of 1 M-NaOH solution was then added to make the solution 0.1 M with respect to alkali and the solution was kept under nitrogen in a stoppered flask for 70 h at 25°C. The solution neutralized with glacial acetic acid, was concentrated to dryness *in vacuo* at 40°C and subjected to acid hydrolysis (6 M-HCl, 105°C, 24 h). Amino acids in the hydrolysate were analysed by the paper chromatographic method of Levy & Chung (1953) as described by Dhar & Bose (1961b). α-Aminobutyric acid was estimated chromatographically according to Opieńska-

-Blauth *et al.* (1960). At the same time, an aliquot of the treated tannase was analysed for sugar alcohols by paper chromatography using *n*-butanol - ethanol - water (10:1:2, by vol). The sugars were spotted by silver reagent (Trevelyan *et al.*, 1950).

RESULTS AND DISCUSSION

Data given in Table 1 show that tannase from *Aspergillus niger* was purified by the procedure of Dhar & Bose (1964) about 100-fold. On further purification by two additional steps, viz. Sephadex G-200 and DEAE-Sephadex A-25 chromatography, specific activity was increased about twofold, i.e. the final purification achieved was 200-fold. The purified tannase was found to be homogeneous on polyacrylamide-gel disc electrophoresis at pH 8.3 (Fig. 1).

Table 1

The purification procedure of tannase of Aspergillus niger

Treatment	Total activity (units)	Total protein (mg)	Specific activity	Yield (%)
Crude extract	8400	1100	7.6	100
Purified preparation*	6130	7.20	851.4	74
Sephadex G-200 chromatography	5000	4.12	1219.5	60
DEAE-Sephadex A-25 chromatography	4050	2.61	1570.0	49

* Dhar & Bose (1964).

A molecular weight of about 55 000 was assigned for the enzyme on the basis of the elution volume from Sephadex G-100 column as compared with those of marker proteins (Fig. 2).

As can be seen from Table 2, the carbohydrate moiety (21.5%) of tannase is composed of about 50 moles of mannose and 16 moles of glucose. As the carbohydrate moiety of tannase was released on acid hydrolysis, a covalent linking to peptide moiety was assumed, suggesting the glycoprotein nature of the enzyme. Many other enzymes, e.g. glucose oxidase, α-amylase, bromelain, were shown to be glycoproteins (Justiz & De La Lllosa, 1972; Pazur & Aronson, 1972).

O-Glycosidic linkage of carbohydrate with serine or threonine is susceptible to alkali (Anderson *et al.*, 1963; Neuberger *et al.*, 1972; Spiro, 1972) and the alkali treatment of glycoproteins containing *O*-glycosidically linked serine or threonine results in β-elimination of carbohydrate and formation of unsaturated amino acids. These, on reduction followed by acid hydrolysis of peptide linkage, yield alanine (from serine) and α-aminobutyric acid (from threonine). When tannase was subjected to alkaline borohydride treatment (Table 3), the content of threonine, serine and mannose was reduced, respectively, by 64, 49 and 67%, with a concomitant increase

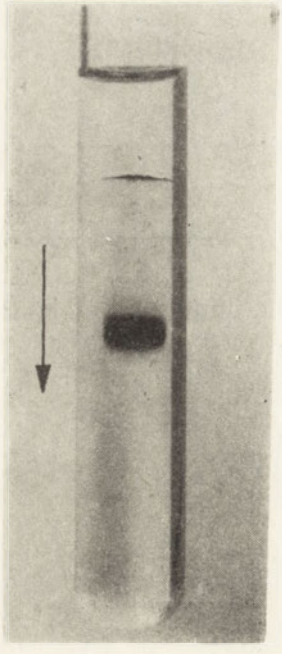

Fig. 1

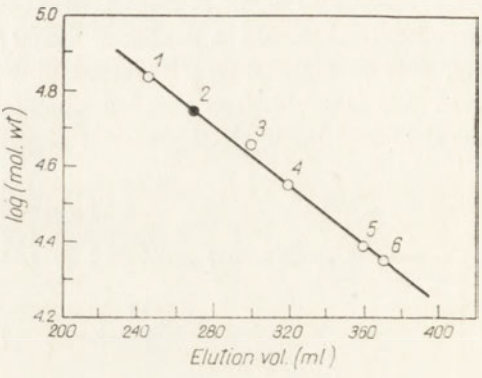

Fig. 2

Fig. 1. Polyacrylamide-gel disc electrophoresis of tannase from *A. niger* (100 µg of protein). For details see Methods.

Fig. 2. Molecular weight determination of tannase from *A. niger*. *1*, Bovine serum albumin; *2*, tannase; *3*, ovalbumin; *4*, pepsin; *5*, trypsin; *6*, α-chymotrypsin. For details see Methods.

in alanine content (23%). There was no significant change in the content of other amino acids and glucose. α-Aminobutyric acid and mannitol were identified in the acid hydrolysate of the treated tannase. These observations imply that mannose was *O*-glycosidically linked to threonine and serine of the polypeptide chains. The linkage of serine with mannose was demonstrated in Taka amylase (Tsugita & Akabori, 1959) and the glycosidic links of mannose with serine and threonine were reported in glucoamylase from *A. niger* (Lineback, 1968).

Table 2

Carbohydrate composition of tannase of Aspergillus niger

Component	g/100 g	Moles/mole of tannase*
Total neutral sugars (expressed as mannose)	21.5	
Mannose	16.2	49.5
Glucose	5.1	15.6

* Based on the molecular weight of 55 000.

Table 3

Effect of alkaline borohydride treatment on the content of amino acids and sugars of tannase

Component	Content (μmoles/mg)		
	before treatment	after treatment	Δ
Aspartic acid	0.84	0.80	−0.04
Threonine*	0.70	0.25	−0.45
Serine*	0.61	0.31	−0.30
Glutamic acid	0.82	0.80	−0.02
Glycine	1.44	1.56	+0.12
Alanine	0.91	1.12	+0.21
α-Aminobutyric acid	0.00	Present**	
Glucose	0.28	0.29	+0.01
Mannose	0.90	0.30	−0.60
Mannitol	0.00	Present**	

* Results uncorrected for losses during acid hydrolysis.
** Not analysed quantitatively.

Eylar (1965) suggested that most of the extracellular proteins are glycoproteins and that the carbohydrate moiety in glycoproteins promotes excretion of the protein into the extracellular environment. However, Marshall (1972) pointed to a number of exceptions. The extracellular enzymes, viz. pepsin, trypsin, chymotrypsin and ox ribonuclease A do not contain carbohydrate components. On the other hand, certain intracellular proteins are glycoproteins. The tannase in *A. niger*, an essentially intracellular protein, was also found to be a glycoprotein.

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GLIKOPROTEIDOWY CHARAKTER TANNAZY Z *ASPERGILLUS NIGER*

Streszczenie

1. Tannazę izolowano z płynu hodowlanego *A. niger* i oczyszczono ok. 200-krotnie. W elektroforezie na żelu poliakryloamidowym enzym stanowił pojedynczy prążek.

2. Ciężar cząsteczkowy enzymu oznaczony przez filtrację na żelu wynosi ok. 55 000. Enzym zawiera 21.5% węglowodanów (mannozy i glukozy)

3. Działanie na tannazę alkalicznym borowodorkiem zmniejszało zawartość treoniny, seryny i mannozy, co wskazuje, że wiązanie węglowodanowo-peptydowe między mannozą a treoniną i/lub seryną jest typu *O*-glikozydowego.

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EFFECT OF SOME LIPIDS ON THE ACTIVITY OF RENIN *IN VITRO*

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A partially purified bovine renin preparation and a homologous renin substrate were used to study the effect of some lipids on the amount of angiotensin formed. It was found that lecithin and the detergents used (deoxycholate and sodium dodecylate) inhibited the renin activity. Palmitic acid, oleic acid and cholesterol had no distinct effect. On the other hand, the reaction was enhanced by prostaglandins E_2 and $F_{2\alpha}$.

The role of the renin-angiotensin system in blood pressure regulation prompted a number of workers to undertake studies on kinetics of the reaction catalysed by renin and participation of inhibitors and activators in regulation of the enzyme activity. It appeared that, as renin inhibitors, may act lower polypeptides resembling in their structure the *N*-terminal sequence of the renin substrate (Skeggs *et al.*, 1964), as well as derivatives of tetrapeptide containing *N*-terminal leucylleucine or C-terminal phenylalanine or tyrosine (Hoobler *et al.*, 1964; Kokubu *et al.*, 1968). The inhibition by tissue phospholipids was first demonstrated by Sen *et al.* (1967) who from acetone-dried kidney isolated a phospholipid, similar in structure to phosphatidylserine, which inhibited renin activity *in vitro*, and lowered blood pressure of chronic renal hypertensive rats.

The structure, properties and physiological role of tissue phospholipids have not been fully elucidated. According to Osmond *et al.* (1969), the phospholipids liberated from tissues are inactive, and are transformed enzymatically into the active inhibitor within the blood vessel. The studies of Pfeiffer *et al.* (1971, 1972) indicate that phosphatidylethanolamine containing polyunsaturated fatty acids is the main component of phospholipid inhibitors. However, some authors question the physiological role of phospholipid inhibitors inferred from experiments *in vitro* (Poulsen, 1971).

In the present paper, the effect of some lipids occurring in normal blood on the course of the reaction catalysed by renin, was studied.

MATERIALS AND METHODS

To determine the inhibitory properties of some natural lipids: oleic and palmitic acids, cholesterol, lecithin, and prostaglandins E_2 and $F_{2\alpha}$, they were incubated with partially purified bovine renin and bovine renin substrate in 0.15 M-phosphate buffer, pH 5.5. To inhibit the activity of angiotensinases, 0.1 μM-diisopropylfluorophosphate (DFP) was used. Changes in the amount of angiotensin formed in relation to the control sample, were taken as a measure of the inhibitory activity of the compounds studied.

Preparation of renin. Renin was isolated from bovine renal cortex as described by Newsome (1969). From several-fold frozen and thawed kidney, a 50% aqueous homogenate was prepared and centrifuged at 6000 *g*. From the supernatant, protein was fractionated with ammonium sulphate at different pH values. The final precipitate was dissolved with water to final ammonium sulphate concentration of 0.8 M, desalted on Sephadex G-25 and then subjected to molecular gel filtration on Sephadex G-100. Renin activity was located in the second fraction, and was further purified by chromatography on CM-Sephadex. The obtained active fraction was concentrated by dialysis against polyethylene glycol and stored at −20°C.

Preparation of renin substrate. From freshly taken bovine blood, serum was separated, cooled to 4°C and subjected to molecular gel filtration on cooled Sephadex G-200 column (50 × 800 mm) equilibrated with 0.1 M-Tris buffer, pH 8, containing 0.2 M-NaCl. The column was eluted with the same buffer under a pressure of 100 mm water. The fraction showing substrate activity for renin was concentrated by dialysis against polyethylene glycol and dialysed against 0.15 M-phosphate buffer, pH 5.7, containing 0.1 M-NaCl, then 20% of glycerol was added and the preparation was stored at −20°C.

Protein was determined by the method of Kingsley in the modification of Weichselbaum (1946) or according to Lowry *et al.* (1951).

Electrophoresis was performed on Whatman no.1 paper in 0.1 M-veronal buffer, pH 8.6, or on polyacrylamide gel according to Davis (1964). Protein was stained with Amido Black 10 B.

Determination of renin activity. This was performed by the modified method of Boucher *et al.* (1964). The composition of the reaction mixture, which always contained DFP, is presented in the respective Tables and legends to Figures. The angiotensin formed during 3-h incubation at 37°C was adsorbed on a column (0.8 × 8 cm) of Dowex WX2, 100 - 200 mesh. The column was then washed with 15 ml of 0.2 M-ammonium acetate, pH 6, and 15 ml of water. Angiotensin was eluted with 15 ml of 0.1 M-diethylamine and then with 15 ml of 0.2 M-ammonia. The pooled eluates were freeze-dried, dissolved in an appropriate volume of physiological saline solution, and the amount of angiotensin was determined by measuring the increase in blood pressure of nephrectomized rats.

Under standard conditions (see below), the procedure used gave reproducible results (Table 1). When the renin activity was about 400 μg of angiotensin formed per sample, standard deviation did not exceed 6.5%. The recovery of added synthetic

angiotensin after 3-h incubation with serum or with the renin substrate preparation, amounted to 80 - 87% (Table 2). With the use of silicone glass, sterile conditions of determinations, and in the presence of DFP, the results were somewhat better than those obtained by the original method.

Table 1

Reproducibility of renin activity determination

The enzyme activity was determined after 3 h of incubation at 37°C and pH 5.5 in the presence of 0.1 M-DFP, and expressed as nanograms of angiotensin formed per sample. Mean values ± S.D. are given, with the number of determinations in parentheses.

Incubation mixture	Activity
Bovine serum, 10 ml (780 mg protein)	35 ± 4.8 (10)
Bovine serum, 10 ml (780 mg protein) + renin, 0.1 ml (110 µg protein)	635 ± 33.0 (10)
Bovine renin substrate, 4 ml (150 mg protein) + renin, 0.1 ml (110 µg protein) + phosphate buffer, 5.9 ml	450 ± 28.0 (6)

Table 2

Recovery of the added angiotensin

To bovine serum and the preparation of bovine renin substrate, synthetic angiotensin was added as indicated and the mixture containing 0.1 M-DFP was incubated at 37°C and pH 5.5 for 3 h; then the amount of angiotensin was determined.

Incubation mixture	Angiotensin (ng)		Recovery (%)
	added	determined	
Bovine serum, 10 ml (780 mg protein)	0	35	
	200	195	80
Bovine renin substrate, 4 ml (150 mg protein) + phosphate buffer, 5.9 ml	0	0	
	100	83	83
	200	175	87.5
	400	350	87.5

The amount of the angiotensin formed was estimated by comparison with the effect of administration of at least two different doses of synthetic angiotensin (Fig. 1). The freeze-dried experimental sample of angiotensin formed was diluted in such a way that 0.1 ml gave an increase in blood pressure within the limits obtained with standard solutions. The amount of angiotensin in the sample was calculated from the equation:

$$\text{Angiotensin formed (ng)} = \frac{\text{Increase in blood pressure after administration of a 0.1 ml sample}}{\text{Increase in blood pressure after administration of 0.1 ml of authentic angiotensin}} \times$$

$$\times \text{ ng of synthetic angiotensin} \times \text{dilution}.$$

Reagents, of analytical grade or pure, were from the following sources: sodium dodecylate (Koch-Light Lab., Colnbrook, Bucks, England), sodium deoxycholate (Polfa, Warszawa, Poland), oleic acid (Merck, Darmstadt, G.F.R.), palmitic acid (Reachim, U.S.S.R.), lecithin (B.D.H., Poole, Dorset, England), prostaglandins E_2 and $F_{2\alpha}$ (Upjohn Co., Kalamazoo, Mich., U.S.A.), synthetic angiotensin (Ciba--Geigy Ltd., Basle, Switzerland).

RESULTS

The renin preparation obtained by the applied procedure was purified 160-fold as compared with the initial aqueous extract of renal cortex; its specific activity was about 4 μg of angiotensin per 1 mg of protein per 3 h. On paper electrophoresis the preparation gave one band with the mobility of albumins, whereas on polyacrylamide-gel electrophoresis it separated into four protein fractions (Fig. 2). Taking into account the low yield of the isolation procedure and low stability of the more highly purified enzyme (Lucas *et al.*, 1970), its further purification was not attempted. The preparation obtained did not lose the activity after 6 months of storage at −20°C.

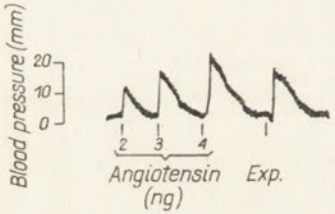

Fig. 1

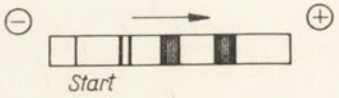

Fig. 2

Fig. 1. Records of rat blood pressure after administration of the indicated amount of synthetic angiotensin, or *Exp.*, 0.1 ml of the sample studied.

Fig. 2. Polyacrylamide-gel electrophoresis at pH 7.9 of the purified renin preparation. To the gel, 30 μg of protein was applied. The protein was stained with Amido Black.

It should be noted that even highly purified renin preparations give on disc electrophoresis 3 - 4 fractions showing enzymatic activity (Newsome, 1969; Skeggs *et al.*, 1967).

The obtained renin substrate incubated with an excess of renin, liberated about 3 ng of angiotensin per 1 mg of substrate protein. The preparation also showed rather high stability and was not destroyed on storage for 6 months at −20°C.

Reaction conditions. Determination of the effect of substrate concentration, enzyme concentration and incubation time on renin activity (Fig. 3), permitted to establish the following optimum conditions: 176 mg of renin substrate protein, 110 μg of renin preparation protein, 0.1 M-DFP, 0.15 M-phosphate buffer, pH 5.5, final volume 10 ml, incubation time 3 h at 37°C; under these conditions about 400 ng of angiotensin was formed.

Effect of detergents on renin activity. Due to the low solubility of the lipids studied, detergents were applied. However, both sodium dodecylate and sodium deoxy-

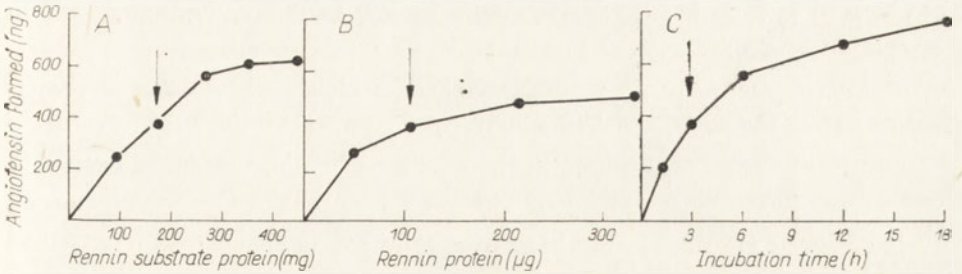

Fig. 3. Dependence of angiotensin formation on: *A*, renin substrate concentration; *B*, renin concentration; *C*, incubation time. The reaction mixture in the final volume of 10 ml contained 0.15 M-phosphate buffer, pH 5.5, 0.1 M-DFP and in *A*: 110 µg of enzyme protein (incubation time 3 h); in *B*: 170 mg of renin substrate (incubation time 3 h); in *C*: 110 µg of enzyme protein and 170 mg of renin substrate. The values adopted in further experiments are shown by arrows.

cholate inhibited the reaction in a degree dependent on their concentration (Table 3). In further experiments, deoxycholate was applied at a concentration of 0.01%, at which the reaction was inhibited by 50%.

Table 3

Effect of detergents on renin activity

The incubation mixture contained: 4 ml of renin substrate (170 mg protein), 0.1 ml of renin (110 µg protein), 4.9 ml of 0.15 M-phosphate buffer, pH 5.5, 0.1 M-DFP and 1 ml of water (control) or 1 ml of the detergent solution of the indicated final concentration. The incubation was carried out for 3 h at 37°C.

Addition	Angiotensin formed		Activity (%)
	ng/sample	ng/µg renin protein	
None (control)	470	4.27	100
Sodium dodecylate			
0.001%	400	3.64	85
0.01%	310	2.82	66
0.1%	22	0.20	5
Sodium deoxycholate			
0.001%	370	3.36	79
0.01%	240	2.18	51
0.1%	101	0.92	21

Effect of oleic and palmitic acids, lecithin and cholesterol. Appropriate amounts of the lipids dissolved in 0.15 M-phosphate buffer, pH 5.5, or in the same buffer containing sodium deoxycholate, were added to the incubation mixture (Table 4). Lecithin was the only compound tested which inhibited the reaction to a greater extent than did the detergent alone. Oleic and palmitic acids inhibited the reaction only by 25 - 30%, and when they were added together with deoxycholate, the inhibition by deoxycholate was even slightly overcome. Similarly, no significant effect

was observed with cholesterol which, due to its low solubility, was tested only in the presence of deoxycholate.

Effect of prostaglandins. In preliminary experiments the direct effect of prostaglandins E_2 and $F_{2\alpha}$ was tested. The addition of the prostaglandins to the incubation mixture containing renin substrate in the absence of renin, caused no change in blood pressure of nephrectomized rats. When the prostaglandins were incubated with the mixture containing the enzyme, the amount of angiotensin formed was increased (Table 5), and the degree of this increase was dependent on the prostaglandin concentration.

Table 4

Effect of some lipids on renin activity

The incubation mixture contained: 4 ml of renin substrate (150 mg protein), 0.1 ml of renin (110 μg protein), 4.9 ml of 0.15 M-phosphate buffer, pH 5.5, 0.1 M-DFP and 1 ml of water (control) or 1 ml of phosphate buffer containing 8 mg of the indicated compounds (except cholesterol, 20 mg); deoxycholate was added at a final concentration of 0.01%. The incubation was carried out for 3 h at 37°C.

Addition	Angiotensin formed		Activity (%)
	ng/sample	ng/μg renin protein	
None (control)	450	4.08	100
Deoxycholate	240	2.18	51
Oleic acid	335	3.04	73
Oleic acid + deoxycholate	265	2.40	59
Palmitic acid	304	2.76	67
Palmitic acid + deoxycholate	270	2.45	60
Lecithin	180	1.64	40
Lecithin + deoxycholate	162	1.47	36
Cholesterol + deoxycholate	270	2.45	60

Table 5

Effect of prostaglandins on renin activity

The incubation mixture contained: 2 ml of renin substrate (115 mg protein), 0.1 ml of renin (174 μg protein), 0.1 M-DFP, 4.9 ml of 0.15 M-phosphate buffer, pH 5.5, and 2 ml of 0.15 M-NaCl or the indicated amount of prostaglandin in NaCl solution. The incubation was carried out for 3 h at 37°C.

Addition	Angiotensin formed		Activity (%)
	ng/sample	ng/μg renin protein	
None (control)	260	1.50	100
PGE_2, 0.5 μg	370	2.13	142
$PGF_{2\alpha}$, 2.5 μg	302	1.74	116
25 μg	425	2.44	163

DISCUSSION

According to Poulsen (1968) and Gould & Green (1971), the methods applied for purification of the renin substrate cause changes in α-globulin conformation, which probably lead to changes in substrate activity. In the present work a simple procedure was applied which may permit to obtain native substrate molecules, and the possible influence of angiotensinase was prevented by adding DFP to the incubation mixture (Poulsen, 1968).

The reaction catalysed by renin is known to yield angiotensin I, which under the influence of the converting enzyme gives angiotensin II possessing pressor activity. *In vivo*, the converting enzyme in lung is able to convert during a single blood circulation an amount of angiotensin I exceeding by 10 000-fold its physiological content (Bakhle, 1968; Huggins *et al.*, 1970). This permits to assume that the estimated changes in blood pressure correspond to the amount of angiotensin I formed in the reaction catalysed by renin *in vitro*.

The presence of sodium deoxycholate, added to increase the solubility of lipids, did not affect the course of biological determination of angiotensin nor the adsorption of angiotensin on the ion exchanger. This was demonstrated by recovery of synthetic angiotensin added to the incubation mixture containing the detergent (not shown). The observed inhibition of renin activity by detergents was probably due to the effect on conformation of globular protein, affecting the conformation of the active site of the enzyme (Jirgensons & Hnilica, 1966; Markovich *et al.*, 1966).

Palmitic and oleic acids and cholesterol at the concentration applied had no distinct effect on renin activity. The two acids in the presence of deoxycholate even decreased the inhibition by this compound by about 20%. It may be supposed that these lipids, by interacting with the detergent, lowered the number of its molecules that affected the conformation of the enzyme protein. Some inhibition of angiotensin formation observed in the presence of fatty acids alone, could be ascribed to non-specific changes in physico-chemical properties of the reaction medium. This interpretation is supported by the results of Pfeiffer *et al.* (1971) showing that fatty acids of phospholipid inhibitors did not participate in inhibition of the reaction catalysed by renin. Lowered renin activity in serum, found by Campbell *et al.* (1973) in experiments *in vivo* on animals fed a high-cholesterol diet, was probably due to lower renin secretion, and not to the effect of cholesterol on the enzymatic reaction itself.

Unlike the fatty acids and cholesterol, lecithin added alone or together with the detergent inhibited the reaction by 60 - 64%. Among phosphoglycerides, phosphatidylserine and phosphatidylethanolamine are considered the natural inhibitors of renin (Sen *et al.*, 1967; Smeby *et al.*, 1967). As reported by Pfeiffer *et al.* (1971, 1972), 1,2-adamantylphosphatidylcholine also shows inhibitory properties. It seems that in the case of lecithin, inhibition of renin activity is dependent on the presence of choline residue and not on the type of fatty acids. The specificity and mechanism of the inhibition of the reaction still remain unclear.

Rather unexpected results were obtained with prostaglandins. Both prostaglandin E_2 and $F_{2\alpha}$, despite their opposite action *in vivo*, under our experimental conditions caused an increase in the amount of angiotensin formed, the increase being dependent on prostaglandin concentration. This activation is difficult to explain, and it is not clear whether it plays any role at all *in vivo*. Recently Kotchen *et al.* (1974) who studied the effect of prostaglandins on the rate of the reaction between renin and renin substrate, both isolated from human tissues, found that prostaglandins A_1 and A_2 significantly inhibited formation of angiotensin, whereas prostaglandins E_1 and E_2 had no effect. Their experiments were performed at high enzyme concentration and very high concentrations of the prostaglandins, therefore they cannot be compared with our results; none the less, the participation of prostaglandins in regulation of renin activity seems very probable.

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WPŁYW NIEKTÓRYCH SUBSTANCJI LIPIDOWYCH NA AKTYWNOŚĆ RENINY *IN VITRO*

Streszczenie

Stosując częściowo oczyszczoną reninę wołową i homologiczny substrat badano wpływ niektórych substancji lipidowych na ilość tworzącej się angiotensyny. Wykazano, że inhibitorem reniny jest lecytyna oraz stosowane w doświadczeniach detergenty: dezoksycholan i dodecylan sodu. Nie stwierdzono istotnego wpływu kwasu palmitynowego, olejowego i cholesterolu. Wzrost wydajności reakcji powodowały natomiast obie badane prostaglandyny: E_2 i $F_{2\alpha}$.

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UPTAKE OF FOLATE AND ITS ANALOGUE - AMETHOPTERIN BY MOUSE L-CELLS

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1. The uptake of folate and amethopterin by the mouse transformed L-cells was found to depend on their extracellular concentration and time of incubation. The uptake conforms to Michaelis-Menten kinetics (K_m values 10^{-4} M and 10^{-6} M for folate and amethopterin, respectively) and is temperature-dependent.

2. The observed differential effect of actinomycin D, cycloheximide and pCMB on the uptake of folate and amethopterin and lack of a pronounced transinhibition in the transport of these compounds indicate the existence in L-cells of two separate transport systems for folate and its analogue.

3. Methionine added to the culture medium enhances the uptake of folate, but not that of amethopterin.

4. Under conditions of methionine-dependent increase of folate uptake the activity of dihydrofolate reductase remains unaltered while that of methionine synthetase is markedly reduced.

5. The efficiency of folate uptake seems to be related rather to the modulations of methionine synthetase activity than to changes in the activity of dihydrofolate reductase.

The uptake of folate, some of its coenzymatic derivatives and analogues by bacteria (Huennekens *et al.*, 1974; Shane & Stokstad, 1975; Huennekens & Henderson, 1976; Zakrzewski & Grzelakowska-Sztabert, 1973) and by normal and leukaemic cells (Hakala, 1965; Divekar *et al.*, 1967; Izak *et al.*, 1968; Corcino *et al.*, 1971; Goldman, 1971, 1973) was extensively studied in the last ten years. Information on the mechanisms involved in the transport of these compounds is of considerable interest because of the established role of folate compounds in cellular metabolism and frequent use of amethopterin in the treatment of various forms of cancer.

In the presented paper basic kinetics of the uptake of folate and amethopterin by L-cells is given and evidence is presented for the occurrence of separate transport systems for the vitamin and its analogue. The effect of intracellular folate pool on the activities of several enzymes involved in C_1 metabolism is also included.

MATERIALS AND METHODS

Cell cultures. Mouse methylcholanthrene-transformed cells (L-cells) were routinely grown as monolayers at 37°C in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated calf serum, L-glutamine (2 μmoles/ml) and antibiotics: penicillin (100 units/ml), streptomycin sulphate (50 μg/ml) and occasionally kanamycin sulphate (50 μg/ml). The Eagle's essential medium contained methionine and folate at concentration of 0.1 μmole/ml and 2.5 nmoles/ml, respectively, but was devoid of vitamin B_{12}. Calf serum supplements the medium with vitamin B_{12} at the final concentration up to 0.032 pmole/ml (Koziorowska, personal communication). The cells were routinely subcultured every 3 - 4 days. In the experiments indicated, they were maintained for 2 - 3 months in the medium to which L-methionine (2 μmoles/ml), folate (2.5 nmoles/ml) or vitamin B_{12} in cyano form (7.7 nmoles/ml) were alternatively added.

Under the experimental conditions used, in two days of active proliferation the number of L-cells per bottle was doubled, then the rate of cell division slowed down and growth stopped 5 - 6 days after subculturing without change in viability of the cells. The addition of folate, vitamin B_{12} or methionine had no effect on mitotic index; however, incorporation of [^{14}C]leucine into trichloroacetic acid-insoluble material was raised by 25 - 50%.

The cells were harvested using the mild trypsin treatment either after 48 h or on the seven consecutive days after seeding, centrifuged at 310 *g* and washed twice with Eagle's medium containing serum; viability of cells was not diminished by this procedure as shown by the trypan blue exclusion test. Then a known amount of cells was suspended, if not otherwise stated, in Goldman's mixture consisting of 2 mM-$CaCl_2$, 1 mM-$MgCl_2 \cdot 6H_2O$ and 26.2 mM-$NaHCO_3$, pH about 7.4 (Lichtenstein *et al.*, 1969). The cells were counted in Neubauer ruling.

The uptake experiments. The cell suspension (0.5 ml) was incubated for 30 min with shaking in glass tubes at defined temperature either with [^{14}C]folate or [^{3}H]amethopterin. The incubation was terminated by cooling in ice-bath followed by the addition of ice-cold isotonic saline solution (10 ml) and immediate centrifugation for 2 min at 4°C. The cells were rewashed with cold saline solution and centrifuged as above. The pellet was then suspended in 2 ml of 0.1 M-maleate, pH 6.8, containing 1% ascorbate, and heated for 5 min at 75°C (Zakrzewski & Grzelakowska-Sztabert, 1973). After cooling, the samples were centrifuged and the radioactivity was determined in supernatant with 90 - 95% recovery.

The uptake of folate or amethopterin was expressed as radioactivity (c.p.m.) incorporated per mg of cell protein. For protein determination according to Lowry *et al.* (1951), the cells were disrupted in maleate buffer without ascorbate, which interferes with determination of protein by this method.

Chromatography of folate metabolites. Supernatants from 4 to 6 cell samples obtained by the procedure described above were pooled, lyophilized and stored frozen at −20°C until used. The lyophilized powder was dissolved in 1 ml of alkalized 0.1% mercaptoethanol (pH 8.0) to which folate (0.6 mg) or 5-formyltetra-

hydrofolate (0.6 mg) were added as standards. The solution was then applied to DEAE-cellulose column (acetate form, 1 × 15 cm) and chromatographed using a linear gradient of 0.05 to 2.0 M-ammonium acetate containing 0.1% mercaptoethanol. Ultraviolet absorption was monitored at 280 nm. Samples of 200 µl of each fraction were used for radioactivity counting.

Enzyme assays. For estimation of the activities of dihydrofolate reductase (7,8-dihydrofolate : $NADP^+$ oxidoreductase, EC 1.5.1.4) and serine hydroxymethyltransferase (5,10-methylenetetrahydrofolate: glycine hydroxymethyltransferase, EC 2.1.2.1) the methods recommended by Scrimgeour & Huennekens (1966) were used. Methionine synthetase (5-methyltetrahydropteroyl-L-glutamate : L-homocysteine 5-methyltransferase) was determined according to Kamely *et al.* (1973).

Chemicals. [2-^{14}C]Folate (54.3 mCi/mmole) and (3′,5′,9(*n*)[^{3}H]methotrexate (5.3 Ci/mmole) were obtained from Amersham-Searle Corporation (England), and culture media from Wytwórnia Surowic i Szczepionek (Lublin, Poland). Folic acid was purchased from B.D.H. (Poole, Dorset, England), amethopterin and calcium leucovorin from Lederle Laboratories Division, American Cyanamid Co. (Pearl River, N.Y., U.S.A.), cycloheximide from Sigma (St. Louis., Mo, U.S.A.), actinomycin D from Serva (Heidelberg, G.F.R.); vitamin B_{12} (cyano form) was from Polfa (Warszawa, Poland). Inorganic salts obtained from commercial sources were of analytical grade.

RESULTS

Kinetics of folate and amethopterin uptake. In the preliminary experiments it was found that the rate of folate and amethopterin uptake by L-cells were higher in Goldman's incubation mixture than in phosphate-buffered saline (PBS) or in Eagle's medium containing 10% calf serum (Table 1).

Table 1

The uptake of folate and amethopterin by L-cells in different media

L-cells were collected 48 h after subculturing and incubated either with [^{14}C]folate (2.5 µCi; 0.92×10^{-4} M) or [^{3}H]amethopterin (12.5 µCi; 1×10^{-6} M) for 30 min at 37°C. Each value is the mean of 3 - 5 samples from two experiments.

Incubation medium	Folate	Amethopterin
	c.p.m./mg protein	
Phosphate-buffered saline	17 940	48 400
Eagle's medium supplemented with calf serum	22 160	56 570
Goldman's mixture	26 590	74 120

The addition of glucose at concentration of 5 and 10 mM resulted in a negligible (about 10%) increase both in folate and amethopterin uptake in contrast to the results obtained with L1210 cells (Huennekens *et al.*, 1974) and bacteria (Henderson & Huennekens, 1974).

The uptake of folate as well as that of amethopterin was found to follow typical Michaelis-Menten kinetics (Fig. 1A,B). The apparent Michaelis constants were 0.4×10^{-4} M and 0.7×10^{-6} M for folate and amethopterin, respectively.

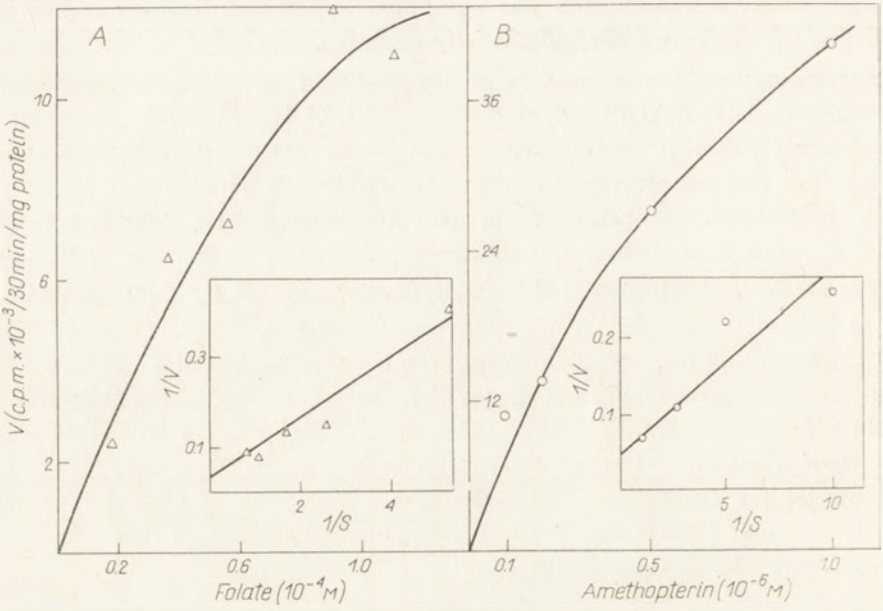

Fig. 1. Kinetics of folate (*A*) and amethopterin (*B*) uptake by L-cells. The cells were collected 48 h after subculturing. Incubation was carried out for 30 min at 37°C.

The rate of folate and amethopterin uptake as a function of time and temperature is presented in Fig. 2A,B. The uptake of folate was linear for 45 min whereas that of amethopterin only for 15 - 20 min. The small amount of radioactivity taken up by the cells at 0°C is probably due to absorption at the cell surface.

Interactions of folate and amethopterin uptake. The effect of amethopterin on folate uptake was measured in the samples to which cold amethopterin, at various concentrations, was added simultaneously with [^{14}C]folate (Table 2.) Inhibition

Table 2

Effect of amethopterin on [^{14}C]*folate uptake*

L-cells were collected 48 h after subculturing. Amethopterin at indicated concentrations and [^{14}C]folate (2.5 μCi; 0.92×10^{-4} M) were added to the sample simultaneously and incubated for 30 min at 37°C. Each value is the mean of 4 - 5 samples from three experiments.

Amethopterin added (M)	Folate	
	c.p.m./mg protein	%
None	37 450	100
1×10^{-5}	34 180	91
1×10^{-4}	28 490	76
1×10^{-3}	28 270	76
5×10^{-3}	28 170	75

of folate uptake by amethopterin used in a 15-fold excess over folate did not exceed 25%, and the inhibition was found to be of a non-competitive type (Fig. 3). In similar experiments it was found that folate added at a concentration by two orders

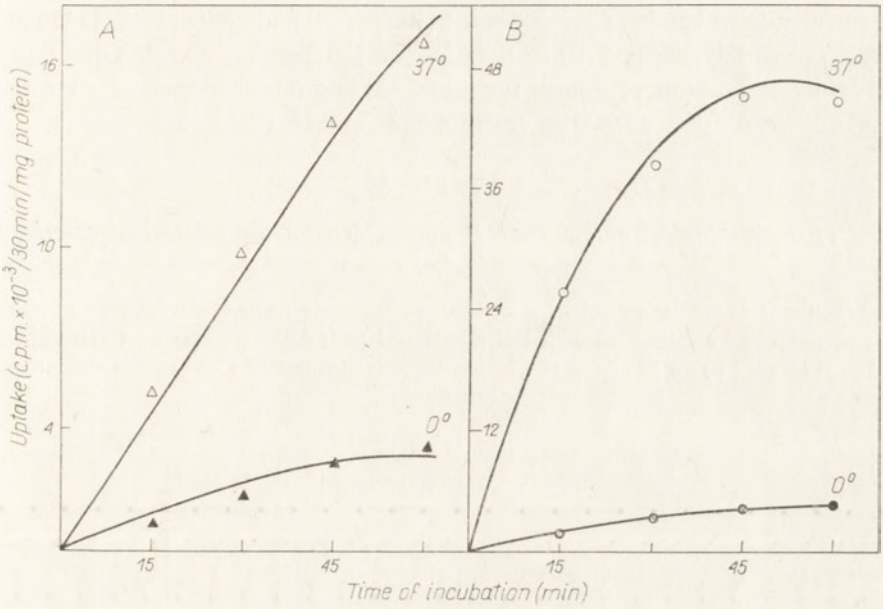

Fig. 2. Time-course of folate (*A*) and amethopterin (*B*) uptake by L-cells at 37° and 0°C. The cells were collected 48 h after subculturing and incubated either with [^{14}C]folate (2.5 μCi, 0.92×10^{-4} M) or [^{3}H]amethopterin (12.5 μCi, 1×10^{-6} M). Each value is an average of at least three separate determinations.

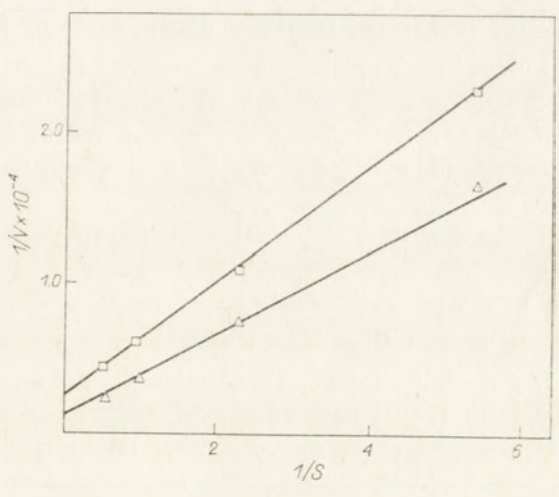

Fig. 3. Inhibition by unlabelled amethopterin of [^{14}C]folate uptake by L-cells. The cells were incubated with [^{14}C]folate in the absence (△) or presence (□) of 1 mM-amethopterin, for 30 min at 37°C. *V* is expressed as c.p.m. of folate taken up per 1 mg of cell protein.

of magnitude (10^{-4} M) higher than that of amethopterin (10^{-6} M) did not inhibit the amethopterin uptake. Thus it appears that folate and amethopterin are transported into L-cells by separate systems. This suggestion was confirmed by the differential effect of cycloheximide, actinomycin D and pCMB[1] on the efficiency of folate and amethopterin uptake. Cycloheximide (100 μg/ml) and actinomycin D (20 μg/ml) almost completely inhibited incorporation of [^{14}C]leucine and [^{14}C]uridine into acid-insoluble fraction of L-cells (unpublished) and inhibited markedly the uptake of folate but did not affect the amethopterin uptake (Table 3).

Table 3

Effect of cycloheximide, actinomycin D and pCMB on the folate and amethopterin uptake

L-cells collected 48 h after subculturing were preincubated with or without inhibitors for 20 min at 37°C, followed by incubation with [^{14}C]folate (2.5 μCi; 0.92×10^{-4} M) or [^{3}H]amethopterin (12.5 μCi; 1×10^{-6} M) for 30 min at 37°C. Each value is the mean of 3 - 5 samples obtained in two experiments.

Preincubation with	Folate		Amethopterin	
	c.p.m./mg protein	%	c.p.m./mg protein	%
None	29 330	100	33 270	100
Cycloheximide (100 μg/ml)	14 770	51	33 880	100
Actinomycin D (20 μg/ml)	19 830	68	29 040	87
None	22 160	100	40 540	100
pCMB (20 μM)	22 370	100	26 020	64

Preincubation of L-cells with pCMB (20 μM) decreased the amethopterin uptake but had no effect on the incorporation of folate (Table 3). This might imply that free SH-groups are involved in the uptake of amethopterin by L-cells.

Effect of culture conditions. The efficiency of the folate and amethopterin uptake by L-cells increased with time of cultivation. The non-proliferating cells from the stationary phase of growth, showing very low mitotic index, accumulated twice as much of these compounds as the cells from actively proliferating cultures (Fig. 4A,B).

The rate of folate uptake was enhanced when L-cells were cultivated in the medium supplemented with methionine (Table 4), but was somewhat decreased in the cells grown in the medium enriched with folate or vitamin B_{12}. Neither methionine, folate or vitamin B_{12} affected amethopterin uptake by L-cells.

The metabolism of folate by L-cells. To examine the conversion of folate into its coenzymatic forms in L-cells under our experimental conditions, the extract of the ^{14}C-labelled cells was chromatographed on DEAE-cellulose as described under Material and Methods. A typical profile is presented in Fig. 5. The bulk of radioactivity, about 70%, eluted from the column, was located in the peak cor-

[1] Abbreviation used: pCMB, *p*-chloromercuribenzoate.

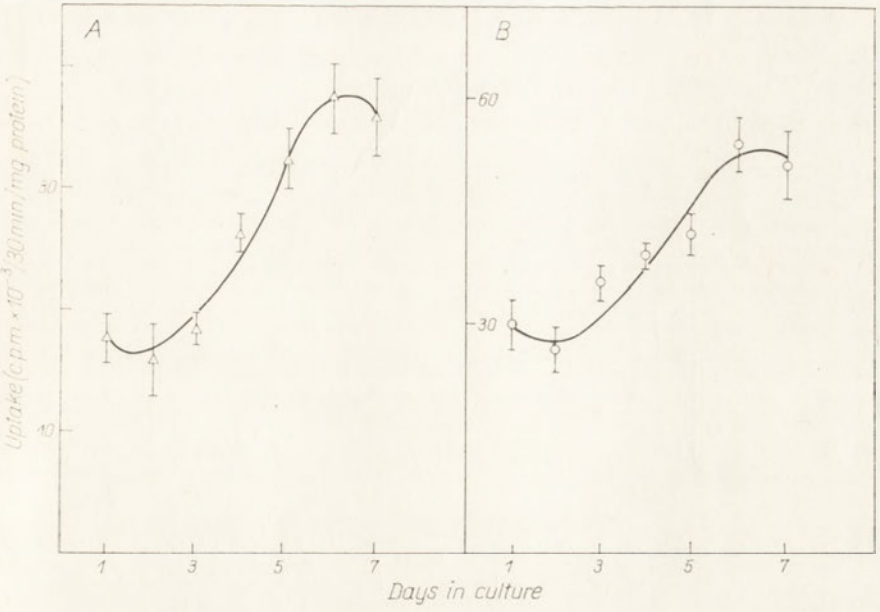

Fig. 4. Uptake of folate (*A*) and amethopterin (*B*) by L-cells cultivated up to seven days without renewal of Eagle's medium. Each day the cells were collected and incubated either with [^{14}C]folate (2.5 μCi; 0.92×10^{-4} M) or [^{3}H]amethopterin (12.5 μCi; 1×10^{-6} M) for 30 min at 37°C. The vertical bars represent S.D.

Table 4

Effect of folate, vitamin B_{12} and methionine on the uptake of folate and amethopterin by L-cells

L-cells cultivated in Eagle's medium either unsupplemented or supplemented with folate, vitamin B_{12} or methionine, were collected 48 h after subculturing. Incubation with [^{14}C]folate (2.5 μCi; 0.92×10^{-4} M) or [^{3}H]amethopterin (12.5 μCi; 1×10^{-6} M) for 30 min at 37°C. The results are expressed as c.p.m./mg protein ± S.D., with the number of experiments in parentheses.

Culture medium	Folate	Amethopterin
Eagle's	22 470 ± 2 530 (9)	26 920 ± 3 330 (3)
+ folate (2.5 μM)	20 890 ± 2 320[b] (7)	25 710 ± 2 420[b] (3)
+ vitamin B_{12} (7.7 μM)	17 510 ± 3 060[a] (5)	25 710 ± 1 820[b] (3)
+ methionine (2.0 mM)	32 710 ± 2 740[a] (4)	24 810 ± 1 210[b] (3)

[a] $0.05 < P < 0.1$; [b] $0.6 < P < 0.9$

responding to folate; besides, two other peaks of radioactivity were detected. The first peak was eluted at the same ammonium acetate concentration as 2-amino-4-hydroxypteridine, a folate degradation product, the second peak at concentration corresponding to 5-formyltetrahydrofolate, tetrahydrofolate or 5-methyltetrahydrofolate. However, it was impossible to identify the tetrahydrofolate derivatives eluted in peak *II* by the applied chromatographic procedure. The presence of

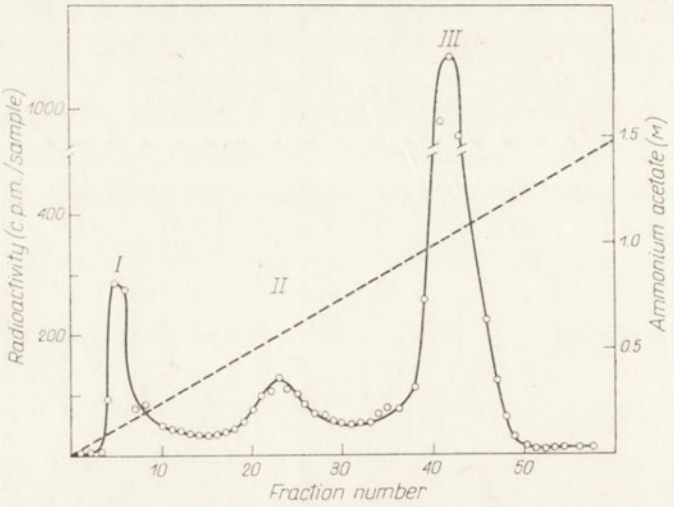

Fig. 5. DEAE-cellulose chromatography of the extracts from the L-cells loaded with [^{14}C]folate. The cells were collected 48 h after subculturing and incubated with [^{14}C]folate (2.5 μCi; 0.92×10^{-4} M) for 30 min at 37°C. The folate and its derivatives were eluted with a linear ammonium acetate gradient (0.05 - 2.0 M) in the presence of 0.1% 2-mercaptoethanol. Fractions of 3 ml were collected. ○, Radioactivity; — — —, ammonium acetate gradient. Peak *III* corresponds to folate.

polyglutamates of any known derivatives of tetrahydrofolate should be excluded since in this chromatographic system they are eluted at higher ammonium acetate concentration (Zakrzewski & Grzelakowska-Sztabert, 1973). It is probable that the concentration of folate applied in our experiments was sufficient to suppress its conversion into coenzymatic forms. Tattersall *et al.* (1974) using the microbiological assay, reported recently that about 50% of folate pool in L-cells is in the form of polyglutamates.

Table 5

The activity of some folate-metabolizing enzymes in L-cells

L-cells cultivated in Eagle's medium either unsupplemented or supplemented with folate, vitamin B_{12} or methionine, were collected 48 h after subculturing. The assays of enzyme activity as described under Materials and Methods. The results are expressed as nmoles of product/mg protein/h ±S.D., with the number of experiments in parentheses.

Culture medium	Dihydrofolate reductase*	Serine hydroxymethyl-transferase**	Methionine synthetase**
Eagle's	152±33 (5)	1950±720 (3)	1.9±0.001 (13)
+folate (2.5 μM)	141±35[d] (5)	3000±420[c] (3)	2.8±0.1[b] (5)
+vitamin B_{12} (7.7 μM)	155±24[d] (6)	2130±510[d] (3)	6.3±1.3[a] (6)
+methionine (2 mM)	149±29[d] (7)	4200±150[c] (3)	0.7±0.1[c] (5)

* Incubation at 20°C; ** Incubation at 37°C.
[a] $P<0.01$; [b] $0.02<P<0.05$; [c] $0.1<P<0.3$; [d] $0.9<P$.

The increase in the uptake of [^{14}C]folate by L-cells cultured in the medium supplemented with methionine did not result in the appearance of folate derivatives other than those found in the cells grown in regular medium. At the same time, the amount of radioactivity in the peak tentatively identified as 5-formyltetrahydrofolate or 5-methyltetrahydrofolate was but slightly elevated.

Effect of folate, vitamin B_{12} and methionine on folate-metabolizing enzymes. The cells cultured in Eagle's medium containing double amounts of folate showed the same activity of dihydrofolate reductase as the cells grown in the unsupplemented medium. The activities of serine hydroxymethyltransferase and methionine synthetase were only slightly enhanced (Table 5). The addition to the growth medium of vitamin B_{12}, a generally known indispensable cofactor in biosynthesis of methionine, increased significantly in L-cells the activity of methionine synthetase but had no effect on the activities of the other two enzymes tested. On the other hand, additional amounts of methionine doubled the activity of serine hydroxymethyltransferase and lowered significantly that of methionine synthetase.

DISCUSSION

The uptake of folate and amethopterin by the mouse fibroblast-like cells of the L-line conforms to Michaelis Menten kinetics and is temperature-dependent, similarly as in reticulocytes (Izak *et al.*, 1968), erythrocytes (Izak *et al.*, 1968; Goldman, 1971), normal (Kessel *et al.*, 1968; Das & Hoffbrand, 1970) and neoplastic leucocytes (Goldman *et al.*, 1968; Kessel *et al.*, 1968; Lichtenstein *et al.*, 1969; Goldman, 1971; Sirotniak & Donsbach, 1972, 1974; Bender, 1975). K_m value for folate found for L-cells, similar to that reported for neoplastic leucocytes, is relatively high as compared with K_m values for folate coenzymes (Goldman, 1971; Huennekens *et al.*, 1974). This confirms the view that folate is not a physiologically active form of this vitamin.

Although it is postulated that folate and its derivatives may be transported into cells by a carrier-mediated process (Goldman, 1971), up to now no carrier proteins for these compounds have been isolated from mammalian cells. Just recently evidence has been presented that in *Lactobacillus casei* cells a folate-binding protein is an active component of the folate transport system (Huennekens & Henderson, 1976; Henderson *et al.*, 1976).

Information concerning the effect of inhibitors of nucleic acid and protein synthesis on the uptake of folate or its derivatives by mammalian cells is rather scanty and not unequivocal. A decrease of the intracellular folate pool by cycloheximide has been reported for some cells of human and hamster origin (Hillcoat & Marshall, 1974; McBurney & Whitmore, 1974). On the other hand, Das & Hoffbrand (1970) observed no effect of inhibitors of RNA and DNA synthesis on the efficiency of folate uptake by phytohemagglutinin-stimulated lymphocytes, in which the maximum of folate uptake preceded the enhanced DNA synthesis. In our experiments it was found that the uptake of folate by L-cells was inhibited by cycloheximide

and actinomycin D. This dependence may imply a limited amount or short duration of mRNA responsible for the synthesis of the folate transport system. In this respect folate transport in L-cells resembles that of thymidine in Novikoff cells (Plagemann *et al.*, 1975) and of uridine in mouse embryo fibroblasts (Hare, 1972) which were reported to be very sensitive to inhibitors of both RNA and protein synthesis. In our experiments there was no response of amethopterin uptake to these inhibitors.

It is of considerable interest that only amethopterin uptake by L-cells was found to be influenced by pCMB, as it was also reported for murine leukemia L1210 cells (Henderson & Huennekens, 1974; Rader *et al.*, 1974). This differential effect of pCMB and of inhibitors of nucleic acid and protein synthesis, as well as the negligible non-competitive inhibition of folate uptake by amethopterin suggest that in L-cells folate and amethopterin are transported by separate systems, as in leukemic L1210 cells (Goldman, 1971; Huennekens *et al.*, 1973; Niethammer & Huennekens, 1973).

The fact that L-cells from non-proliferating cultures took up amethopterin more efficiently than the cells from actively proliferating cultures was rather unexpected as the activity of dihydrofolate reductase, still considered the main target enzyme for amethopterin, is very low at this phase of growth. These results suggest that concentration of amethopterin accumulated intracellularly exceeds the content of the dihydrofolate reductase protein. A similar conclusion was drawn from the results on the mechanism of amethopterin action on DNA synthesis in L-cells (Goldman, 1974, 1975) as well as in L1210 cells (Sirotniak & Donsbach, 1972, 1974). Apart from dihydrofolate reductase, the presence of other amethopterin-binding proteins has been demonstrated in some mammalian tissues (Newbold & Harding, 1971).

Low intensity of folate uptake by L-cells from proliferating cultures did not correlate with the activity of dihydrofolate reductase (Grzelakowska-Sztabert *et al.*, 1976). Moreover, under conditions of enhanced folate uptake by L-cells cultivated in the medium enriched in methionine, level of dihydrofolate remained unchanged. In contrast, under the above conditions the activity of serine hydroxymethyltransferase was doubled whereas that of methionine synthetase was lowered by 60%. One may presume therefore that although the synthesis of the immediate precursor of methyltetrahydrofolate was enhanced, the utilization of 5-methyltetrahydrofolate in L-cells was impaired limiting in this way the amount of free tetrahydrofolate available in the cells for activation of one-carbon units. The enhanced uptake of folate under conditions impairing utilization of 5-methyltetrahydrofolate might therefore reflect the increased demand of the cells for tetrahydrofolate. Moreover, L-cells showing high activity of methionine synthetase, induced by the addition of vitamin B_{12} to the growth medium, took up folate less efficiently that the cells grown in control medium. Thus it seems probable that the efficiency of folate uptake may be rather related to the modulations of methionine synthetase activity than to changes in the activity of dihydrofolate reductase.

It is also not to be excluded that the differences in the intensity of folate and amethopterin uptake found in L-cells from growing and stationary cultures might

be at least partially due to the differences in protein turnover in plasma membranes (Ceccarini *et al.*, 1975; Roberts & Yuan, 1974, 1975).

The autor wishes to thank Prof. Zofia Zielińska for her constant interest in this study and mgr M. Landman for the estimation of serine hydroxymethyltransferase and methionine synthetase activity. The efficient technical assistance of Mrs. Katarzyna Kropiwnicka, Miss Irmina Zając and Mr. Maciej Fedorowicz is gratefully acknowledged.

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POBIERANIE FOLIANU I JEGO ANALOGU, AMETOPTERYNY, PRZEZ MYSIE KOMÓRKI L

Streszczenie

1. Pobieranie folianu i ametopteryny przez mysie transformowane komórki L zależy od stężenia tych związków w środowisku inkubacyjnym, temperatury i czasu trwania inkubacji. Oznaczone wartości stałych Michaelisa są w przypadku folianu i ametopteryny rzędu 10^{-4} i 10^{-6} M.

2. Różny efekt aktynomycyny D, cykloheksoimidu i pCMB na pobieranie folianu i ametopteryny oraz brak wzajemnego hamowania transportu tych związków sugerują obecność w komórkach L odrębnych układów transportujących folian i ametopterynę.

3. Metionina dodana do środowiska hodowlanego podwyższa efektywność transportu folianu do komórek, nie wywiera natomiast wpływu na pobieranie ametopteryny.

4. Przy wzmożonej intensywności pobierania folianu przez komórki L, rosnące w środowisku wzbogaconym w metioninę, stwierdzono obniżenie aktywności syntetazy metioninowej przy niezmienionym poziomie reduktazy dwuhydrofolianowej.

5. Efektywność pobierania folianu przez komórki L raczej wydaje się mieć związek ze zmianami aktywności syntetazy metioninowej niż ze zmianami aktywności reduktazy dwuhydrofolianowej.

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ANDRZEJ PASZEWSKI and JERZY GRABSKI

ON SULFHYDRYLATION OF *O*-ACETYLSERINE AND *O*-ACETYLHOMOSERINE IN HOMOCYSTEINE SYNTHESIS IN YEAST *

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Considerable differences in the activities of cysteine and homocysteine synthase (EC 4.2.99.8), and in the ratio of these activities were observed in yeast strains representing different genera and species. This suggests that homocysteine synthesis directly from *O*-acetylhomoserine may predominate in some strains, whereas in the others homocysteine is formed from cysteine *via* cystathionine.

Recently, Yamagata *et al.* (1974) proved unequivocally that sulfhydrylation of *O*-acetylserine[1] and *O*-acetylhomoserine in *Saccharomyces cerevisiae* is catalysed by the same enzymatic protein, namely cysteine-homocysteine synthase (EC 4.2.99.8), (OAS and OAH lyase, adding hydrogen-sulphide). This implies that this protein may be involved in both pathways of homocysteine synthesis described in fungi (see review by Flavin, 1975): in direct formation of homocysteine by sulfhydrylation of OAH, or by sulfhydrylation of OAS yielding cysteine, from which homocysteine is formed *via* cystathionine as an intermediate. The physiological role of these two pathways has been a subject of discussion (Cherest *et al.*, 1969; Savin & Flavin, 1972; de Robichon-Szulmajster *et al.*, 1973). It might be assumed that in each yeast strain both pathways may operate, with one of them predominating. This may be reflected in the variation of the Cys/Hcy-synthase activity ratio from one strain or species to another. This ratio has been found to be subject to mutation (Yamagata *et al.*, 1975). Therefore it was conceivable that the ratio could vary in natural yeast populations. In the present work considerable differences in both activities and in their ratio were observed in some yeast strains representing different species and genera.

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[1] Abbreviations: OAS, *O*-acetylserine; OAH, *O*-acetylhomoserine; Cys, cysteine; Hcy, homocysteine.

MATERIAL AND METHODS

Biological material. Yeast strains *Candida tropicalis C.t 2*, *Candida utilis C.t 5*, and *Saccharomycopsis lipolytica S.l 1* were from the collection of the Institute of Fermentation Industry (Warsaw). *Candida utilis CCY 29-38-18* was obtained from the Institute of Chemistry, Slovac Academy of Sciences (Bratislava). *Candida tropicalis W-1* and *Candida utilis Ż-1* were from our collection. All the strains are prototrophs.

Growth conditions and preparation of extracts. Yeasts were grown in GO minimal medium (Galzy & Słonimski, 1957) containing in 1 litre of water: KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 5 g; NaCl, 0.1 g; $CaCl_2$, 0.1 g; $(NH_4)_2SO_4$, 50 g; H_3BO_3, 0.5 μg; $MnSO_4 \cdot 5H_2O$, 0.4 μg; $ZnSO_4 \cdot 7H_2O$, 0.4 μg; $FeCl_2 \cdot H_2O$, 0.2 μg; $Na_2MoO_4 \cdot 2H_2O$, 0.2 μg; KI, 0.1 μg; $CuSO_4 \cdot 7H_2O$, 0.04 μg; nicotinamide, pyridoxine, thamine and calcium panthotenate, 0.4 μg each; *p*-aminobenzoic acid and riboflavin, 0.2 μg each; inositol, 2 μg; folic acid and biotin, 0.002 μg each; and glucose, 20 g. Erlenmayer flasks of 300 ml containing 150 ml of GO minimal medium, or the medium supplemented with 2 mM-methionine, were inoculated with 2×10^6 cells and incubated for 20 h at 30 - 31°C in a rotary shaker. The cells were harvested at the early stationary phase by centrifugation, and washed once with cold water. Extracts were prepared by mixing the cell paste with an equal amount of glass powder and grinding in a mortar with gradual addition of 0.1 M-potassium phosphate buffer, pH 7.5 (1 ml per 1 g of yeast paste). The homogenate was then centrifuged for 15 min at 15 000 *g* and the supernatant used for the enzyme assay.

Enzyme assay. Cys-synthase activity was assayed according to Pieniążek *et al.* (1973) with 60 mM-OAS concentration in the reaction mixture. Hcy-synthase activity was determined as described previously (Paszewski & Grabski, 1973). Protein was determined by the method of Lowry *et al.* (1951).

Reagents. L-Serine, DL-homoserine and L-methionine were obtained from Sigma Chemical Co. (St.Louis, Mo., U.S.A.). *O*-Acetyl-L-serine and *O*-acetyl-DL-homoserine were prepared according to Sakami & Toennies (1942). All inorganic compounds were reagent grade products.

RESULTS AND DISCUSSION

The results given in Table 1 show that both the synthase activities and the Cys/Hcy-synthase ratio varied considerably in the yeast strains. Large differences in the specific activities of Hcy-synthase are found in *S. cerevisiae* strains of different origin (Cherest *et al.*, 1973). Far more interesting are variations in the Cys/Hcy-synthase activity ratio which ranges from 4.85 in *C. utilis CCY* to 0.27 in *S. lipolytica*. Despite these differences, Cys-synthase and Hcy-synthase are repressed by methionine practically to the same extent. This implies a bifunctional character of a single enzymatic protein, similarly as it was found in *S.cerevisiae* (Yamagata *et al.*, 1974). The Cys/Hcy-synthase activity ratio might determine which of the alternative pathways for homocysteine synthesis predominates in

a given strain. The data obtained suggest that in *C.utilis* the cystathionine pathway is the main one whereas in *S.lipolytica* direct synthesis of homocysteine prevails. In the two *C. tropicalis* strains studied both pathways may contribute to a similar extent to homocysteine synthesis. It is also noteworthy that yeast strains belonging to the same species show practically the same Cys/Hcy-synthase activity ratio, but more strains should be examined to see whether this ratio is species specific.

Table 1

Cysteine and homocysteine synthase activities in various yeast species

Yeast cells were grown on the minimal medium (GO) of Galzy & Słonimski (1957), supplemented where indicated with 2 mM-L-methionine (Met). The results are mean values from at least 3 determinations and are expressed as nmoles of cysteine or homocysteine formed per minute per 1 mg protein.

Strain	Cys-synthase			Hcy-synthase			Cys/Hcy-synthase activity ratio in GO medium
	GO	GO+ Met	repression (%)	GO	GO+ Met	repression (%)	
C. tropicalis C.t 2	215.6	136.3	36.8	259.3	154.0	40.4	0.83
C. tropicalis W-1	309.1	131.0	58.0	348.7	168.0	52.0	0.88
C. utilis C.t 5	399.6	74.4	81.4	86.2	14.7	83.0	4.63
C. utilis CCY	273.9	105.6	61.5	56.4	26.3	53.4	4.85
C. utilis Ż-1	278.6	56.5	89.7	109.4	15.3	86.0	2.54
S. lipolytica S.l 1	160.2	53.9	66.4	584.9	102.9	82.4	0.27

Although the contribution of the two alternative pathways in homocysteine synthesis may differ, it seems unlikely that either pathway should be obligatory. An extensive genetical analysis of a hundred methionine mutants of *S.cerevisiae* (Masselot & de Robichon-Szulmajster, 1975) representing 21 complementation groups, revealed no mutant which would grow on homocysteine but not on cysteine, except *met-2* which is known to be deficient in homoserine transacetylase. Such mutants, very common in Neurospora and Aspergillus, are blocked at cystathionine γ-synthase or β-cystathionase.

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SULFHYDRYLACJA *O*-ACETYLOSERYNY I *O*-ACETYLOHOMOSERYNY W SYNTEZIE HOMOCYSTEINY U DROŻDŻY

Streszczenie

Stwierdzono znaczne różnice w aktywności sulfhydrylazy *O*-acetyloseryny i *O*-acetylohomoseryny oraz w stosunku tych aktywności w drożdżach należących do różnych gatunków i rodzajów. Wyniki sugerują, że u jednych szczepów drożdży synteza homocysteiny przebiega głównie poprzez bezpośrednią sulfhydrylację *O*-acetylohomoseryny, u innych zaś homocysteina syntetyzowana jest głównie z cysteiny poprzez cystationinę.

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PYRUVATE METABOLISM IN RAT BRAIN MITOCHONDRIA*

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1. Oxidation of pyruvate by rat brain mitochondria was stimulated in state 3 by malate or, succinate up to 250 nmoles O_2/mg protein/min. Oxidation of malate, succinate, 2-oxoglutarate or glutamate as the sole substrates, was 1/4 - 1/5 that observed with pyruvate.

2. Maximum oxygen consumption in state 3 was observed at pH 6.90 - 7.20, whereas in state 4 it was not affected by changes in pH.

3. In state 4, in the absence of exogenous acceptor of acetyl residues, acetate was the main oxidation product, corresponding to about 80% of the amount of pyruvate utilized. Malate did not affect the rate of pyruvate utilization but lowered acetate concentration and raised concentration of citrate and 2-oxoglutarate.

4. In state 3, pyruvate and malate were converted mainly to 2-oxoglutarate, its concentration being three times as high as that of citrate.

5. Formation of citrate, 2-oxoglutarate and acetate from pyruvate in brain is considered as a function of availability of the acceptor of acetyl residues and the energy state of mitochondrion.

In brain, pyruvate is the main source of acetylcholine (Tuček & Cheng, 1970) and fatty acids (Carey & Cantrill, 1975). Inhibition of intramitochondrial pyruvate metabolism by the diet low in thiamine pyrophosphate (Cheney *et al.*, 1969) or by administration of pyruvate dehydrogenase inhibitors (Gibson *et al.*, 1975) lowers acetylcholine synthesis in cytoplasm of nervous cells (Hebb, 1972). This points to the decisive role of pyruvate dehydrogenase in supplying acetyl residues for synthetic processes in cytoplasm. So far, the mechanism of transport of acetyl residues from mitochondrion to cytoplasm in nervous tissue has not been elucidated (Tuček & Cheng, 1974). In liver, about 80% of acetyl residues synthesized from pyruvate in mitochondria is transported to cytoplasm in the form of citrate (Daikuhara *et al.*, 1968). Participation of citrate in generation of acetyl residues depends on the activity of ATP citrate (*pro*-3*S*)lyase (EC 4.1.3.8). According to D'Adamo & D'Adamo (1968) and Tuček (1967), very low activity of citrate lyase in brain

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excludes citrate as a source of cytoplasmic acetyl-CoA. Poor incorporation of citrate into acetylcholine *in vivo* found by Tuček & Cheng (1974) supports this view. However, our results (Szutowicz *et al.*, 1974, 1975) indicate that the activity of citrate lyase in soluble fraction of rat brain homogenate is 10 - 20 times as high as the activity found by Tuček (1967) in sheep and rabbit, and by D'Adamo & D'Adamo (1968) in rat. To elucidate this discrepancy, more information is required on concentration of pyruvate metabolites in brain mitochondria. In the present paper it has been demonstrated that, depending on concentration of the acceptor of acetyl residues and energy state of mitochondria, the main products of intramitochondrial pyruvate metabolism are: citrate, 2-oxoglutarate or acetate.

MATERIALS AND METHODS

Reagents. Pyruvate, monopotassium salt of 2-oxoglutaric acid, phosphoenolpyruvate (PEP), NADH, EGTA, lactate dehydrogenase (EC 1.1.1.27), hexokinase (EC 2.7.1.1), L-malate dehydrogenase (EC 1.1.1.37), citrate (*pro*-3*S*)lyase (EC 4.1.3.6), sodium salt of β-hydroxybutyric acid and β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) were from Sigma Chem. Co. (St.Louis, Mo., U.S.A.). L-Glutamate dehydrogenase (EC 1.4.1.2) and acetate kinase (EC 2.7.2.1) were from Boehringer und Soehne GmbH (Mannheim, G.F.R.). L-Malic acid, ethyl ester of acetoacetic acid, Tris and bovine serum albumin, fraction V, were from B.D.H. (Poole, Dorset, England). Ficoll (Pharmacia, Uppsala, Sweden) was dialysed and freeze-dried before use. All other chemicals were from POCh (Gliwice, Poland).

Preparation of rat brain mitochondria. White Wistar rats weighing 180 - 260 g were used for the experiments. Brain mitochondria were isolated according to Clark & Nicklas (1970). The final mitochondrial pellet was suspended in the medium containing 250 mM-sucrose, 10 mM-Tris-HCl buffer, pH 7.4, and 0.5 mM-EDTA, supplemented with 0.1% of albumin. The obtained fraction was devoid of ATP-citrate lyase; the activity of lactate dehydrogenase did not exceed 0.4% of the total activity, and the activity of Na- and K-dependent ATPase was about one-tenth that of Mg-dependent ATPase. No oxygen consumption was observed in the presence of NADH as an exogenous substrate. The respiratory control ranged from 7 to 15 in the presence of pyruvate *plus* malate.

Analytical methods. Pyruvate was assayed according to Rosenberg & Rush (1966), 2-oxoglutarate by the method of Bergmeyer & Bernt (1965), citrate after Dagely (1965), and β-hydroxybutyrate according to Williamson & Mellanby (1965). Acetate was determined by the kinetic method of Knowles *et al.* (1974) and acetoacetate according to Walker (1954). ATPase activity was estimated as described by Nishimura *et al.* (1962). The rate of oxygen consumption was measured in the Warburg apparatus, or with the aid of Clark type oxygen electrode (E5046), and PHM 71 pH-meter (Radiometer, Copenhagen) provided with I 37-1 registrator (U.S.S.R.).

Protein was determined by the biuret method (Layne, 1957) with bovine serum albumin as a standard.

Incubation conditions. All experiments were performed at 37°C and pH 7.05±0.05. The incubation medium for measuring oxygen consumption with the oxygen electrode, contained in the final volume of 3 ml: 120 mM-sucrose, 50 mM-KCl, 20 mM-Tris-HCl buffer, 5.0 mM-potassium phosphate, 0.5 mM-EGTA, pyruvate and malate, 1.0 mM each, and brain mitochondria (about 1 mg protein). State 3 was induced by addition of 1.66 mM-ADP.

The pyruvate metabolites were determined in the reaction mixture incubated in the Warburg apparatus, with simultaneous measurement of oxygen uptake. Metabolism of pyruvate in state 4 was studied in the same medium (2 ml) as used for determination of oxygen consumption with oxygen electrode, except that concentration of pyruvate was 2.5 mM, and brain mitochondria 3 - 4 mg protein. After 3 min incubation, the reaction was stopped by the addition of 0.2 ml of 30% $HClO_4$. The sample was centrifuged for 3 min at 20 000 *g* and the supernatant neutralized with K_2CO_3 was used for determination of the metabolites.

Metabolism of pyruvate in mitochondria in state 3 was studied in the medium containing in a final volume of 2 ml: 20 mM-potassium phosphate, 10 mM-Tris-HCl buffer, 50 mM-sucrose, 60 mM-glucose, 2 mM-$MgCl_2$, 0.5 mM-EGTA, 1.25 mM-ADP, 5.0 mM-pyruvate, 0.5 or 5.0 mM-malate and about 1 mg of mitochondrial protein. After 3 min preincubation, hexokinase (60 μg) was added from a side vessel. After 20 min incubation, the reaction was stopped by the addition of 0.2 ml of 30% $HClO_4$ from another side vessel. The deproteinized and neutralized supernatant was used for determination of the metabolites.

Formation of acetate in state 3 was not determined due to high concentration of pyruvate in the incubation medium.

RESULTS

Oxygen consumption by rat brain mitochondria

As it appears from the results presented in Fig. 1, the rate of oxygen consumption by rat brain mitochondria in state 4 in the presence of pyruvate *plus* malate, was unaltered over pH range from 5.8 to 8.0 whereas in state 3 a distinct optimum was observed at pH 6.90 - 7.20. Further experiments were therefore performed at pH 7.05±0.05.

The rate of oxygen consumption by rat brain mitochondria in state 3 (Table 1) was the lowest with pyruvate as a sole respiratory substrate, and it was at least doubled (about 50 nmoles of O_2/min/mg protein) with malate, 2-oxoglutarate, succinate or glutamate. The addition of pyruvate to the incubation mixture containing a dicarboxylic substrate distinctly stimulated oxygen consumption. In the presence of malate and succinate, this stimulation was almost fivefold, whereas on addition of 2-oxoglutarate and glutamate, about threefold.

Oxygen consumption was not further stimulated when pyruvate concentration was raised to 4 mM and that of dicarboxylic substrate to 6 mM.

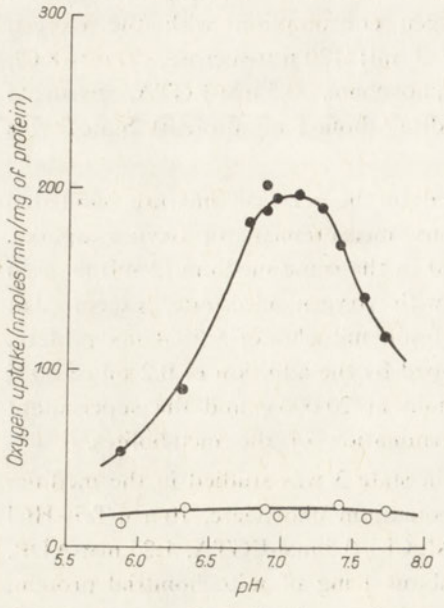

Fig. 1.

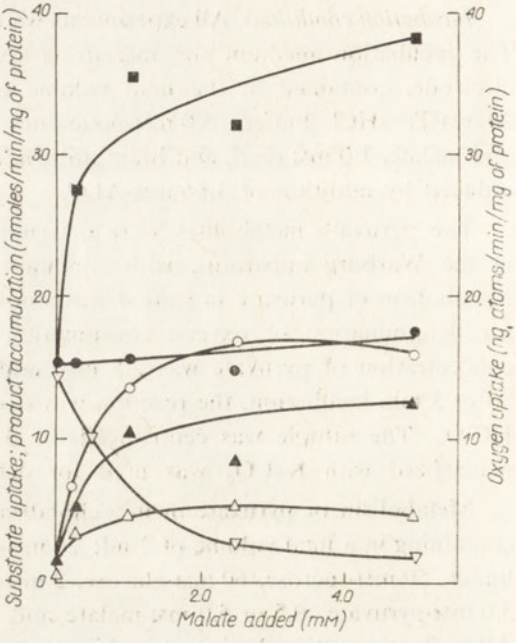

Fig. 2.

Fig. 1. Effect of pH on the rate of oxygen consumption by rat brain mitochondria. The incubation mixture contained: 1 mM-pyruvate, 1 mM-malate, 2 mM-$MgCl_2$, and about 1 mg of mitochondrial protein. Mitochondria: ○, in state 4; ●, in state 3 (induced by 1.66 mM-ADP).

Fig. 2. Effect of malate on pyruvate metabolism in rat brain mitochondria in state 4. The incubation medium contained: 2.5 mM-pyruvate and 3.5 mg of mitochondrial protein. Consumption of: ■, oxygen; ●, pyruvate; ○, malate. Formation of: ▲, citrate; △, 2-oxoglutarate; ▽, acetate.

Table 1

Effect of pyruvate on oxygen consumption by rat brain mitochondria in state 3

The incubation medium contained 2 mM-$MgCl_2$ and about 1 mg of mitochondrial protein.

Substrate	Oxygen consumption (nmoles O_2/min/mg protein)	
	without pyruvate	with 1 mM-pyruvate
None	—	21
Malate (1 mM)	55	260
2-Oxoglutarate (2 mM)	46	158
Succinate (2 mM)	53	285
Glutamate (2 mM)	61	169

Pyruvate metabolism in rat brain mitochondria

Mitochondria in state 4. In the absence of malate in the incubation medium, pyruvate was converted in mitochondria in state 4 in a one-step reaction to acetate, which accounted for about 80% of utilized pyruvate; the ratio of oxygen consumed

to pyruvate reacted equalled unity (Table 2). 2-Oxoglutarate, citrate and malate appeared in only trace amounts. On addition of ATP, the consumption of oxygen was doubled whereas utilization of pyruvate was but slightly increased. At the same time, acetate concentration decreased by two-thirds. Mg^{2+} added together with ATP lowered conversion of pyruvate whereas acetate concentration was twice as high as that found in the presence of ATP without Mg^{2+}. Addition of oligomycin to the medium containing pyruvate and ATP, caused a parallel inhibition of oxygen and pyruvate consumption by about 30%. Simultaneously, formation of 2-oxoglutarate and malate was raised, and that of acetate decreased to one-fifth. On addition of 2 mM-Mg^{2+}, inhibition of oxygen and pyruvate consumption caused by oligomycin, was partially relieved: formation of 2-oxoglutarate and malate was decreased to control values, and that of acetate corresponded to 70% of the control values. The addition of 0.5 mM-malate to the incubation medium containing 5 mM-ATP, 2 mM-Mg^{2+} and pyruvate resulted, as compared with the control values, in a more than fourfold stimulation of oxygen consumption with a simultaneous twofold enhancement of pyruvate conversion. The amount of acetate formed corresponded only to about 1.5% of pyruvate utilized. Formation of 2-oxoglutarate was higher by a factor of 15, and that of citrate was increased fourfold (Table 2).

Table 2

Effect of ATP and Mg on pyruvate metabolism in rat brain mitochondria in state 4

The incubation mixture contained 2.5 mM-pyruvate, about 3.5 mg of mitochondrial protein, and where indicated 5 mM-ATP, 2 mM-$MgCl_2$, oligomycin (1 µg/ml) or malate (0.5 mM). Oxygen consumption is expressed in ng atoms O_2/min/mg protein, other results in nmoles/min/mg protein.

Additions	Consumption of		Formation of			
	oxygen	pyruvate	2-oxo-glutarate	citrate	acetate	malate
None (control)	17.9	16.4	0.2	0.5	14.0	0.4
ATP	39.0	20.5	0.3	0.3	4.3	0.4
ATP+Mg^{2+}	33.5	16.4	0.2	0.5	7.7	0.5
ATP+oligomycin	12.7	11.7	1.0	0.2	2.7	0.7
ATP+Mg^{2+}+oligomycin	16.9	13.2	0.4	0.3	9.8	0.4
ATP+Mg^{2+}+malate	77.0	31.0	3.4	2.3	0.5	—

As can be seen from the results presented in Fig. 2, the rate of pyruvate conversion in mitochondria in state 4 in the absence of ATP and Mg^{2+}, was independent of the concentration of malate added. Oxygen consumption reached the maximum value at a malate concentration of about 1 mM, and was under these conditions twice as high as pyruvate utilization. At higher malate concentrations, over 1 mM, utilization of pyruvate and malate was equimolar, which indicates that, under these conditions, citrate synthesis proceeded at the maximum rate and was twice as high as formation of 2-oxoglutarate. Synthesis of acetate decreased rapidly with the increase in malate concentration in the medium.

Mitochondria in state 3. Consumption of oxygen and pyruvate by rat brain mitochondria in state 3 (Fig. 3A,B) was linear with time in the presence of both 0.5 and 5 mM-malate in the incubation medium. The increase in malate concentration from 0.5 to 5.0 mM caused a parallel 50% stimulation of both oxygen consumption and pyruvate utilization. After 10 min incubation of mitochondria with 0.5 mM-malate, 90% of this amount was utilized concomitantly with the equimolar amount of pyruvate. The sum of substrates utilized and the reaction products formed corresponded to oxygen consumption. Since, on further incubation, the level of malate, citrate and 2-oxoglutarate remained unchanged and oxygen consumption continued to increase, one may anticipate a several-fold turnover of malate in the tricarboxylic acids cycle. In the presence of 0.5 mM-malate in the incubation medium, the ratio of citrate to 2-oxoglutarate equalled 0.62.

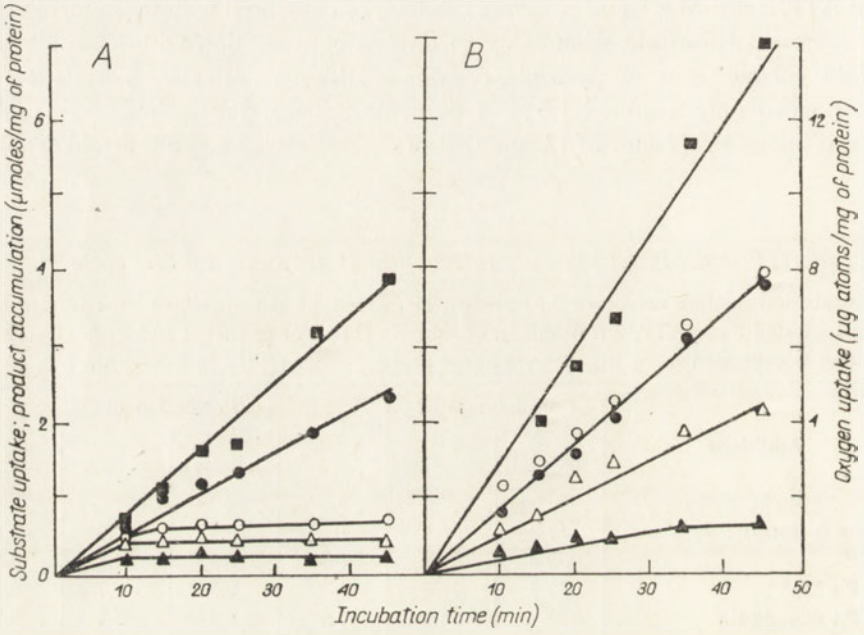

Fig. 3. Effect of malate on pyruvate metabolism in rat brain mitochondria in state 3. The incubation medium contained: 5.0 mM-pyruvate, 1 mg of mitochondrial protein and malate at final concentration of: 0.5 mM (*A*) or 5.0 mM (*B*). Consumption of: ■, oxygen; ●, pyruvate; ○, malate. Formation of: △, 2-oxoglutarate; ▲, citrate.

In the presence of 5 mM-malate, utilization of pyruvate was equimolar with that of malate, and was consistent with oxygen consumption (Fig. 3B). The ratio of citrate to 2-oxoglutarate was 0.38.

β-Hydroxybutyrate was not formed in rat brain mitochondria either in state 3 or 4, and only trace amounts of acetoacetate could be detected. No formation of lactate was observed.

DISCUSSION

The rate of oxygen consumption by rat brain mitochondria with pyruvate and malate depended to a large extent on pH of the incubation medium and the metabolic state of mitochondria: in state 3, in contrast to liver mitochondria (Tobin *et al.*, 1972), a distinct pH optimum (6.90 - 7.20) was observed; in state 4, changes in pH of the medium did not affect the rate of oxygen consumption. Hydrogen ion concentration in brain tissue *in vivo* was found to be 9.1×10^{-8} M, i.e. pH 7.04 (Siesjö *et al.*, 1972).

Low consumption of oxygen by rat brain mitochondria with pyruvate as a sole substrate is presumably related to the deficiency of oxaloacetate and to low activity of pyruvate carboxylase, reported by Wilbur & Patel (1974). Oxygen consumption with pyruvate is stimulated several-fold by malate and succinate, and to a smaller extent by 2-oxoglutarate and glutamate, in correspondence to the efficiency of their conversion to oxaloacetate. Accumulation of 2-oxoglutarate observed in state 3 suggests that oxoglutarate dehydrogenase (EC 1.2.4.2) is a step limiting the rate of pyruvate oxidation in the tricarboxylic acids cycle. From the studies of LaNoue *et al.* (1970, 1972) performed on heart mitochondria it is known that succinyl-CoA accumulates in state 3 and might be responsible for inhibition of oxoglutarate dehydrogenase. In state 3, the 0.5 mM concentration of malate was insufficient to assure the maximum rate of pyruvate oxidation (see Fig. 3A). As it was found by Nicklas *et al.* (1971), low level of oxaloacetate — the acceptor of acetyl residues — may result in accumulation of acetyl-CoA, and consequently in inhibition of pyruvate dehydrogenase (Erfle & Sauer, 1969). On the other hand, at low concentration of oxaloacetate, succinate dehydrogenase is not inhibited (Ackrell *et al.*, 1974). The ratio of citrate to 2-oxoglutarate at 0.5 mM concentration of malate was half that found at 5 mM concentration of malate. It might be, therefore, concluded that the increase in malate concentration results in accumulation of succinyl-CoA and thus in inhibition of oxoglutarate dehydrogenase.

Preferential accumulation of citrate observed in state 4 implies that under these conditions isocitrate dehydrogenase limits the oxidation of pyruvate in the tricarboxylic acids cycle due to the increased intramitochondrial level of ATP and NADH (Chen & Plaut, 1963). In rat brain mitochondria, in contrast to other tissues, the activity of NAD-dependent isocitrate dehydrogenase accounts for about 60% of the total activity of this enzyme in mitochondria (Watanabe *et al.*, 1974). Therefore one may anticipate that pyruvate metabolism is regulated by changes in intramitochondrial ratios of ATP/ADP and NADH/NAD, allosteric effectors of isocitrate dehydrogenase.

Under the conditions studied, pyruvate carboxylation is low, as evidenced by low concentration of malate when pyruvate serves as a sole oxidative substrate (cf. Table 2). Carboxylation is stimulated by the addition of ATP and ATP+ oligomycin, and thus might be responsible for concomitant lowering of acetate formation. Addition of Mg^{2+} in the presence of ATP raised acetate concentration since by inducing the activity of Mg^{2+}-dependent mitochondrial ATPase (Kimelberg &

Bourke, 1973) it lowered concentration of ATP accessible for carboxylation process (Patel & Tilgham, 1973). Presumably, oligomycin does not inhibit completely the activity of mitochondrial Mg^{2+}-dependent ATPase (Slater, 1967), as oxygen consumption and pyruvate utilization are higher in the presence of Mg^{2+} than in its absence (cf. Table 2).

As it has been shown in this paper, citrate, 2-oxoglutarate and acetate were the main metabolites of pyruvate in brain mitochondria and malate played an essential role in incorporation of pyruvate into tricarboxylic acids cycle.

In the absence of malate or its low level, pyruvate is metabolized mainly to acetate in the reaction described by Knowles *et al.* (1974) catalysed by acetyl-CoA hydrolase. Formation of acetate decreases rapidly parallelly to the increased rate of pyruvate utilization, even when pyruvate oxidation is stimulated by ATP and Mg^{2+} at low concentration of malate (cf. Table 2).

In the presence of malate, not acetate but citrate and 2-oxoglutarate were the main products of pyruvate metabolism. *In vivo*, the level of oxaloacetate precursors, succinate and malate, is several-fold higher than that of pyruvate (Norberg & Siesjö, 1975). It seems therefore that the lack of acetyl residues *in vivo* is improbable and a possible shortage of oxaloacetate might be only due to impaired transport of this precursor into mitochondrion (Minn *et al.*, 1975) or to its conversion to aspartate. The data presented imply that in brain *in vivo* citrate may serve as a carrier of acetyl-CoA from mitochondria to cytosol. Generation of acetyl-CoA seems to be possible as the activity of citrate lyase in brain cytosol was shown to be high enough to catalyse this reaction (Szutowicz *et al.*, 1975, 1976).

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METABOLIZM PIROGRONIANU W MITOCHONDRIACH MÓZGU SZCZURA

Streszczenie

1. Utlenianie pirogronianu przez mitochondria mózgu szczura w stanie 3 jest stymulowane przez jabłczan lub bursztynian (250 nmoli O_2/min/mg białka). Zużycie tlenu w obecności samego jabłczanu, bursztynianu lub oksoglutaranu czy też glutaminianu było 4 - 5 -krotnie niższe.

2. Maksymalne zużycie tlenu w stanie 3 obserwowano w pH 6.90 - 7.20. Natomiast w stanie 4 nie obserwowano wpływu pH na szybkość zużycia tlenu.

3. W stanie 4 przy braku egzogennego akceptora reszty acetylowej spalanie pirogronianu zachodziło jednoetapowo; głównym produktem przemiany był octan, który stanowił około 80% ilości zużytego pirogronianu. Jabłczan nie miał wpływu na szybkość zużycia pirogronianu, natomiast obniżał stężenie octanu i zwiększał stężenie cytrynianu i oksoglutaranu.

4. W stanie 3 głównym produktem przemiany pirogronianu i jabłczanu był oksoglutaran, którego stężenie było około trzykrotnie wyższe od stężenia cytrynianu.

5. Powstawanie cytrynianu, oksoglutaranu i octanu w mózgu jest zależne od dostępności akceptora reszty acetylowej i stanu energetycznego mitochondrionu.

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ANNA LIPIŃSKA, WANDA KRAJEWSKA, ZOFIA KILIAŃSKA, RYSZARD WIERZBICKI and LEOKADIA KŁYSZEJKO-STEFANOWICZ

STUDIES ON HISTONES AND NUCLEAR PHOSPHOPROTEINS OF RAT LIVER AND MORRIS HEPATOMA*

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1. The chemical composition of chromatins obtained from Buffalo rat liver and Morris hepatoma strain 5123 was investigated.

2. The presence of an additional subfraction within the very lysine-rich histone (*f1*) was stated in both kinds of tissues. It amounted to about 8% and 5% of *f1* in rat liver and Morris hepatoma, respectively.

3. Nuclear phosphoproteins (phenol-soluble) from normal and tumour tissues in polyacrylamide gel in SDS showed some qualitative differences in the range of molecular weights of about 40 000 - 70 000 and over 100 000 daltons.

Studies of histones have generally indicated their similarity in normal and neoplastic cells. Recently, evidence has been obtained for the presence in several hepatomas of a minor histone fraction (Lea *et al.*, 1974) previously reported to be absent in tumours (Panyim & Chalkley, 1969a). Non-histone chromatin proteins differ at various development stages (LeStourgeon & Busch, 1973; Vidali *et al.*, 1973) and from tissue to tissue of a given organism (Wu *et al.*, 1973; Bekhor *et al.*, 1974). It has also been demonstrated that non-histone proteins of tumour tissues differ both qualitatively and quantitatively from these proteins of normal tissues (Kostraba & Wang, 1972; Wierzbicki *et al.*, 1974; Chae *et al.*, 1974).

In this paper the results obtained in the course of examination of histones and nuclear phosphoproteins (phenol-soluble non-histone nuclear proteins) from Morris hepatoma strain 5123 are reported. This kind of tumour is metabolically very similar to normal liver.

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MATERIAL AND METHODS

Materials. The experiments were performed on the Buffalo rats. Morris hepatoma strain 5123 was inoculated in the thigh muscles at the Institute of Oncology (Gliwice) and 6 weeks were allowed for tumour growth.

Isolation of cell nuclei and chromatin. Nuclei were isolated by the saccharose method. Normal liver or disintegrated tumour were homogenized in 0.25 M-saccharose - 0.8 mM-KH_2PO_4 - 1 mM-$CaCl_2$, pH 6.7, and the nuclei were purified twice by centrifugation through 0.4 M-saccharose - 0.18 mM-$CaCl_2$ - 0.8 mM-KH_2PO_4, pH 6.7, at 700 *g* for 5 min. The nuclei were examined for purity by methyl green--pyronine staining as well as by phase microscopy. The purified nuclei were used for preparation of chromatin by the method of Spelsberg & Hnilica (1971).

Isolation of histones and nuclear phosphoproteins. Total histone was obtained by extraction of chromatin with 0.2 M-H_2SO_4. Histone *f1* was isolated in the course of the fractionation of total histone according to the method of Oliver *et al.* (1972). Nuclear phosphoproteins were obtained by the method of Teng *et al.* (1971).

Electrophoresis. Total histone and histone *f1* were fractionated by electrophoresis in 15% acrylamide, 2.5 M-urea, 0.9 M-acetic acid, pH 2.7, according to Panyim & Chalkley (1969b). Phosphoproteins were characterized by electrophoretic analysis in 10% polyacrylamide gel containing 1% sodium dodecyl sulphate and 0.14 M--2-mercaptoethanol, pH 7.4 (Teng *et al.*, 1971). The approximate molecular weights of the phosphoprotein fraction were estimated against the relative migrations of standard proteins: α-phosphorylase (mol.wt. 94 000), bovine serum albumin (68 000), subunits of catalase (60 000), ovalbumin (43 000), deoxyribonuclease I (31 000) and cytochrome *c* (12 000).

Gels after staining were scanned at 560 nm using an ERJ 65 densitograph (Carl Zeiss, Jena, G.D.R.).

Chemical analysis of chromatin. Protein content was determined by the microbiuret method (Itzhaki & Gill, 1964), RNA by orcinol method (Schneider, 1957) and DNA according to Burton (1956).

Reagents. Tris came from Loba-Chemie (Wien, Austria). α-Phosphorylase and deoxyribonuclease I were purchased from Worthington Biochemicals Corp. (Freehold, N.J., U.S.A.). bovine serum albumin, catalase, ovalbumin and cytochrome *c* were products of Serva Feinbiochemica (Heidelberg, G.F.R.). Sodium dodecyl sulphate (SDS) specially pure, perchloric acid, calf thymus DNA and yeast RNA were supplied by B.D.H. Chemicals Ltd (Poole, Dorset, England).

Acrylamide was a product of Koch-Light Lab. Ltd (Colnbrook, Bucks, England); *N,N'*-bisacrylamide was from Fluka AG (Buchs S.G., Switzerland), and 1,4-tetra--methylethylenediamine (TEMED) from K and K Lab.Inc. (Plainview, N.Y., U.S.A.); naphthalene Black 10B from George T. Gurr (High Wykombe, Bucks, England) and Coomassie Brilliant Blue R from Sigma Chem. Co. (St.Louis, Mo., U.S.A.). Other reagents were analytical grade products supplied by POCh (Gliwice, Poland).

RESULTS AND DISCUSSION

The chromatin preparations obtained from liver and Morris hepatoma showed identical spectra in the ultraviolet range (Table 1) and, typical for chromatin, low A_{320}/A_{260} values and high A_{260}/A_{290} values.

Table 1

Ultraviolet absorption spectra of chromatins isolated from rat liver and Morris hepatoma

Limit values from 7 experiments are given.

Spectral ratios	Source of chromatin	
	Rat liver	Morris hepatoma
A_{320}/A_{260}	0.05 - 0.08	0.06 - 0.07
A_{280}/A_{260}	0.63 - 0.68	0.63 - 0.68
A_{260}/A_{230}	0.77 - 0.97	0.76 - 0.93
A_{260}/A_{270}	1.11 - 1.15	1.09 - 1.13
A_{260}/A_{280}	1.46 - 1.57	1.47 - 1.56
A_{260}/A_{290}	2.52 - 2.98	2.58 - 2.92

Chemical analysis of chromatin preparations showed that the ratios of total protein to DNA were in both cases practically identical (Table 2), in agreement with the u.v. spectra. The values of the ratio of histone to non-histone protein indicate the lack of essential difference in the content of both these proteins between normal and neoplastic tissues. Our results do not quite agree with the data given by Chae *et al.* (1974). They have found significantly higher content of non-histone protein in hepatoma chromatin in comparison with normal tissue. The discrepancy does not seem to be caused by the different ways of chromatin isolation as total protein to DNA ratio in chromatin preparations from rat liver in our and their experiments are almost identical.

Table 2

Chemical composition of rat liver and Morris hepatoma chromatins

The results are expressed as w/w ratios. Limit values from 3 experiments are given.

	Source of chromatin	
	Rat liver	Morris hepatoma
Total protein/DNA	1.67 - 1.75	1.62 - 1.76
RNA/DNA	0.09 - 0.11	0.09 - 0.10
Histone/non-histone protein	0.73 - 0.83	0.62 - 0.64

Electrophoretic analysis of total histone indicated the presence of an additional subfraction *f1a* (Fig. 1) which has also been observed by Lea *et al.* (1974). This subfraction was extracted together with histone *f1* by 0.5 M-$HClO_4$ (Panyim & Chalkley, 1969a; Lipińska & Klyszejko-Stefanowicz, 1975). Subfraction *f1a* (Fig. 2)

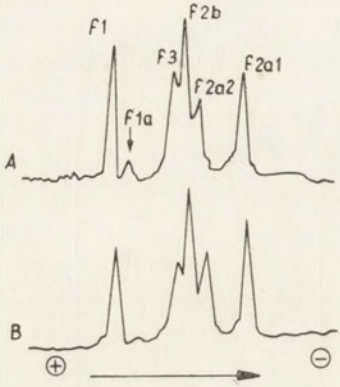

Fig. 1

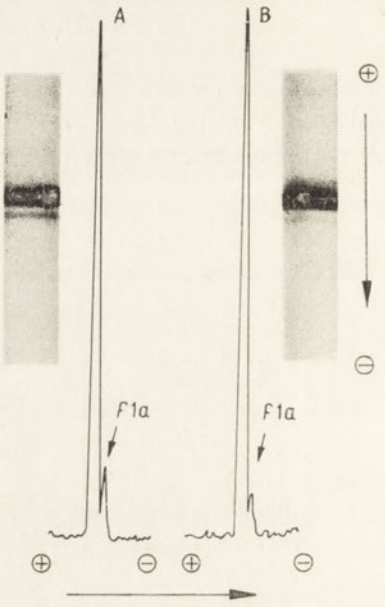

Fig. 2

Fig. 1. Densitometric tracings of total histone from rat liver (*A*) and Morris hepatoma (*B*) after gel electrophoresis according to Panyim & Chalkley (1969b). Electrophoresis was run for 3.5 h at pH 2.7 and 1.5 mA per gel. The protein was stained with 1% Naphthalene Black 10B in 40% ethanol and 7% acetic acid.

Fig. 2. Polyacrylamide-gel electrophoresis of histone *f1* from rat liver (*A*) and Morris hepatoma (*B*) obtained according to Oliver *et al.* (1972). Electrophoresis was run for 3.5 h at pH 2.7 and 1.5 mA per gel. The protein was stained with 1% Naphthalene Black 10B in 40% ethanol and 7% acetic acid; 20 μg of protein was applied per gel.

represented about 8% and 5% of histone *f1* in rat liver and Morris hepatoma, respectively (as calculated from densitometric tracing).

The nuclear phosphoprotein isolated from liver and Morris hepatoma, separated on polyacrylamide-gel electrophoresis into 17 - 20 distinct bands corresponding to polypeptide chains of different size (Fig. 3). They revealed many similarities. A number of common bands were present, which may be in agreement with the concept of "limited heterogeneity" for these proteins (Elgin & Bonner, 1970). However, there were some striking differences. In the case of tumour phosphoproteins a few additional fractions were observed in the range of molecular weight between 40 000 - 70 000 daltons as well as a fraction with molecular weight over 100 000 daltons.

The above-mentioned additional fractions seem to correspond to the fractions observed previously by us (Wierzbicki *et al.*, 1974) in the total non-histone protein of Morris hepatoma. The similarity of differences between total non-histone protein and phosphoprotein of hepatoma and normal liver suggests that the changes concern mainly nuclear phosphoproteins. It is worth noting that differences observed by Chae *et al.* (1974) in the electrophoretic patterns of hepatoma non-histone proteins,

concerned also components of similar molecular weight. The occurrence of additional fractions in non-histone chromatin protein of Novikoff hepatoma has been demonstrated by Yeoman *et al.* (1973) also by means of two-dimensional electrophoresis in polyacrylamide gel.

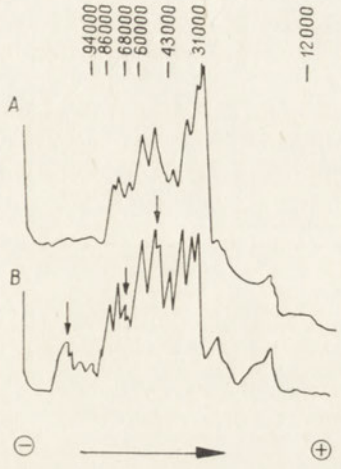

Fig. 3. Densitometric tracings of nuclear phosphoproteins from rat liver (*A*) and Morris hepatoma (*B*) after sodium dodecyl sulphate gel electrophoresis at pH 7.4 according to Teng *et al.* (1971). About 150 µg of protein were applied per gel. Electrophoresis was run for 7 h at pH 7.4 and 5 mA per gel. The protein was stained with 0.02% Coomassie Blue in 12.5% trichloroacetic acid.

Preliminary experiments on the functional significance of nuclear phosphoprotein in cell-free RNA synthesizing system showed that phenol-soluble non-histone protein both from normal and tumour tissue stimulated RNA synthesis.

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BADANIA HISTONÓW I FOSFOPROTEIDÓW JĄDER KOMÓRKOWYCH WĄTROBY SZCZURA I WĄTROBIAKA MORRISA

Streszczenie

1. Analizowano skład chemiczny chromatyny wątroby szczura Buffalo i wątrobiaka Morrisa 5123.

2. W obrębie histonu silnie lizynowego (*f1*) wykazano obecność dodatkowej podfrakcji *f1a*, która stanowiła około 8% i 5% histonu *f1*, odpowiednio w wątrobie szczura i wątrobiaku Morrisa.

3. Analiza w żelu poliakryloamidowym zawierającym siarczan dodecylu sodu rozpuszczalnych w fenolu fosfoproteidów jąder komórkowych tkanki prawidłowej i nowotworowej wykazała występowanie jakościowych różnic w zakresie mas cząsteczkowych od 40 000 do 70 000 daltonów oraz ponad 100 000 daltonów.

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A C T A B I O C H I M I C A P O L O N I C A

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PROTEINS OF YEAST RIBOSOMAL SUBUNITS: NUMBER AND GENERAL PROPERTIES *

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1. *Saccharomyces cerevisiae* at the early stationary phase of growth accumulate 80 S ribosomes, easily dissociating into subunits, which retain full activity in phenylalanine polymerization *in vitro*. A simplified and efficient technique for large-scale preparation of yeast ribosomal subunits is proposed.

2. Presence of 34 proteins in 40 S subunit and 42 proteins in 60 S subunit was demonstrated by two-dimensional acrylamide-gel electrophoresis. Both ribosomal subunits contain acidic proteins: three in 60 S and six or seven in 40 S subunit. It seems that two of them correspond to prokaryotic proteins, L7 and L12. The total number of yeast ribosomal proteins is similar to those obtained for other Eukaryota.

Recent studies on eukaryotic ribosomes revealed at least 70 different protein components (Sherton & Wool, 1974a; Kruiswijk & Planta, 1974; see also Traugh & Traut, 1973), whereas the simplest prokaryotic ribosomes, e.g. of *E. coli* contain 55 proteins (Garret & Wittmann, 1973; Wittmann, 1976). Thus far, much work has been done on isolation and characterization of *E. coli* ribosomal proteins and their spatial arrangement in the ribosome particle (for review see Garret & Wittmann, 1973; Wittmann, 1976). Studies on eukaryotic ribosomal proteins are less advanced though attempts have been made to separate and characterize them by two-dimensional polyacrylamide-gel electrophoresis (Welfle *et al.*, 1971; Martini & Gould, 1971; Bickle & Traut, 1971; Sherton & Wool, 1972; Westermann & Bielka, 1973; Howard *et al.*, 1975; Terao & Ogata, 1975).

Unequivocal identification of the ribosomal proteins requires highly purified ribosomal subunits depleted of any cytoplasmic contaminants. The basic method for isolation of active and pure ribosomal subunits from Eukaryota was developed by Martin & Wool (1968). According to this method, ribosomes are treated with KCl

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at concentration up to 1.0 M (Martin *et al.*, 1969). However, dissociation of ribosomes into subunits at high concentration of KCl depends on removal of the nascent peptide chain, e.g. by incubation of polysomes *in vitro* with puromycin (Blobel & Sabatini, 1971) or without puromycin but under conditions favourable for termination of polypeptide chain synthesis (Falvey & Staehelin, 1970).

In 1972 Van der Zeijst *et al.* described a very efficient method for isolation of yeast ribosomal subunits from protoplasts of *Saccharomyces carlsbergensis* treated with 1 mM-NaN_3, which inhibits initiation of protein and leads to accumulation of the salt-labile 80 S ribosomes.

This paper describes another modified, large-scale procedure for isolation of yeast ribosomal subunits. We have taken advantage of the fact that *Saccharomyces cerevisiae*, grown under strictly controlled conditions, accumulate at an early stationary phase of growth the "run off" 80 S ribosomes easily dissociable in buffered 0.5 M-KCl solution. The accumulated ribosomes as well as the subunits derived from them, when recombined, were very active in polypeptide synthesis directed by polyuridylic acid. We report also on separation of individual ribosomal proteins from both ribosomal subunits using a slightly modified technique of two-dimensional polyacrylamide-gel electrophoresis.

MATERIAL AND METHODS

Yeast cultures. Saccharomyces cerevisiae, *As*-strain, was cultivated aerobically in a rich medium of Cooper *et al.* (1962). The growth medium was inoculated with one tenth its volume of the 12 - 14 h growing yeast culture ($A_{450nm}=3.5$ - 4.0) and yeast cells were cultivated for the time indicated. The cultures reached the mid-log phase ($A_{450nm}=1.8$) and the early stationary phase ($A_{450nm}=3.5$) of growth after 4.5 - 5.0 h and 6 - 7 h, respectively. Growth was routinely determined for each yeast culture. The cells were harvested, at the above mentioned growth phases, washed once with saline and twice with buffer *A* (50 mM-Tris-HCl, pH 7.5, 10 mM-KCl, 10 mM-$MgCl_2$ and 6 mM-mercaptoethanol). The cell mass was used immediately or stored frozen at −15°C.

Preparation of cell-free extraets, ribosomes, and elongation factors. Yeast cell-free extracts were obtained by mechanical rupturing of the cell wall with corundum or alumina as the abrasives (Grankowski *et al.*, 1974). Ribosomes were isolated as follows: the postmitochondrial extract was treated with Triton X-100 added slowly, with continuous stirring, to a final concentration of 1%. The mixture was allowed to stand in an ice-bath for 30 min and was centrifuged for 2 h at 150 000 *g*. The resulting ribosome pellets were used for dissociation experiments or were further purified by washing twice with buffer *B* (50 mM-Tris-HCl, pH 7.5, 0.5 M-KCl, 50 mM-$MgCl_2$, 6 mM-mercaptoethanol) as described previously (Grankowski & Gąsior, 1975).

Partly purified preparations of yeast elongation factors were obtained by the method of Richter & Lipmann (1974). ^{14}C-labelled yeast phenylalanyl-tRNA preparations were kindly supplied by T. Jakubowicz from this Department.

Dissociation of ribosomes and separation of ribosomal subunits. Crude, Triton-treated ribosome pellets were suspended in buffer *C* (50 mM-Tris-HCl, pH 7.5, 0.5 M-KCl, 5 mM-$MgCl_2$ and 6 mM-mercaptoethanol) by gentle homogenization, and the aggregates were removed by low-speed centrifugation. The suspension was kept in ice until it was loaded into a MSE super-speed 65 ultracentrifuge zonal rotor B-XIV. All operations were performed according to the MSE Instruction Manual (MSE Technical Publication No 49). The rotor was filled with buffer *C* followed by: a ribosomal sample (1100 - 1900 A_{260nm} units in a volume of 20 ml), a linear gradient, by volume, of 10 - 30% sucrose, generated with the use of MSE gradient former, and finally a cushion of 50% sucrose solution. All solutions were made in buffer *C*. Ribosomal subunits were separated by centrifugation at 14°C for 6 - 7 h at 30 000 r.p.m. and 10 ml fractions were collected after displacement of the gradient with 60% sucrose. The optical density profile of the gradient was measured spectrophotometrically at 260 nm. The collected fractions corresponding to 40 S and 60 S subunits were pooled separately and after dilution, each with one volume of buffer *D* (50 mM-Tris-HCl, pH 7.5, 20 mM-$MgCl_2$ and 20 mM-mercaptoethanol), were centrifuged for 12 h at 150 000 *g* in an aluminium MSE angle rotor at 2 - 4°C.

The resulting 60 S ribosomal subunits, slightly contaminated by their 40 S counterparts, were subjected to the second sucrose gradient centrifugation under conditions described above. Under the applied conditions of zonal rotor centrifugation, 40 S ribosomes were not contaminated by 60 S subunits; nevertheless, they were routinely washed with buffer *B* to remove any cytoplasmic protein bound to ribosomal particles. Both 40 S and 60 S ribosome preparations were stored at −20°C as a suspension in buffer *E* (50 mM-Tris-HCl, pH 7.5, 10 mM-KCl, 15 mM-$MgCl_2$, 6 mM-mercaptoethanol and 40% glycerol).

Assay of ribosome and ribosomal subunit activities. The incubation mixture contained in a total volume of 0.25 ml: 100 mM-Tris-acetate, pH 6.5, 70 mM-NH_4Cl, 10 mM-magnesium acetate, 0.4 mM-GTP, 25 μg of poly U, 180 - 200 μg of 80 S ribosomes, or 60 μg and 120 μg, respectively, of 40 S and 60 S subunits; approximately 20 μg of [^{14}C]phenylalanyl-tRNA (spec. act. 4×10^5 c.p.m./mg tRNA) and 50 - 80 μg of elongation factors protein. The samples were incubated for 20 min at 30°C and counted using a Packard liquid scintillation spectrometer.

Analysis of RNA. Samples containing 4 - 5 mg ribosomal subunits/ml were incubated with 0.1% (final concentration) SDS[1] for 15 min at 37°C according to Sherton *et al.* (1974). Then 0.1 ml aliquots were layered on analytical sucrose gradient (5 - 20% sucrose in a 50 mM-Tris-HCl buffer, pH 7.5) and centrifuged at 4°C for 3 h at 48 000 r.p.m. using a MSE SW (3×6.5 ml) rotor.

Isolation and preparation of ribosomal proteins for gel electrophoresis. Proteins were extracted from ribosomes and ribosomal subunits with 67% acetic acid according to Hardy *et al.* (1969). Lyophilized proteins (1.0 mg) were dissolved in 0.1 ml of 8.0 M deionized urea containing 0.1 M-mercaptoethanol (pH was adjusted to 8.0 with Tris) and the sample was incubated for 2 h at 37°C or 5 - 6 h at room tempera-

[1] Abbreviation: SDS, sodium dodecyl sulphate.

ture to assure reduction of proteins (Ventimiglia & Wool, 1974). The preincubated samples were supplemented with sucrose (10%, final concentration) and used for gel electrophoresis.

Two-dimensional polyacrylamide-gel electrophoresis. Electrophoresis in the first dimension was run at pH 8.6 on a 4 × 160 mm column of 8% acrylamide and 0.2% bis-acrylamide. Other constituents and buffers were as in the original procedure of Kaltschmidt & Wittmann (1970). Then equal amounts of protein sample were applied directly at the top of two separate gels; neither spacer nor sample gel was used (Howard & Traut, 1973). Electrophoresis of one gel was from the anode to catode and of the other gel, in the opposite direction. Both gels were run for 16 - 17 h at a current of 2 mA per tube. The method enabled good resolution of both acidic and basic ribosomal proteins.

After electrophoresis in the first dimension, the gels were kept for 5 min in 0.3 M-HCl and then washed several times with cold distilled water. Electrophoresis in the second dimension was run for 22 - 24 h on 18% acrylamide gel slabs (4 × 150 × × 170 mm) at pH 4.6 and a current of 35 mA per slab. All runs were performed at 4°C. The gels were stained with 0.5% Amido Black in 7% acetic acid and destained in 7% acetic acid. The protein spots from each subunit were numbered separately along horizontal lines beginning at the upper left of the gel slab (Sherton & Wool, 1972). The 60 S and 40 S ribosomal proteins were designated by L (large) and S (small), respectively.

Other methods. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard, and RNA spectrophotometrically. Concentration of ribosomes and ribosomal subunits was calculated after Van der Zeijst *et al.* (1972).

Reagents. Mercaptoethanol, Triton X-100, K-salt of poly U were from Serva Feinbiochemica (Heidelberg G.F.R.), Tris was the product of Koch-Light Lab. (Colnbrook, Bucks, England), acrylamide, bis-acrylamide and sucrose were purchased from British Drug Houses (Poole, Dorset, England), *N,N,N',N'*-tetramethylethylenediamine from Fluka A.G. (Buchs, Switzerland). GTP-sodium salt was from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and [^{14}C]phenylalanine (spec. act. 270 mCi/mmole), from the Institute of Radioisotopes (Prague, Czechoslovakia).

RESULTS

Dissociability of 80 S yeast ribosomes. A comparative study was made on the ability of 80 S yeast ribosomal preparations, isolated from different growth phases, to dissociate at high concentration of KCl. It appeared (Fig. 1) that ribosomes from an early stationary phase underwent complete dissociation into subunits. The dissociation occurred in buffered 0.5 M-KCl solution and did not require any previous pretreatment of the ribosome preparation (e.g. with puromycin). This might mean that *Saccharomyces cerevisiae* entering the stationary phase of growth accumulated the 80 S "run off" ribosomes, whereas ribosomes from the cells harvested in mid-log phase (data not shown) required preincubation with puromycin for complete dissocia-

tion. Table 1 shows that the ribosome preparation isolated from the early stationary phase of growth retained practically the same activity in polyphenylalanine synthesis as the log-phase ribosomes.

Thus, the stationary phase ribosomes appeared to be a convenient material for large-scale preparation of yeast ribosomal subparticles.

Separation of ribosomal subunits by zonal centrifugation. Under conditions favouring dissociation into subunits (see Material and Methods) approximately 200 mg of ribosomes could be efficiently separated in the zonal rotor (Fig. 2). The

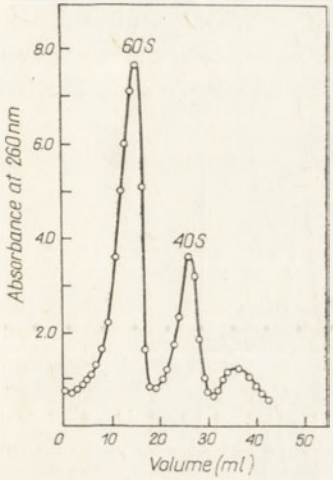

Fig. 1

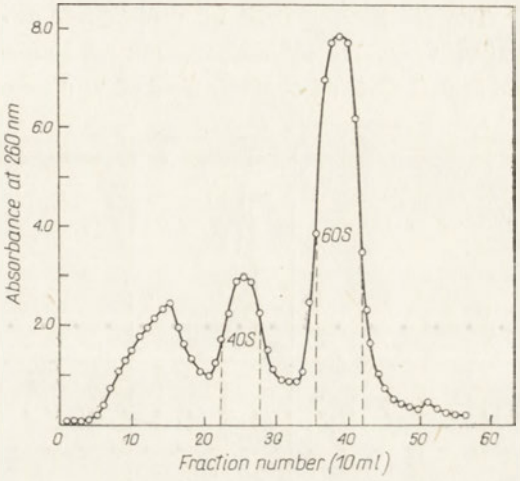

Fig. 2

Fig. 1. Sedimentation of ribosomal subunits in sucrose gradient. Ribosomes from the early stationary phase of growth were suspended in buffer *C* and 100 $A_{260\ nm}$ units were applied to a linear (10 - 30%) sucrose gradient in the same buffer. Ribosomal subunits were separated by centrifugation at 10°C for 15 h at 18 000 r.p.m. using a MSE SW rotor (3 × 70 ml).

Fig. 2. Separation of ribosomal subunits by zonal centrifugation. Yeast ribosomal subunits from the early stationary phase (1100 $A_{260\ nm}$ units) were separated by centrifugation at 14°C for 6 h at 30 000 r.p.m. in a MSE B-XIV zonal rotor using a 10 - 30% linear sucrose gradient in buffer *C*. The fractions (10 ml) indicated by broken lines were pooled and ribosomal subunits were recovered by centrifugation.

Table 1

Activity of ribosomes isolated from a log and an early stationary phase of growth

Ribosomes from both growth phases, purified by successive washing with buffered KCl solutions and ultracentrifugation were free of elongation factors and devoid of endogenous activity. For details see Materials and Methods.

Source of ribosomes	[^{14}C]Phenylalanyl-tRNA incorporated (c.p.m.)
Log-phase ribosomes	2080
Stationary phase ribosomes	1700

obtained fractions containing 40 S and 60 S subunits were pooled separately and concentrated by high-speed centrifugation. The isolated 40 S particles, when analysed in analytical gradient, sedimented as a single peak (Fig. 3A); 60 S ribosomes were slightly contaminated with 40 S subunits (Fig. 3B), and therefore were always purified by second centrifugation in the zonal or SW (3 × 70 ml) rotor. Additional purification gave one symmetrical peak of 60 S subunits (Fig. 3C). The same results were obtained when rRNA isolated from the corresponding preparations of ribosomal subparticles was analysed (Fig. 4A, B and C). Data presented show that rRNAs were not degraded during preparation of the ribosomal subunits.

The results presented in Table 2 indicate that both ribosomal subunits were active, when recombined, in phenylalanine polymerization. Their activity was the same as of the control, purified and non-dissociated 80 S ribosomes. The lack

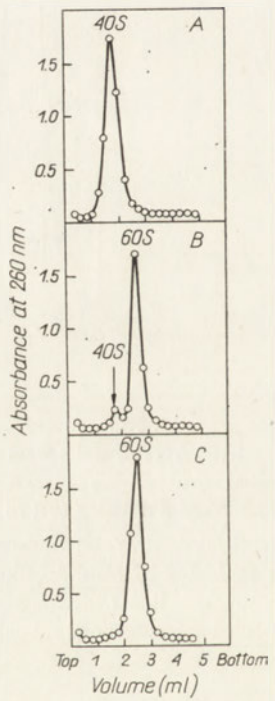

Fig. 3

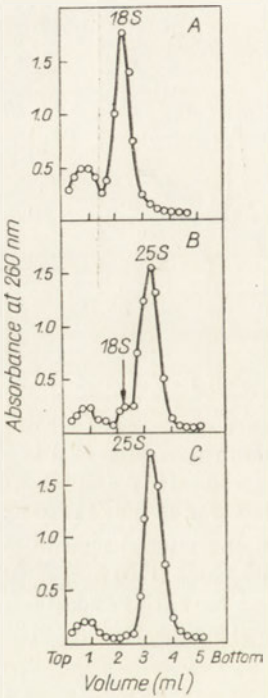

Fig. 4

Fig. 3. Sedimentation of ribosomal subunits in sucrose gradients. The subunit fractions collected from zonal centrifugation depicted in Fig. 2 were analysed by analytical sucrose density gradient after pelleting the subunits and resuspension of the pellets in buffer *C*. The samples (approximately 0.2 and 0.4 mg of 60 S and 40 S ribosomal subunits, respectively) were sedimented in a 10 - 30% sucrose density gradient in buffer *C* at 20°C for 1 h at 48 000 r.p.m. using MSE, SW (3 × 6.5 ml) rotor. Recovery of 40 S (*A*) and 60 S (*B*) subunits after zonal centrifugation; 60 S ribosomal subunits after purification in a second sucrose gradient (*C*).

Fig. 4. Sedimentation in sucrose gradients of rRNAs isolated from ribosomal subunits. Ribosomal RNA isolated from subunits as described under Materials and Methods was centrifuged in a 5 - 20% sucrose gradient in 50 mM-Tris-HCl, pH 7.5, at 4°C for 3 h at 48 000 r.p.m. using a MSE, SW (3 × 6.5 ml) rotor. *A*, *B* and *C* correspond to the samples *A*, *B* and *C* depicted in Fig. 3.

of activity of 60 S or 40 S subparticles tested alone gave additional evidence that isolated subunits were not cross-contaminated. As one can see in Table 2, the recombined subunits did not display any activity if elongation factors were not added. This indicates that ribosomal subunits prepared by the adopted technique were depleted of supernatant proteins and can be used for determination of protein components of ribosomal subunits.

Table 2

Activity of ribosomes and ribosomal subunits

Non-dissociated 80 S purified ribosomes and the ribosomal subunits obtained therefrom were isolated from an early stationary phase of growth. Both ribosomal subunits were purified by two sucrose gradient centrifugations. Incorporation of [^{14}C]phenylalanine was measured as described under Materials and Methods. Proteins containing elongation factors were added as indicated.

Ribosomes	Elongation factors	[^{14}C]Phenylalanyl-tRNA incorporated (c.p.m.)
40 S	+	55
60 S	+	40
40 S + 60 S	+	1895
40 S + 60 S	−	45
80 S	+	1690
80 S	−	75

The yeast ribosomal subunits suspended in buffer *E* could be stored at −20°C for more than one week without loss of activity.

Electrophoretic separation of ribosomal proteins. Figure 5 shows the two-dimensional polyacrylamide-gel pattern of 40 S ribosomal proteins. The total number of resolved proteins exceeded 30. Some of the spots were faint as for instance S18 and S25 which might cast doubt on their belonging to the structural ribosomal proteins. Occasionally we observed spots which were not visible on photographs of the stained gel slabs (Fig. 6). Possibly, these were the degradation products of protein, and they were not taken into account in numbering the spots on the electrophoretogram. It is worth noting that the small ribosomal subunit contained 6 or 7 acidic proteins (Fig. 5, left side). Resolution of all these proteins was quite satisfactory under our experimental conditions, except the double spot containing S1 and S2 proteins. In a separate experiment we demonstrated, using the original technique of Kaltschmidt & Wittmann (1970), only one protein spot (results not shown) corresponding to this double spot. The exact identification and/or separation of these proteins need further experiments.

Electrophoretic pattern of 60 S ribosomal proteins revealed the presence of 43 spots (Fig. 7). Four fast migrating basic proteins (L40, L41, L42, L43) could be seen as round-shaped, distinct spots only when electrophoresis in the second dimension was run not longer than 12 hours (Fig. 7, right side, and Fig. 8). Several spots (Fig. 8) appeared on the electrophoretogram occasionally, e.g. L26. The 60 S ribosomal subunit contains three acidic proteins (L2, L3 and L21 in Fig. 7,

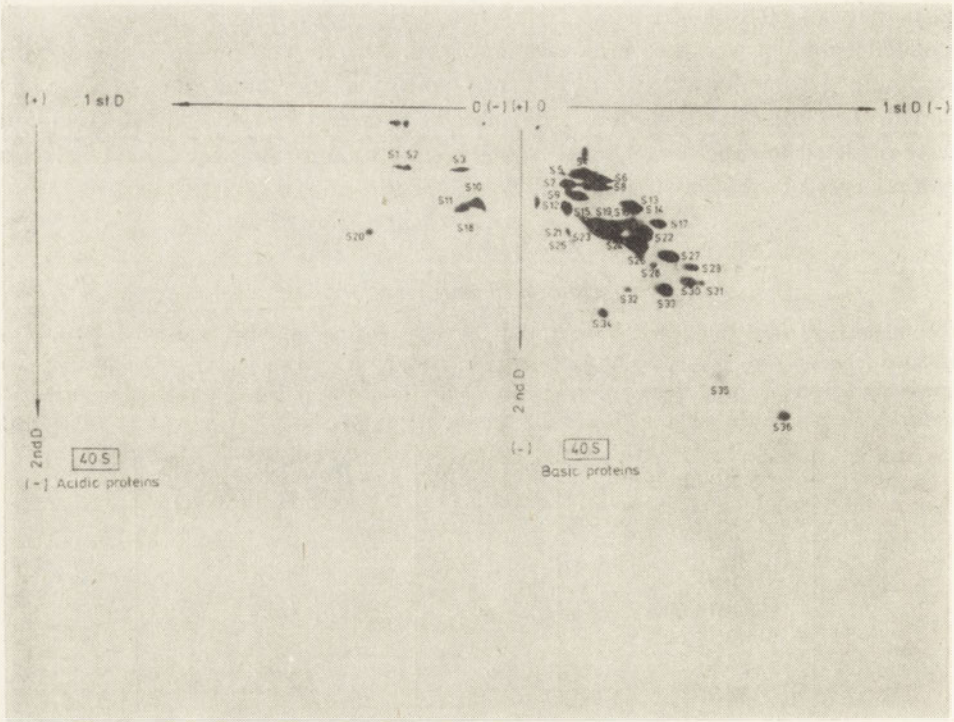

Fig. 5. Two-dimensional gel electrophoresis of 40 S ribosomal proteins. Acidic and basic proteins were run separately in two dimensions. Protein samples of 1 mg were subjected to electrophoretic separation.

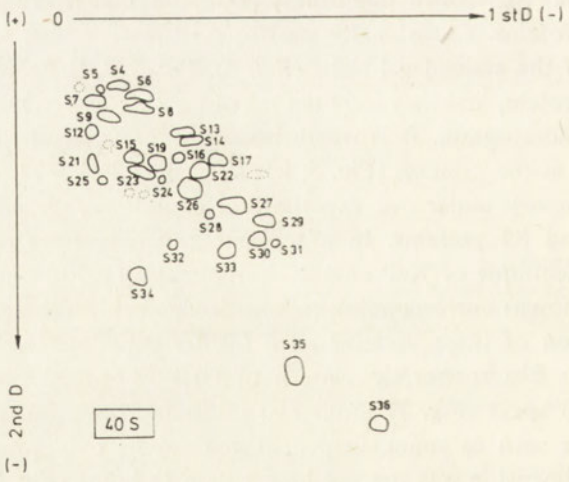

Fig. 6. Schematic two-dimensional electrophoretogram of 40 S basic ribosomal proteins. The dotted-circled spots represent the proteins occasionally visible on gel slabs.

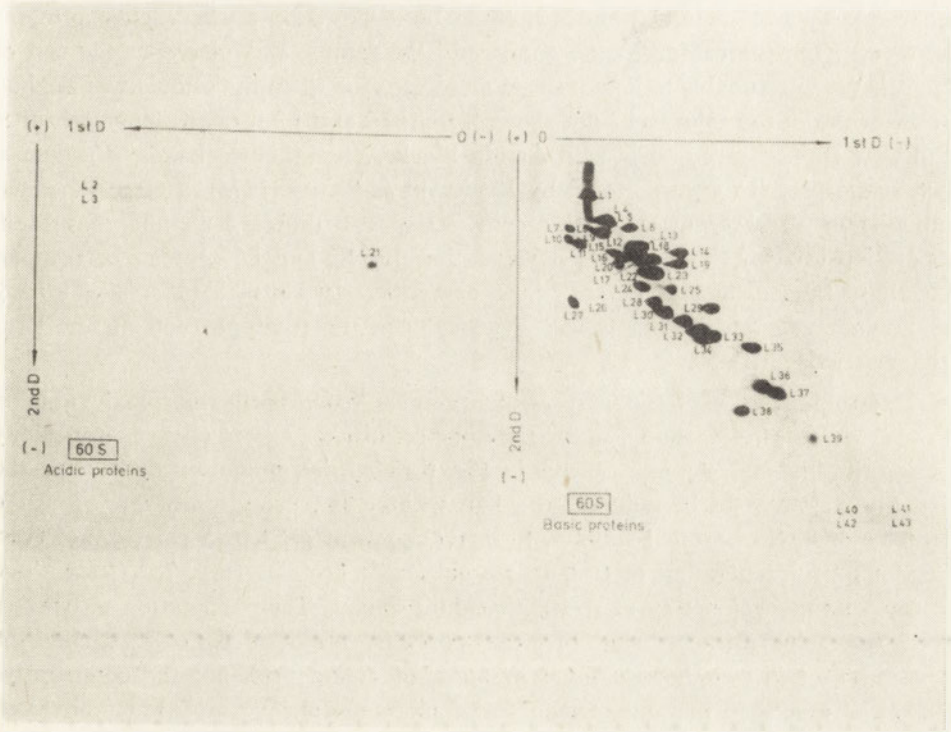

Fig. 7. Two-dimensional gel electrophoresis of 60 S ribosomal proteins. As in Fig. 5, the acidic and basic proteins were run separately. The amount of protein applied for electrophoresis was about 1 mg.

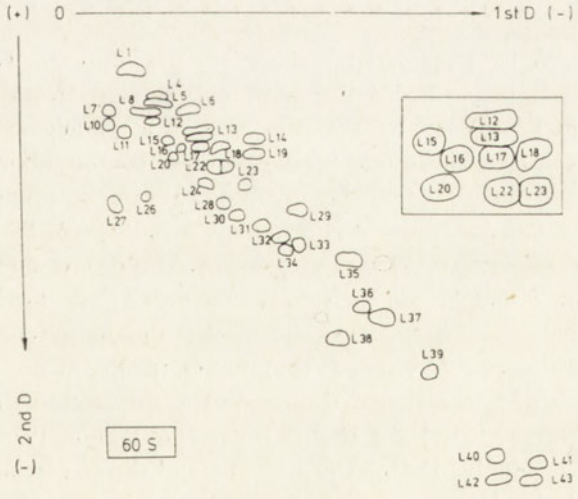

Fig. 8. Schematic two-dimensional electrophoretogram of 60 S basic ribosomal proteins. The dotted-circled spots represent the occasionally observed proteins. The inset shows proteins poorly resolved on two-dimensional gel.

left side). Two of them, L2 and L3, could be detected only when acidic proteins were run from the catode to the anode and the sample was placed at the top of the gel. We were unable to demonstrate these proteins when the sample was applied in the center of the gel (results not shown). Both L2 and L3 formed elongated zones and were stained very poorly with Amido Black. When this work was in progress, two acidic proteins were detected by Kruiswijk & Planta (1975) in 60 S ribosomal subunits of *Saccharomyces carlsbergensis*. Despite a different method of protein separation used by these authors it seems that our L2 and L3 proteins correspond exactly to their acidic proteins; in both cases these two proteins displayed strong acidic character and were localized practically in the same position on the two-dimensional gel slabs.

Electrophoresis of basic and acidic proteins from both ribosomal subunits was performed, in this study, under the same conditions, except changes in polarity during the first run. As one can see in Fig. 5 (left side), migration distance in the first dimension could be reduced to a half without impairing resolution of acidic proteins of 40 S ribosomal subunit. Such reduction of gel length cannot, however, be applied for separation of 60 S ribosomal acidic proteins (Fig. 7, left side) due to the long migration way of strongly acidic proteins. Their separation on shorter gel would require shorter time of electrophoresis. We decided not to change the conditions of electrophoresis for separation of acidic proteins; for comparative study the described method seemed to be more useful. We are aware, however, that our technique needs modification for application on micro scale. In recent years several such methods of two-dimensional gel electrophoresis have been described (Howard & Traut, 1973; Ventimiglia & Wool, 1974; Lin *et al.*, 1976).

DISCUSSION

We have presented in this paper the improved, multistep procedure for isolation of highly purified active ribosomal subunits from yeast, and separation of ribosomal proteins by two-dimensional acrylamide-gel electrophoresis. The results obtained indicate that yeast ribosomes contain about 76 individual proteins, which approximates the number of ribosomal proteins found in other eukaryotic organisms. For *Saccharomyces carlsbergensis*, Kruiswijk & Planta (1974, 1975) have reported the presence of 71 unique proteins. The discrepancy between their results and our data might be due to some differences in the method of extraction of ribosomal proteins and in the technique of electrophoretic separation. It is now well documented (Sherton & Wool, 1974b) that extraction of proteins from eukaryotic ribosomes with 67% acetic acid (this paper) is superior to the method involving the use of 2-chloroethanol and HCl (Kruiswijk & Planta, 1974). It seems to us that reduction of disulphide linkages in proteins and direct placing of samples at the top of disc gel also increase the number of proteins resolved on gel slabs.

The number of ribosomal proteins has been determined for a great variety of eukaryotic organisms as well as for different organs within a given species. The

ribosomes from relatively distant organisms showed quite extensive differences in protein composition (Delaunay & Schapira, 1974). The results of Kruiswijk & Planta (1974, 1975) and the data presented here indicate that yeast ribosomes, and particularly 40 S subunits, contain more acidic proteins than mammalian ribosomes (Sherton & Wool, 1972, 1974a; Terao & Ogata, 1975; Howard *et al.*, 1975; Martini & Gould, 1975). The presence of as many as eleven acidic proteins has been found also in *E. coli* ribosomes (Garret & Wittmann, 1973). These comparative data have led Kruiswijk and Planta to assume that the number of acidic ribosomal proteins decreases from primitive to higher organisms. Very recently Collatz *et al.* (1976) reported on the presence of 8 acidic proteins in each ribosomal subunit of rat liver, after group fractionation of ribosomal proteins on carboxymethyl cellulose. Since, however, all these proteins stained poorly, identification and final proof of their ribosomal origin is difficult to achieve. Thus far, it is well established that two strongly acidic proteins of 60 S ribosomal subunit of higher organisms are undoubtedly structural ribosomal proteins (Sherton & Wool, 1974a; Stöffler *et al.*, 1974; Möller *et al.*, 1975). They are antigenically related to bacterial proteins L7 and L12 and play an important role in protein synthesis (Wittmann, 1976). As it is shown in this paper and in the report of Kruiswijk & Planta (1975), yeast 60 S ribosomal subunit contains also two such acidic proteins which seem to correspond to those found in other eukaryotes. These properties of strongly acidic proteins are of great interest. We have recently found (W. Kudlicki, N. Grankowski & E. Gąsior, in preparation) that L2 and L3 are the only proteins of 60 S subunit which undergo extensive phosphorylation *in vitro* in the presence of GTP-dependent protein kinase from yeast, with GTP or ATP as phosphoryl donors. We have also found that acidic protein(s) of 40 S yeast ribosomal subunit (double spot — S1, S2; Fig. 5, left side) is strongly phosphorylated with this kinase. Significance of specific phosphorylation of the ribosome proteins for their biological function is unknown.

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BIAŁKA PODJEDNOSTEK RYBOSOMOWYCH DROŻDŻY: LICZBA I OGÓLNE WŁASNOŚCI

Streszczenie

1. Wykazano, że *Saccharomyces cerevisiae* akumulują we wczesnej fazie stacjonarnej rybosomy 80 S, które łatwo dysocjują na podjednostki i jednocześnie zachowują pełną aktywność w polimeryzacji fenyloalaniny *in vitro*. W pracy przedstawiono uproszczoną i wydajną metodę preparatywnej izolacji podjednostek rybosomowych drożdży.

2. Przy pomocy dwuwymiarowej elektroforezy na żelu poliakrylamidowym wykazano obecność 34 białek w podjednostce 40 S i 42 białka w podjednostce 60 S. Obie podjednostki rybosomowe zawierały kwaśne białka: trzy z nich znajdowano w podjednostce 60 S i sześć lub siedem w podjednostce 40 S. Wydaje się, że dwa białka kwaśne rybosomów drożdży odpowiadają prokariotycznym białkom L7 i L12. Całkowita liczba białek rybosomowych drożdży jest podobna do liczby białek znajdowanych u innych organizmów eukariotycznych.

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FLUORESCENCE OF RADIATION-INDUCED COLLAGEN AGGREGATES

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Tropocollagen shows higher ultraviolet fluorescence (emission max. about 305 nm) and lower blue fluorescence (emission max. about 420 nm) than the dimers and tetramers isolated from irradiated collagen. This indicates dityrosine structures in the radiation-induced collagen aggregates.

Collagen in solution exposed to ionizing radiation under anaerobic conditions forms defined molecular aggregates (Dancewicz, 1975; Majewska & Dancewicz, unpublished). The mechanism of aggregation and the nature of the new intermolecular bonds appearing during this process are unknown. It is probable that the radiation-induced new aldehyde compounds found in irradiated collagen solution (Jeleńska *et al.*, 1975) might participate in formation of cross-links accounting for aggregation. An increase in the number of borohydride-reducible components in irradiated collagen (Jeleńska & Dancewicz, 1975) suggests another type of intermolecular bonding with covalent bonds formed due to stabilization of aldimine structures.

The fluorescence measurements presented in this paper indicate one more possible type of cross-links in collagen aggregates, namely formed by dimerization of radiation-induced phenyl radicals located on the neighbour molecules.

MATERIALS AND METHODS

Collagen aggregates. Rat skin collagen was isolated according to the procedure of Bornstein & Piez (1964). Collagen dissolved in 0.05 M-acetic acid was purified by repeated 5 h centrifugation at 110 000 *g*. Each time the upper half of the solution was collected for further processing. Deaerated solution was irradiated in nitrogen with 5 to 15 krad dose of X-rays using Siemens Stabilipan 250 apparatus operated at 200 kV and 20 mA and provided with 0.5 mm Cu filter. On Sepharose 6B column the irradiated collagen was separated into three fractions. The first one contained

tetramers, the second dimers, and the third unchanged monomers of tropocollagen. Each fraction was purified by repeated chromatography of peak portions.

Fluorescence measurements. Fluorescence of 0.15% solutions of collagen or its aggregates was measured at ambient temperature using Turner Model 430 spectrofluorimeter equipped with the recorder; the fluorescence spectra were presented for comparative purposes and were not corrected for sensitivity of the instrument.

RESULTS AND DISCUSSION

The ultraviolet fluorescence maximum of collagen aggregates excited at 275 nm did not differ from that of tropocollagen (Fig. 1A). However, the relative intensity of emission fluorescence of aggregates was lower than that of non-irradiated collagen.

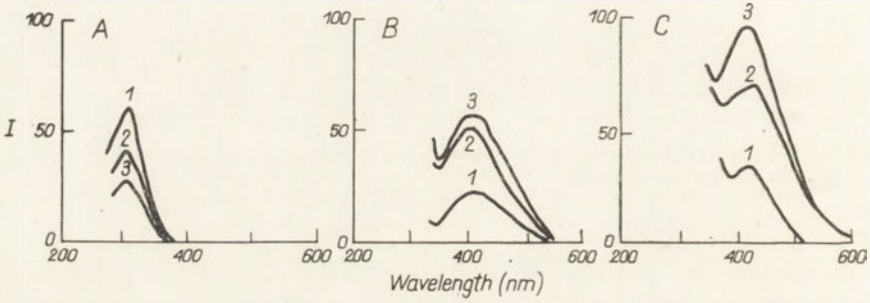

Fig. 1. Ultraviolet (*A*) and blue (*B*, *C*) fluorescence emission spectra of tropocollagen (*1*) and its dimers (*2*) and tetramers (*3*), dissolved in 0.05 M-acetic acid (1.5 mg/ml). Excitation wavelength: *A*, 275 nm; *B*, 313 nm; *C*, 350 nm. *I*, relative fluorescence intensity.

The decrease in ultraviolet fluorescence can be ascribed to conformational changes around aromatic amino acid residues or to structural changes in these amino acid residues themselves. Cowgill (1968) has demonstrated that changes of helicity in muscle proteins are reflected in fluorescence changes. Our studies showed that the content of helical structure in collagen aggregates is lower than in non-irradiated tropocollagen (Majewska & Dancewicz, unpublished), which might contribute to the decreased ultraviolet fluorescence of collagen aggregates. This decrease in fluorescence is probably due, in part, to dimerization of phenyl radicals which are induced in protein and peptides by ionizing radiation as well as by ultraviolet irradiation (Wheeler & Montalo, 1959; Yamamoto, 1972, 1973).

According to Lehrer & Fasman (1967) dimerization resulting from interaction of two phenyl radicals leads to structures such as dityrosine, found among photoproducts of tyrosine or polytyrosine solutions. Dityrosine has been demonstrated among products of tyrosine oxidation by peroxidase (Gross & Sizer, 1959). LaBella *et al.* (1968) concluded that radical interaction presents a basic mechanism of dityrosine formation also during the oxidation process.

Dityrosine has an intense blue fluorescence (Vladimirov, 1965; Lehrer & Fasman, 1967). We have noted similar blue fluorescence in solutions of tropocollagen and

its radiation-induced aggregates excited at 313 or 350 nm ultraviolet light (Fig. 1B,C), the intensity of fluorescence being higher in solutions containing collagen aggregates. An increase in blue fluorescence of collagen solution has been observed also upon its exposure to ultraviolet light (Fujimori, 1966). The increased blue fluorescence of radiation-induced collagen aggregates and the fact that aromatic amino acid residues form phenyl radicals upon exposure of protein to ionizing radiation, present circumstantial evidence for the existence of dityrosine type of cross-links in these aggregates.

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FLUORESCENCJA AGREGATÓW KOLAGENU POWSTAŁYCH POD WPŁYWEM PROMIENIOWANIA

Streszczenie

Fluorescencja ultrafioletowa (maksimum emisji ok. 305 nm) tropokolagenu była większa, zaś fluorescencja niebieska (maksimum emisji ok. 420 nm) mniejsza aniżeli dimerów i tetramerów wyizolowanych z napromienionego kolagenu. Wskazuje to na istnienie struktur bityrozynowych w agregatach kolagenu powstałych pod wpływem promieniowania.

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FORMATION OF LIPID-BOUND *N*-ACETYLHEXOSAMINE DERIVATIVES IN YEAST PARTICULATE FRACTIONS*

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1. Particulate fraction (105 000 *g*) from *Saccharomyces cerevisiae* catalyses the transfer of *N*-acetylglucosamine from UDP-*N*-acetyl[^{14}C]glucosamine into lipid fraction as well as to insoluble polymer.

2. The evidence presented is in favour of the lipid containing the *N*-acetylglucosamine mono-, di- and tri-saccharide derivatives of dolichyl diphosphate.

3. The presence of a transferase synthesizing dolichyl-linked sugars in mitochondrial fraction is also reported.

The role of yeast dolichyl phosphate as an acceptor of mannosyl residues, and its participation in the formation of yeast cell wall polymannan protein complex has been recently reviewed (Babczinski & Tanner, 1973; Sharma *et al.*, 1974; Lehle & Tanner, 1974).

The work by Nakajima & Ballou (1974a,b) on the linkage region between the polymannan part and protein revealed that a mannosyl di-*N*-acetylglucosamine unit attached to an asparagine residue of protein is present in the core region of the mannoprotein complex.

This paper reports on the formation of dolichyl phosphate mono- and di-*N*-acetylglucosamine derivatives and their participation in biosynthesis of polymannan protein complex in yeast, and describes the fate of *N*-acetylglucosamine in the yeast particulate fraction and distribution of UDP-*N*-acetylglucosamine transferase in subcellular fractions.

When this work was in progress, the paper on the formation of lipid-bound oligosaccharides in yeast by Lehle & Tanner (1975) was published. The results obtained by these authors are consistent with our results and provide evidence for the role of dolichyl diphosphate di-*N*-acetylglucosamine as an acceptor for the mannosyl unit transferred from GDP-mannose.

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MATERIALS AND METHODS

Reagents. Di-*N*-acetylchitobiose was a gift from Dr. F. Hemming of the University of Liverpool. UDP-*N*-[U-^{14}C]acetylglucosamine (spec. act. 286 mCi/mmole) was supplied in aq. 2% (v/v) ethanol from The Radiochemical Centre (Amersham, Bucks, England) and was stored at −20°C.

Standard oligosaccharides were obtained by partial acid hydrolysis of dextran (Thoma & French, 1957) and purified on Sephadex G-10 column. Prenyl phosphate was synthesized according to Mańkowski *et al.* (1975).

All other materials were either obtained and purified as described by Richards & Hemming (1972) or were commercial products.

Preparation of yeast particulate fraction. Saccharomyces cerevisiae (strain KL 184, diploid) was grown in the medium containing 2% of glucose, 1% of yeast extract and peptone to the early stationary phase.

The particulate fraction was prepared by mechanical disruption of yeast cells in a hydraulic press in 0.25 M-sucrose containing 0.05 M-Tris-maleate, pH 7.1. The broken cells were centrifuged for 5 min at 1000 *g* to remove unbroken cells and cell debris, then centrifuged at 20 000 *g* for 20 min and subsequently at 105 000 *g* for 70 min. The final pellet (105 000 *g*) was washed with 0.4 M-NaCl in Tris-maleate buffer, pH 7.2, and was then resuspended in the same buffer, at a concentration of protein 35 - 40 mg/ml, and stored at −18°C.

Preparation of mitochondria was based on the method of Kováč *et al.* (1968). The method involved disruption of yeast by grinding with Ballotini beads in 0.6 M-sorbitol containing 0.05 M-Tris-HCl, pH 7.2, and 0.25 M-EDTA. The 1000 *g* supernatant was centrifuged at 20 000 *g* for 30 min and the pellet was resuspended in 0.6 M-mannitol containing 2 mM-EDTA and 0.1% of bovine serum albumin, followed by centrifugation at 2000 *g* for 10 min. The supernatant obtained was centrifuged for 10 min at 20 000 *g*. The pellet was resuspended in 0.6 M-mannitol containing 2 mM-EDTA (5 vol.) and centrifuged again at 2000 *g* for 5 min. Finally, the supernatant was centrifuged at 29 000 *g* for 20 min, washed with 0.4 M-NaCl and then resuspended in Tris-maleate, pH 7.2, and stored at −18°C.

Incubation conditions and recovery of lipids. The incubation was performed in small centrifuge tubes, routinely for 30 min at 30°C, with shaking, in a medium containing in a final volume of 250 µl: $MnCl_2$ (2 µmoles), $MgCl_2$ (2 µmoles), EDTA (0.25 µmole), 2-mercaptoethanol (1 µmole), NaF (8 µmoles), 100 µl of enzymatic fraction (about 4 mg of protein) in Tris-maleate buffer, pH 7.2, and UDP-*N*-acetyl [^{14}C]glucosamine (0.1 µCi, 286 mCi/mmole). Incubation was terminated by shaking with chloroform - methanol - 4 mM-$MgCl_2$ (3:2:1, by vol.) and lipids were recovered as described previously (Palamarczyk & Hemming, 1975).

Chromatography. Chromatography of lipids on DEAE-cellulose acetate was performed as described by Barr & Hemming (1972); the lipid fraction was applied to a column (0.8 × 10 cm) and eluted successively with 20 ml portions of: chloroform - methanol (2:1, by vol.); methanol; and chloroform - methanol (2:1) containing 0.01, 0.05 and 0.1 M-ammonium acetate.

T.l.c. of lipid-linked sugars was carried out as described previously (Palamarczyk & Hemming, 1975) on silica gel (Merck) precoated plates using either system *A* (chloroform - methanol - water; 65:25:4, by vol.) or system *B* (diisobutyl ketone - acetic acid - water; 20:15:2, by vol.). Products of mild acid treatment of lipid-linked sugars were chromatographed on cellulose (Whatman CC41) predeveloped in 0.1 M-$ZnSO_4$ in system C (butan-1-ol - pyridine - water; 6:4:3, by vol.; Palamarczyk & Hemming, 1975), and in system *D* (*n*-propanol - nitromethane - water; 5:2:3, by vol.; Oliver & Hemming, 1975).

Unlabelled standard sugars were detected with alkaline silver spray reagent or diphenylamine aniline (Dawson *et al.*, 1969).

Radioactivity was located with a Berthold strip scanner or by radioautography.

Hydrolysis of lipid-linked sugars. Mild hydrolysis with HCl and NaOH was performed as described by Richards & Hemming (1972), and treatment with ammonium hydroxide according to Lehle & Tanner (1975).

RESULTS

Formation of ^{14}C-*labelled lipids and polymer.* When the particulate fraction from yeast (105 000 *g*) was incubated with UDP-*N*-acetyl[^{14}C]glucosamine, radioactivity was incorporated into the lipophilic fraction as well as into the methanol-insoluble polymer (Fig. 1). The radiochemical purity of the lipid was examined by chromatography on a DEAE-cellulose anion exchange column. The elution pattern always revealed the presence of two different lipids: lipid A and lipid B. Lipid A was eluted as a single peak with 0.1 M-ammonium acetate but was retained on the anion exchanger on elution with chloroform - methanol (2:1), methanol and 0.01 M- and 0.05 M-ammonium acetate in chloroform - methanol (2:1). Lipid B was eluted with chloro-

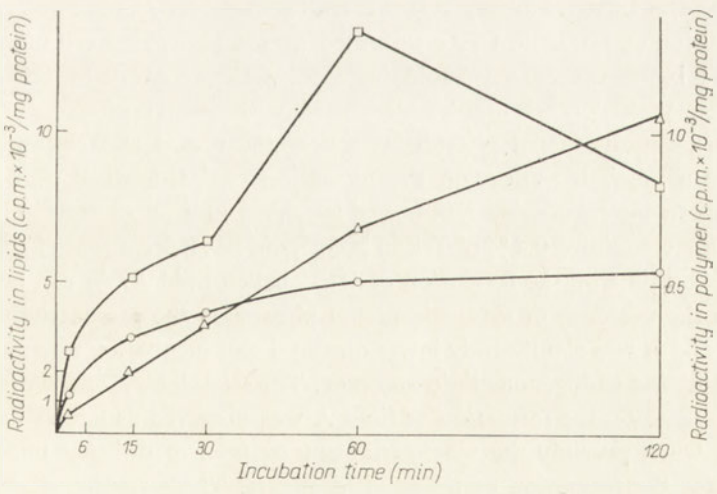

Fig. 1. Time-course of ^{14}C transfer from UDP-*N*-acetyl[^{14}C]glucosamine into lipid A (○), lipid B (△), and insoluble polymer (□). The results refer to the protein of 105 000 *g* particulate fraction.

form - methanol (2:1). The amount of both lipids formed depended on time and temperature of incubation and fluctuated from one enzyme preparation to another; 40 - 80% of total ^{14}C-labelled lipids was identified as lipid A, depending on enzyme preparation.

Time-course of ^{14}C incorporation into both lipids A and B and the polymer was found to be different (Fig. 1). Formation of lipid A was more rapid than of lipid B within 30 min of incubation, an reached a plateau by 1 hour. At 15 and 30 min the labelling was approximately 40 and 80%, respectively, of the maximum value. Finally, after 120 min of incubation, the amount of lipid B was twice as high as that of lipid A. Incorporation of ^{14}C into insoluble polymer also reached a maximum by 1 hour; at 15 and 30 min, the amount of radioactivity was, respectively, 43 and 50% of the maximum. The extension of incubation period to 2 hours caused a decrease of radioactivity recoverable in insoluble polymer, probably due to hydrolytic activity accompanying the *N*-acetylglucosamine transferase activity.

Addition of an excess of cold-UDP-*N*-acetylglucosamine (20 nmoles) decreased slightly the total amount of radioactivity in the lipid fraction and had no effect on the amount of ^{14}C transferred to the polymer. GDPmannose (20 nmoles) did not affect the kinetics of ^{14}C transfer.

Incorporation of ^{14}C into lipids took place both in the presence of Mg^{2+} and Mn^{2+}, but the amount of ^{14}C incorporated was three times higher in the presence of Mg^{2+}. The *N*-acetylglucosamine transfer to the polymer obligatorily required Mn^{2+}.

The optimum pH for ^{14}C transfer was 7.2 - 7.4 but about 80 - 90% of the activity was found at pH 6 and 8.

To get more information on the nature of the lipid acceptor of *N*-acetylglucosamine, exogenous prenyl phosphates were added to the incubation mixture together with Triton X-305, at final concentration of 0.4%. This detergent was found to be the least inhibitory for the endogenous reaction.

The addition of synthetic C_{55} α-saturated prenyl phosphate (50 nmoles) to the incubation mixture caused a twofold increase in the recovery of ^{14}C in lipid A as compared with control non-supplemented with prenyl phosphate (Table 1). The other exogenous prenyl phosphates tested (ficaprenyl and castaprenyl phosphates) had a negligible effect on the formation of ^{14}C-labelled lipid (Table 1). Introduction to the incubation mixture of lecithin emulsion (2 and 5 mM) in Tris-maleate buffer containing 0.4% of Triton X-305, did not increase the effect of synthetic prenyl phosphate on the formation of ^{14}C-labelled lipid A. As can be seen from Table 2, in the presence of synthetic prenyl phosphate the radioactivity recovered in lipids A and B was significantly lower during 6 min incubation as compared with control containing endogenous lipid acceptor. The stimulatory effect of exogenous prenyl phosphate on the formation of lipid A was observed as late as at 24 min of incubation, when possibly the whole endogenous pool of dolichyl phosphate had been used for the formation of lipid-linked sugars. These results suggest that the *N*-acetylglucosamine transferase preferentially used the endogenous pool of dolichyl phosphate for the formation of *N*-acetylglucosamine derivatives. The chromatogra-

phic profiles of these derivatives on DEAE-cellulose acetate and t.l.c. in systems *A* and *B* were identical with endogenous and exogenous lipid acceptors.

A double-labelling experiment with UDP-*N*-acetyl[^{14}C]glucosamine and ^{3}H-labelled α-end of C_{55} α-saturated prenyl phosphate revealed formation of a ^{3}H- and ^{14}C-labelled compound in which the ^{3}H/^{14}C ratio was 1:1.

Characterization of ^{14}C*-labelled lipid-linked sugars.* [^{14}C]Lipid A was eluted from DEAE-cellulose acetate column in the manner expected for prenyl diphosphate sugar, i.e. by 0.1 M-ammonium acetate. As can be seen from Fig. 2, the elution profiles of lipid A and C_{55} α-saturated diphosphate *N*-acetylglucosamine are identical, and distinct from dolichyl monophosphate [^{14}C]mannose. The monophosphate derivatives were eluted from the column by 0.05 M-ammonium acetate.

Since ^{14}C-labelled lipid B was not retained on the anion exchange column, it might be assumed that it lacks negative charge. Mild alkali treatment of lipid B with dilute sodium hydroxide rendered water-soluble 100% of the ^{14}C label. On the other hand, mild treatment with HCl resulted in 90% of ^{14}C remaining in the

Table 1

Effect of exogenous prenyl phosphates on incorporation of N-acetyl[^{14}C]*glucosamine into lipid A*

Prenyl phosphates (50 nmoles) were added at zero time of incubation and the reaction was carried out for 24 min with the 105 000 *g* particulate fraction as the source of enzyme.

Addition	Radioactivity (c.p.m./mg of protein)
None	827
C_{55} α-saturated phosphate	1660
Castaprenyl phosphate	956
Ficaprenyl phosphate	792

Table 2

The time-dependent effect of exogenous C_{55} *α-saturated prenyl phosphate on formation of lipids A and B*

Prenyl phosphate (50 nmoles) was added at zero time of incubation and the reaction was carried out for the time indicated. Chromatography on DEAE-cellulose acetate was used for separation of lipids A and B (see Methods). Figures in parentheses indicate the amount of radioactivity incorporated into the lipids when the incubation was performed without the exogenous prenyl phosphate.

Time of incubation (min)	Radioactivity (c.p.m./mg of protein)	
	lipid A	lipid B
3	560 (960)	960 (1200)
6	400 (1400)	960 (1200)
24	2200 (1080)	2600 (2600)

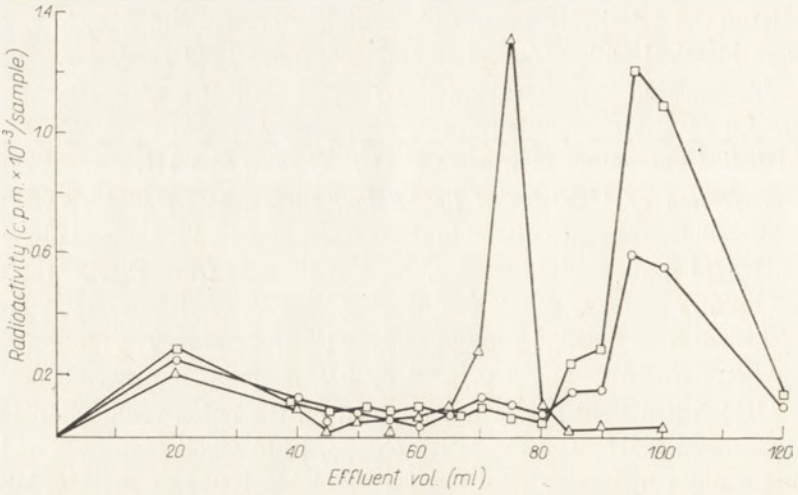

Fig. 2. DEAE-cellulose acetate chromatography of lipid A (○), dolichyl phosphate mannose (△), and C_{55} α-saturated diphosphate *N*-acetylglucosamine (□).

lipid fraction. Treatment of lipid A with dilute acid rendered water-soluble 100% of ^{14}C, as was expected of a prenyl disphosphate sugar.

The nature of ^{14}C-labelled lipids obtained on incubation of the yeast particulate fraction with UDP-*N*-acetyl[^{14}C]glucosamine was further examined by t.l.c. It was shown that the lipids were separated in chromatographic system *A* into three compounds with R_F values of about 0.15, 0.30 and 0.40 (Fig. 3, right side); the corresponding value for dolichyl monophosphate mannose was 0.48 (not shown). When

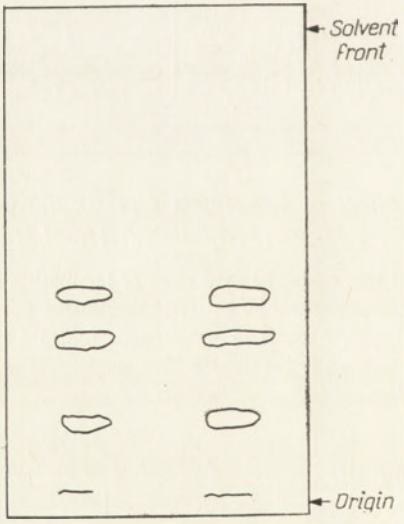

Fig. 3. T.l.c. pattern of ^{14}C-labelled lipids. UDP-*N*-acetyl[^{14}C]glucosamine was incubated with mitochondria (left) and 105 000 *g* particles (right). Chromatographic separation was performed in system *A* as described under Materials and Methods.

total lipids were separated on DEAE-cellulose acetate column into lipid A and B and chromatographed alongside, the R_F value 0.40 corresponded to the lipid B, showing that it was lipid A which on t.l.c. separated into two components. The chromatographic pattern obtained is very similar to that reported by Lehle & Tanner (1975). The non-identified by these authors peak 1 corresponds to our lipid B. Thin-layer chromatography in system *B* instead of system *A* led to separation of only two ^{14}C-labelled substances: lipid A and lipid B.

The diphosphate linkage suggested by the elution pattern on DEAE-cellulose was confirmed by t.l.c. of [^{14}C]lipid A treated with ammonium hydroxide. The water-soluble hydrolysate chromatographed in system *C* revealed the presence of sugar phosphates. The same chromatographic system applied for separation of the water-soluble ^{14}C-labelled product of mild acid treatment yielded four radioactive areas (Fig. 4). The major compound (about 50% of total ^{14}C) had R_F 0.38, the compound with R_F value 0.47 contained 28% of total radioactivity, two other compounds containing 22% of radioactivity had R_F values 0.23 and 0.30. The major component was identified by cochromatography with a standard as *N*-acetylglucosamine. The R_F value 0.30 corresponds with a standard of di-*N*-acetylchitobiose. To confirm this conclusion, the products of mild acid treatment of dolichyl diphosphate-*N*-acetylglucosamine derivatives were subjected to chromatography

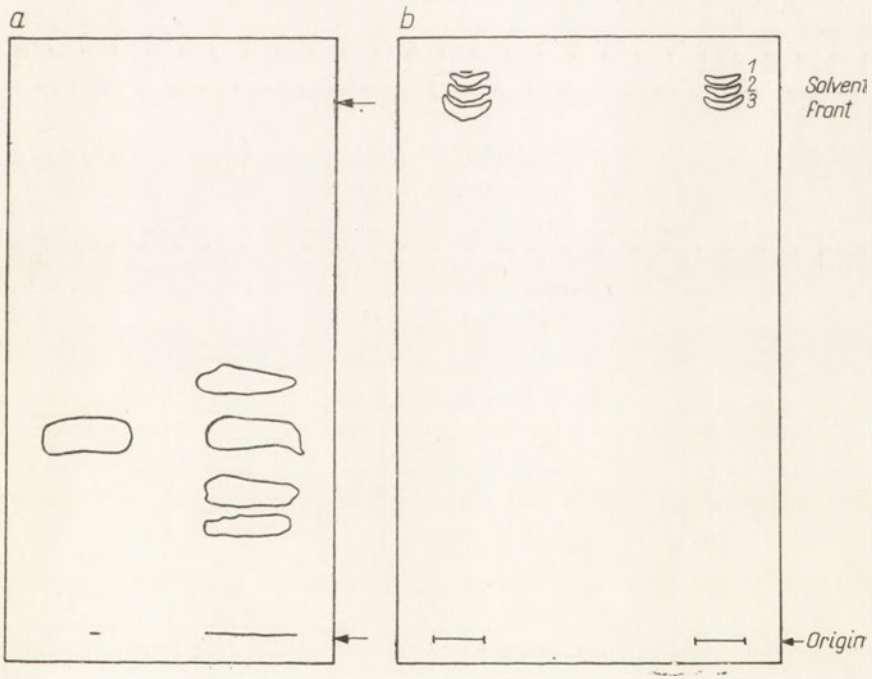

Fig. 4. T.l.c. pattern of the products of mild acid hydrolysis of ^{14}C-labelled lipid A. Chromatography was performed on cellulose (Whatman CC41) plates precoated with 0.1 M-$ZnSO_4$ in system *C* (Fig. 4a) or on Kieselguhr-Kieselgel G (1:3) in system *D* (Fig. 4b). Standard *N*-acetyl[^{14}C]glucosamine was run for comparison in system *C*, and mono-, di- and tri-saccharides (*1*, *2*, *3*) in system *D*.

in system *D*, alongside with standard mono, di- and tri-saccharides. The chromatographic pattern revealed the presence of three radioactive areas which corresponded to mono-, di- and tri-saccharides.

The t.l.c. mobility in system *C* of the compound with R_F value of 0.48 is similar to *N*-acetylmannosamine. The conversion of *N*-acetylglucosamine into *N*-acetylmannosamine when attached to dolichyl diphosphate in pig liver system was described previously (Palamarczyk & Hemming, 1975). It seems probable that a similar reaction can be catalysed by yeast particulate fraction.

UDP-N-acetylglucosamine transferase activity. Most of the experiments were carried out using the fraction pelleted at 105 000 *g* as a source of the enzyme. The *N*-acetylglucosamine transferase activity was stable during storage of yeast particulate fraction for several weeks, at −18°C.

Solubilization of the enzyme from 105 000 *g* pellet was also attempted. The extraction procedure based on the method of Osborn *et al.* (1972) involved subsequent treatment of the particulate fraction with non-ionic detergents followed by centrifugation at 105 000 *g*; the activity was determined both in the supernatant and in the pellet (Table 3). The most satisfactory results were obtained when Triton X-305 was used as a solubilizer, since almost 50% of activity and of total protein was solubilized. Triton X-100 and Polytergent were less effective (not shown). However, the solubilized activity concerned mainly formation of lipid B; the activity of interest, i.e. toward formation of lipid A, remained bound with the particles (Table 3).

Table 3

Solubilization of UDP-N-acetylglucosamine transferase from the 105 000 g particulate fraction

Extraction was performed by two successive 5-min treatments of the 105 000 *g* particles with Triton X-305 at final concentration of 0.5%. The enzymatic activity was determined in the pellet and in the combined supernatants. Lipids A and B were separated on DEAE-cellulose acetate column as described under Methods.

Source of enzyme	Radioactivity (%)	
	lipid A	lipid B
Particles		
non-treated	41	59
treated with Triton X-305	70	30
Supernatant	28	72

Since the enzymatic yeast preparation so far used (Lehle & Tanner, 1975) contained mitochondria and our preparation was devoid of this fraction, additional experiments were performed with yeast mitochondria and the fraction pelleted at 105 000 *g* from the post-mitochondrial supernatant. As shown in Table 4, the specific activity of the transferase forming dolichyl phosphate *N*-acetylglucosamine and dolichyl phosphate mannose was about fivefold higher in the mitochondrial fraction than in the 105 000 *g* particles. The ^{14}C-labelled lipids obtained from

the incubation mixture when the mitochondrial or 105 000 *g* fraction were used as a source of enzyme, had identical chromatographic properties on t.l.c. in system *A* (Fig. 3) and *B* (not shown).

Table 4

The UDP-N-acetyl[^{14}C]glucosamine and GDP[^{14}C]mannose transfer into the lipid and insoluble polymer

Mitochondria and the 105 000 *g* particles from post-mitochondrial supernatant were used.

Fraction	Incorporation (c.p.m./mg of protein) from			
	UDP-*N*-acetyl[^{14}C]glucosamine		GDP[^{14}C]mannose	
	lipid	polymer	lipid	polymer
Mitochondria	3 903	973	18 491	32 051
105 000 *g* particles	726	395	2 765	9 461

DISCUSSION

The results presented and those of Lehle & Tanner (1975) proved unequivocally that yeast particulate fractions catalyse incorporation of *N*-acetyl[^{14}C]glucosamine both into lipid and polymer fractions. On t.l.c., the lipid product separates into three compounds; two of them appear to be dolichyl diphosphate derivatives of *N*-acetylglucosamine.

The mobility of ^{14}C-labelled lipid A on DEAE-cellulose acetate is in favour of its being a dolichyl diphosphate sugar. The radioactivity runs just behind the region for dolichyl monophosphate sugars, consistent with extra negative charge on the ^{14}C-labelled lipid. Further evidence for the dolichyl diphosphate sugar nature of lipid A is the formation of sugar phosphates on alkaline hydrolysis. The dolichyl diphosphate nature of the lipid moiety was confirmed by the stimulatory effect of C_{55} α-saturated prenyl phosphate and the formation of the double-labelled product on incubation of UDP-*N*-acetyl[^{14}C]glucosamine and [^{3}H]C_{55} α-saturated prenyl phosphate. The transfer was not stimulated by other exogenous prenyl phosphates: ficaprenyl and castaprenyl phosphates. This confirms the requirement for an α-saturated bond in the lipid acceptor. The length of the C_{55} α-saturated prenyl phosphate as well as the presence of the α-saturated bond resemble the native yeast dolichyl phosphate which contains 60 - 85 carbon atoms (Babczinski & Tanner, 1973). The preferential "choice" of the endogenous prenyl phosphate suggests high specificity of the tested transferase toward the lipid acceptor.

The mobility of lipid B on DEAE-cellulose acetate as well as the resistance to mild acid treatment suggest that the sugar residue is not attached to the lipid moiety by a 1-phosphate bond. The chromatographic mobility on t.l.c. plates is consistent with that of the lipid described by Lehle & Tanner (1975) as unidentified peak 1. The chromatographic properties, resistance to mild acid and sensitivity to dilute alkali suggest the presence of an *O*-hydroxylic bond between lipid moiety and sugar residue.

Studies on the sugar portion, obtained after mild acid treatment of lipid A, showed that *N*-acetylglucosamine accounted for 50% of the product, and 22% was in the form of disaccharide identified by t.l.c. as di-*N*-acetylchitobiose, and trisaccharide. The formation of mono- di- and tri-*N*-acetylglucosamine derivatives of dolichyl diphosphate may be accompanied by epimerization of *N*-acetylglucosamine when attached to dolichyl phosphate; the product was identified tentatively by t.l.c. as *N*-acetylmannosamine.

The formation of dolichyl phosphate sugar derivatives by the yeast particulate fraction is of great interest since there is good evidence that dolichyl phosphate mannose is a donor for the first mannosyl residue attached to the protein in the yeast cell wall mannoprotein complex (Babczinski & Tanner, 1973; Sharma *et al.*, 1974). For this reason the transferase activity was studied so far in the membrane fraction. The results of present experiments show that formation of dolichyl phosphate sugars is even more pronounced in the mitochondrial fraction.

The formation of dolichyl diphosphate derivatives of *N*-acetylglucosamine by yeast particulate fraction is of considerable interest in the light of reports by Nakajima & Ballou (1974a,b) on the presence of a mannosyl-di-*N*-acetylglucosamine region linked to asparagine in a core region of yeast protein - polymannan complex. In this respect the process is very similar to the involvement of dolichyl diphosphate oligosaccharides in glycoprotein biosynthesis in mammalian tissues, described by Levy *et al.* (1974) and Palamarczyk *et al.* (1975).

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SYNTEZA ZWIĄZANYCH Z LIPIDAMI POCHODNYCH *N*-ACETYLOHEKSOZAMINY PRZEZ FRAKCJE CZĄSTKOWE DROŻDŻY

Streszczenie

1. Frakcja cząstek (105 000 *g*) wyizolowana z *Saccharomyces cerevisiae* katalizuje przenienie reszty *N*-acetyloglukozaminy z UDP-*N*-acetylo[^{14}C]glukozaminy do frakcji lipidowej i nierozpuszczalnego polimeru ściany.

2. Przedstawione dane sugerują, że powstająca frakcja lipidowa składa się z mono-, dwu- i trójsacharydowych pochodnych dwufosforanu dolichylu.

3. Wykazano występowanie transferazy syntetyzującej pochodne cukrowe dwufosforanu dolichylu we frakcji mitochondrialnej drożdży.

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THE PRESENCE OF SERINE PROTEASE IN PEA EMBRYO CHROMATIN*

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1. It has been shown that chromatin from pea seedlings contains a proteolytic enzyme, similar to that of mammalian chromatin.

2. The protease was isolated from chromatin by acid extraction and partly characterized. It is a serine-type enzyme, sensitive to DFP, of low mol. wt. (about 18 000 - 20 000), with optimum pH at about 8 with [^{3}H]*N*-acetylated histone as a substrate.

3. In chromatin complex, histones *f1* and *f3* are preferentially degraded.

Animal tissues contain chromatin-bound proteolytic enzyme(s) (Bartley & Chalkley, 1970; Kurecki *et al.*, 1971; Carter & Chae, 1976) showing high affinity to histones as a substrate and preferential degradation of histones *f1* and *f3* in chromatin complex.

The protease from calf thymus chromatin has been characterized as a low molecular weight (about 16 000) serine endopeptidase, closely resembling trypsin in respect to peptide bond specificity and sensitivity to inhibitors (Kurecki & Toczko, 1974; Kurecki *et al.*, 1975; Kowalska-Loth *et al.*, 1976).

In this paper the results are reported on the presence of serine protase in chromatin of pea embryo axis; so far the chromatin-bound proteolytic enzyme has not been described in higher plants.

MATERIALS AND METHODS

Materials. Pea embryo axes from 3-day-old pea seedlings (*Pisum sativum*, var. Kujawski) germinated in the dark, were used immediately after harvesting.

Isolation of chromatin. From 100 g of pea embryo axes, nuclei were isolated according to Sadgopal & Bonner (1970), washed twice with 40 ml of 0.01 M-Tris-HCl

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buffer, pH 7.8, containing 1 mM-$MgCl_2$, and for the third time with 40 ml of 2.5 mM--EDTA, pH 7.4. Each time the chromatin pellet was separated by centrifugation at 6000 *g* for 10 min. Finally, the chromatin sediment suspended in 6 - 8 ml of 0.01 M-Tris-HCl buffer, pH 7.8, was swelled overnight, sheared in Virtis-type homogenizer at 150 V for 3 min and centrifuged at 6000 *g* for 20 min. The supernatant (solubilized chromatin) was then diluted with 0.01 M-Tris-HCl buffer, pH 7.8, to obtain 5 $O.D._{260nm}$ units/ml. All procedures were carried out at 0 - 4°C.

Protease preparations. The solubilized chromatin from pea seedlings was treated with 0.1 vol. of 2.5 M-HCl for 2 min with stirring, at 0 - 4°C, and centrifuged at 6000 *g* for 10 min. Cold acetone (4 vol.) was added immediately to the supernatant and the mixture was stored overnight at −18°C. The resulting sediment (protease preparation) was then spun down at 2000 *g* for 10 min and fractionated on Sephadex G-75 column. The column (0.8 × 46 cm) was equilibrated and eluted either with 0.01 M-sodium acetate, pH 4.2, containing 0.5 M-NaCl, or with 0.1 M-Tris-HCl buffer, pH 7.8, containing 0.5 M-NaCl. The effluent was collected in 1 ml fractions at a flow rate 10 ml/h.

Neutral protease from calf thymus chromatin was prepared as described by Kurecki & Toczko (1974).

Preparation of [^{3}H]*N-acetylated histones from calf thymus.* Histones were labelled with [^{3}H]acetic anhydride according to Carter & Chae (1976) with some modifications. A sample of 100 mg of histone preparation from calf thymus in 1 ml of 0.1 M-Tris-HCl buffer, pH 7.8, was heated at 90°C for 10 min to inactivate endogenous proteolytic activity. The preparation was cooled to 4°C and stirred for 2 h with [^{3}H]acetic anhydride (100 μCi in 0.2 ml). After overnight dialysis against the same buffer, proteins were precipitated with 2 M-$HClO_4$ (1:1, v/v), centrifuged at 10 000 *g* for 6 min and dissolved in 0.1 M-Tris-HCl buffer, pH 7.8. The acetylated histones were dissolved in 4 ml of 0.1 M-Tris-HCl buffer, pH 7.8, and *N*-acetylated histones were reprecipitated with 2 M-$HClO_4$, with concomitant splitting of *O*-acetyl groups. The procedure was repeated once more for total removal of *O*-acetyl substituents.

[^{3}H]*N*-Acetylated histones were then diluted to 0.1% concentration (1×10^5 c.p.m./ml) in 0.01 M-Tris-HCl buffer, pH 7.8, and stored at −10°C until use.

Assay of proteolytic activity. [^{3}H]*N*-Acetylated histones (0.1 ml) were added to 0.4 ml of solubilized chromatin or to each of the fractions separated on Sephadex G-75, and were incubated at 37°C for 20 h under toluene. Samples of 0.2 ml were withdrawn before (control) and after incubation, cooled to 0°C, treated with 2 M--$HClO_4$ (0.2 ml) and, after standing on ice-bath for 15 min, centrifuged at 10 000 *g* for 6 min. To 0.1 ml of the supernatant, 0.8 ml of water, 0.1 ml of 1 M-KOH and 10 ml of Bray scintillation fluid (Bray, 1960) were added. Radioactivity of the supernatant was measured in Liquid Scintillation Counter.

Labelling of protease with [^{3}H]*DFP.*[1] Each fraction separated on Sephadex G-75 column was adjusted to pH about 7 with 0.1 M-Tris-HCl buffer, mixed with

[1] Abbreviations: DFP, diisopropylfluorophosphate; PPO, 2,5-diphenyloxazol; POPOP, 1,4-bis(5-phenyl-2-oxazolyl)-benzol.

5 µl of [^{3}H]DFP (1 µCi) and incubated for 20 h at room temperature. Aliquots of 0.1 ml were applied on Whatman no.3 paper discs and the excess of [^{3}H]DFP was removed by sequential washing with 10 ml portions of: ethanol - ethyl ether (9:1, v/v); isopropanol; ethylene glycol; isopropanol; and ethanol- ethyl ether (9:1, v/v). The discs were air-dried and ^{3}H radioactivity was counted in toluene scintillation fluid, containing PPO and POPOP.

In some experiments chromatin was labelled with [^{3}H]DFP as follows: 10 ml of solubilized chromatin (5 $O.D._{260nm}$ units/ml) was incubated with [^{3}H]DFP (50 µCi in 0.25 ml) at 4°C for 20 h and dialysed exhaustively against 0.01 M-Tris-HCl buffer, pH 7.8. The histone fraction was extracted twice from chromatin with HCl at final concentration of 0.25 M, and spun down at 6000 *g* for 10 min. The final sediment was dissolved in 10 ml of NCS Tissue Solubilizer. The radioactivity of 1 ml aliquots was counted in Bray scintillation fluid.

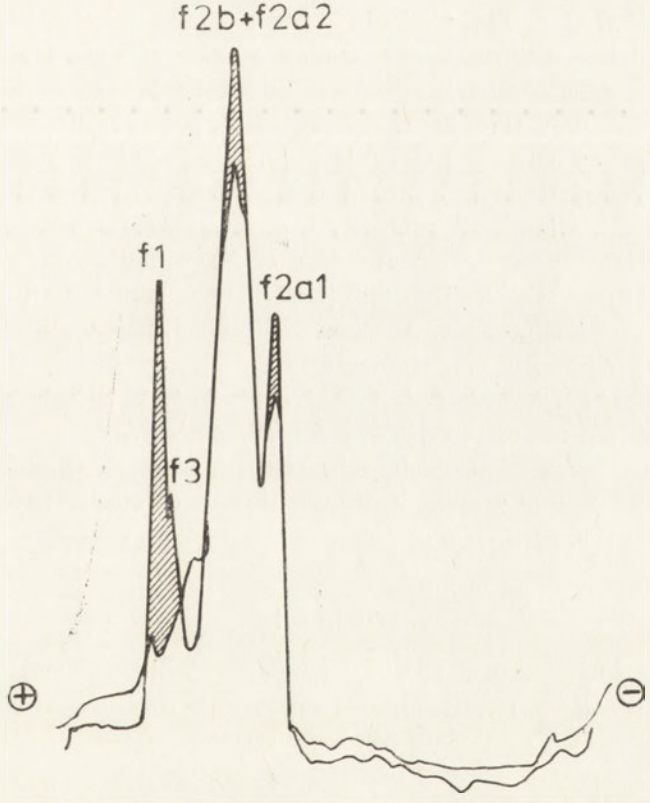

Fig. 1. Changes in electrophoretic pattern of histones on incubation of chromatin from pea embryo axis. Solubilized chromatin was incubated at 37°C for 12 h. Histones were extracted with 0.25 M-HCl (final concentration) and subjected to polyacrylamide-gel electrophoresis as described under Materials and Methods. Shaded fields represent the difference in content of particular histone components in chromatin before and after incubation. Migration is from left (+) to right (—).

Polyacrylamide-gel electrophoresis of histones was carried out according to Panyim & Chalkley (1969).

Reagents. [^{3}H]DFP, [^{3}H]acetic anhydride and NCS Tissue Solubilizer were purchased from The Radiochemical Centre (Amersham, England); POP and POPOP from Fluka AG (Buchs SG, Switzerland), Sephadex G-75 from Pharmacia (Uppsala, Sweden), polyacrylamide from Serva (Heidelberg, G.F.R.). All other reagents were of analytical grade and were obtained from POCh (Gliwice, Poland).

Histone preparation was obtained from chromatin by acid extraction according to Bonner *et al.* (1968).

RESULTS AND DISCUSSION

The presence of chromatin-bound proteolytic activity in pea seedlings was demonstrated by electrophoretic analysis of histone pattern following autolysis of chromatin. Densitometer scans presented in Fig. 1 demonstrate that incubation of chromatin at 37°C for 12 h results in almost complete disappearance of histones *f1* and *f3*, and minor differences in the content of other histones. It was also shown that exogenous [^{3}H]*N*-acetylated histones added to pea chromatin underwent progressive degradation (Fig. 2). Hydrolysis of histones was found to be pH-dependent with pH optimum at 7.8 - 8.0 (Fig. 3). These results indicate the presence of a proteolytic enzyme similar to that found previously in mammalian chromatin.

The pea chromatin-bound protease, like thymus chromatin-bound enzyme, is sensitive to DFP, which at concentration of 5 mM inhibits completely hydrolysis of histones (not shown). Therefore [^{3}H]DFP was used in further experiments as an indicator of this protease. As shown in Table 1, nearly all radioactivity incorporated into chromatin was recovered in the histone fraction (I extract). The amount of radioactivity extracted with the second portion of HCl and that remaining in the sediment did not exceed 6% of total radioactivity.

The protease preparation obtained by acid extraction of chromatin from pea embryo axis and neutral protease from calf thymus chromatin were subjected to gel filtration on Sephadex G-75 column and the fractions eluted were analysed for

Table 1

Distribution of [^{3}H]*DFP labelling in pea chromatin*

Histones were extracted from chromatin labelled with [^{3}H]DFP and solubilized as described under Materials and Methods.

Preparation	Radioactivity	
	c.p.m./l $OD_{260\,nm}$	%
Chromatin	5200	100
I extract	4600	89
II extract	160	3
Sediment	180	3

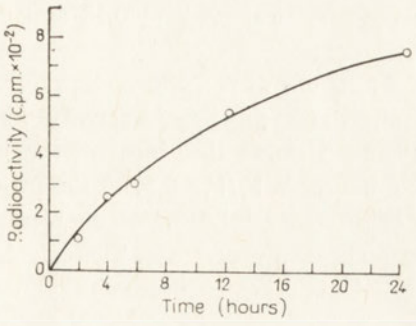

Fig. 2

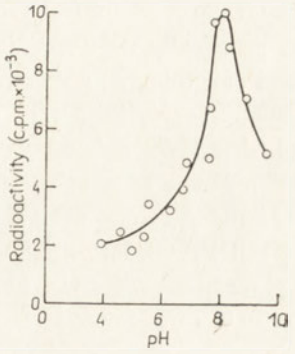

Fig. 3

Fig. 2. Time-course of degradation of exogenous [^{3}H]*N*-acetylated histones by chromatin of pea embryo axis. Solubilized chromatin (1 ml, 5 O.D.$_{260nm}$ units) was incubated with 0.2 ml (2×10^4 c.p.m.) of [^{3}H]histones at 37°C, and proteolytic activity was assayed as described under Materials and Methods.

Fig. 3. pH optimum of proteolytic activity of solubilized pea chromatin. Proteolytic activity war assayed using [^{3}H]*N*-acetylated histones (1×10^5 c.p.m./ml) as substrate, as described undes Materials and Methods. The following buffers were used: 0.1 M-sodium acetate (pH 3 - 5), 0.1 M--sodium phosphate (pH 5.5 - 7.0), 0.1 M-Tris-HCl (pH 7.2 - 10.0). The reaction was carried out at chromatin concentration equivalent to 1 O.D.$_{260nm}$ unit/sample, at 37°C for 20 h under toluene.

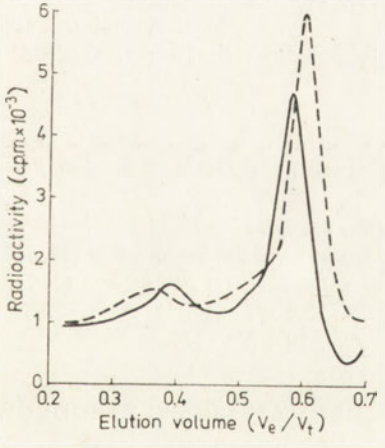

Fig. 4

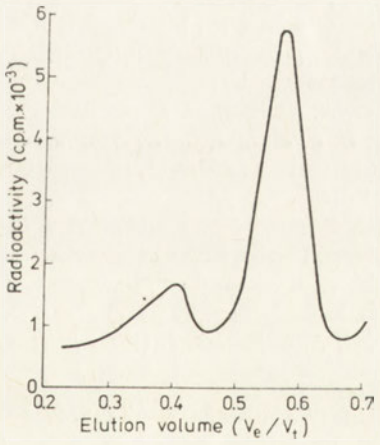

Fig. 5

Fig. 4. Gel filtration of pea (——) and calf thymus (— ---) protease preparation on Sephadex G-75 column. The column was equilibrated and eluted with 0.01 M-sodium acetate, pH 4.2, containing 0.5 M-NaCl. [3H]DFP-protein bound radioactivity was counted on paper discs as described under Materials und Methods.

Fig. 5. Gel filtration of pea protease preparation on Sephadex G-75. The column was equilibrated and eluted with 0.1 M-Tris-HCl buffer, pH 7.8, containing 0.5 M-NaCl. Fractions from the column were assayed for proteolytic activity with [^{3}H]*N*-acetylated histones as substrate, as described under Materials and Methods.

their ability to incorporate [^{3}H]DFP. As shown in Fig. 4, the radioactivity incorporated was practically recovered in one symmetrical peak of 0.58 and 0.60 V_e/V_t for pea and calf thymus, respectively.

In a separate experiment each of the fractions of pea chromatin protease eluted from Sephadex G-75 column was analysed for proteolytic activity with [^{3}H]*N*--acetylated histones as a substrate. Figure 5 shows that most of the proteolytic activity was eluted as one peak showing the same $V_e/V_t=0.57$ as the protein labelled with [^{3}H]DFP (cf. Fig. 4). The use of Sephadex gel filtration enabled evaluation of the apparent molecular weight of pea chromatin protease. The calculated value of 18 000 - 20 000 was found to be slightly higher than that for calf thymus protease. In addition to this major peak, a minor peak of protein material eluted at $V_e/V_t=$ 0.4 (cf. Figs. 4 and 5) was found to digest histones and bind [^{3}H]DFP. Since this protein corresponds to molecular weight of 42 000, one may assume that it could be a dimer of the enzyme studied.

The data presented give evidence for the presence in pea chromatin of a low molecular weight serine protease which closely resembles the calf thymus chromatin enzyme. The occurrence of a proteolytic enzyme in chromatin of both higher plants and animals suggests the essential function of this enzyme in control of gene expression in Eukaryotes.

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WYSTĘPOWANIE PROTEAZY SERYNOWEJ W CHROMATYNIE OSI ZARODKOWYCH GROCHU

Streszczenie

1. Wykazano, że w chromatynie zarodków grochu występuje enzym proteolityczny, podobny do enzymu występującego w chromatynach ssaków.

2. Enzym wyizolowano z chromatyny na pomocą ekstrakcji kwaśnej i określono jako proteazę serynową (wrażliwą na DFP), o niskiej masie cząsteczkowej (około 18 000 - 20 000) i pH optimum około 8 w stosunku do [^{3}H]histonów jako substratu.

3. Wykazano silne powinowactwo proteazy do histonów *f1* i *f3* w kompleksie chromatyny.

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OXIDATION OF METHANOL BY FACULTATIVE AND OBLIGATE METHYLOTROPHS

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1. The newly isolated methanol obligate *Methylomonas* sp. and the methanol facultative *Pseudomonas* sp. oxidize methanol at an unchanged rate over concentration range from 0.1 to 600 mM; the oxidation rate by the obligate methylotroph is 2.5 times higher (300 nmoles O_2/min/mg dry wt.). Low-molecular alcohols, formaldehyde and formate serve as respiratory substrates for the intact cells of both methylotrophs.

2. Methanol dehydrogenase of both methylotrophs isolated should be classified as the phenazine methosulphate-dependent pteridine-type enzyme of double methanol- and formaldehyde-dehydrogenase function. This soluble enzyme is stimulated about 10-fold by NH_4^+, which results in enhancement of V_{max}, and shows the same specificity and the same affinity toward methanol and formaldehyde (K_m about 5×10^{-5} M). Heat-inactivation of the 10-fold purified enzyme is associated with the release of a watersoluble pigment with maximum fluorescence at 420 - 430 nm.

3. NAD-dependent formate dehydrogenase was found to catalyse the third step of methanol oxidation in both methylotrophs.

Methanol is oxidized in a three-step process providing energy for assimilation of C_1 unit at the oxidation level of formaldehyde, formate or carbon dioxide (Ribbons *et al.*, 1970; Quayle, 1972). The number of ATP molecules generated during oxidation of methanol is not precisely known as the enzymes involved in this process have not been fully characterized nor the components of respiratory chain unequivocally elucidated (Anthony, 1975; van Dijken & Harder, 1975; Widdowson & Anthony, 1975). It seems that bacterial methanol dehydrogenases are pteridine-containing proteins (Anthony & Zatman, 1967), whereas the fungal enzymes cooperate with FAD (Tani *et al.*, 1972; Sahm & Wagner, 1973). Much less is known on oxidation of formaldehyde. The NAD- and glutathione-dependent dehydrogenase has been reported in *Pseudomonas methanica* (Harrington & Kalio, 1960). On the other hand, evidence has been presented for the identity of methanol and formaldehyde dehydrogenases in *Methylococcus capsulatus* (Patel & Hoare, 1971; Patel *et al.*, 1972). The formate dehydrogenases so far described are the NAD-

dependent enzymes, but their properties are poorly understood due to great lability of these enzymes (Johnson & Quayle, 1964).

It is believed that information on the enzymatic system oxidizing methanol is of great importance in characterization of each organism able to grow on methanol as a sole carbon source. This paper presents a preliminary account of the dehydrogenase system oxidizing methanol in the facultative and obligate methylotrophs isolated by us from the sludge produced during biological purification of industrial sewage.

MATERIALS AND METHODS

Reagents. NAD, NADP, phenazine methosulphate[1] and puromycin were obtained from Sigma Chem. Corp. (Rockford, Ill., U.S.A.), formic acid was from Fluka AG (Buchs, Switzerland), paraformaldehyde from Chemapol (Prague, Czechoslovakia) and Triton X-100 from Rohm & Haas (Philadelphia, Pa., U.S.A.). Phospholipase D from cabbage (0.5 i.u./mg) was purchased from Calbiochem AG (Lucerne, Switzerland), aminopterin from Koch-Light Lab. Ltd (Colnbrook, Bucks, England), amethopterin (methotrexate) from Lederle (Munich, G.F.R.) and actinomycin D from Boehringer (Mannheim, G.F.R.). Other reagents were supplied by POCh (Gliwice, Poland).

Strains and culture conditions. The facultative methylotroph *Pseudomonas ZB1 J26* and obligate methylotroph *Methylomonas ZB36 Pl1* were isolated from activated sludge by the liquid enrichment technique (Raczyńska-Bojanowska *et al.*, 1977). From the former strain, *Mut-7*, *Mut-9*, *Mut-12* and *Mut-15* were obtained on selection towards higher growth rate on methanol as a sole source of carbon and energy. *Mut-11* was isolated from *Methylomonas ZB36 Pl1* by the two-step selection. The first two letters and numbers refer to the collection, further to the phenotypic character of methanol utilization of *Pseudomonas* and *Methylomonas* isolates.

All the strains were cultivated on the liquid mineral medium of Tonge *et al.* (1972) in 500 ml Ehrlenmayer flasks on the rotatory shaker (240 rev./min) at 30°C. To the sterilized medium methanol was added to a final concentration of 1.5% (v/v) before inoculation with 48 h culture (1%). The exponentially growing cells (18 h of growth) or the cells from the stationary phase of growth (40 h of growth) were used for the experiments.

Oxidation of low-molecular alcohols, formaldehyde and formate by the intact cells. The cells from the late logarithmic phase of growth were separated by centrifugation and washed with 50 mM-sodium phosphate buffer, pH 7.0. Oxygen consumption was measured polarographically with the aid of Clark electrode (Yellow Springs Instrument Co., Inc.) using 2 ml of the cell suspension corresponding to 0.22 mg of dry wt. of cellular material.

Preparation of extracts. The separated cells were washed with 15 mM-KCl, suspended in 50 mM-phosphate buffer, pH 7.0 (1:5, w/v) and sonicated (5 × 1 min) using MSE sonicator (20 Kc). The sonicates were centrifuged for 15 min at 12 000

[1] Abbreviation: PMS, phenazine methosulphate.

rev./min and the supernatant was used for enzymatic determinations. To the sonicates used for the assay of formate dehydrogenase activity, 2-mercaptoethanol (0.1 mM) was added to stabilize the enzyme.

Preparation of the purified methanol dehydrogenase. The crude extract (15 mg protein/ml) was recentrifuged at 109 000 *g* for 60 min. The enzyme was precipitated at 0.4 - 0.8 ammonium sulphate saturation. The purification factor was about 10 and specific activity about 2 μmoles/min/mg protein.

Dehydrogenase assay. The activity toward methanol and formaldehyde was measured with the oxygen electrode of Clark type using PMS as the electron acceptor. The reaction mixture contained in a volume of 2 ml: Tris-HCl buffer, pH 9.5, 100 μmoles; NH_4Cl, 30 μmoles; PMS, 3.3 μmoles , and methanol, 25 μmoles or formaldehyde, 20 μmoles. Formate dehydrogenase was assayed according to Johnson & Quayle (1964) in the reaction mixture containing in 1 ml: Tris-HCl buffer, pH 8.4, 20 μmoles; NAD, 0.5 μmole; sodium formate, 25 μmoles and 0.05 ml of the enzyme extract. The enzymatic assays were performed at 30°C.

Determination of protein and dry weight. Protein was determined by the method of Lowry *et al.* (1951) using fraction V of bovine serum albumin as a standard. Dry weight was calculated from the turbidimetric measurements at 650 nm with the Beckman 24/25 spectrophotometer previously calibrated against dry weight of cells/ml of bacterial suspension. One extinction unit at 650 nm corresponded to 0.56 mg of dry wt.

RESULTS AND DISCUSSION

Oxygen consumption by intact cells of methylotrophs

Cells of methylotrophs of both types, i.e. facultative *Pseudomonas J26* and obligate *Methylomonas P11*, oxidized methanol, ethanol, propanol, formaldehyde and formate (Table 1). The rate of oxidation of alcohols and formate by the methanol obligate strain was about twice as high as that by the facultative methylotrophic strain. Formaldehyde was oxidized practically at the same rate by both isolates. The oxidation rate of methanol in both isolates was significantly higher than oxidation rate of formaldehyde and formate, similarly as it was found in the obligate methylotroph *Methylococcus capsulatus* (Patel *et al.*, 1972).

Both the methanol obligate and facultative methylotrophs maintained the same oxidation rate of methanol over a very large concentration range (from 0.1 mM to 600 mM, Fig. 1): the oxidation of methanol by the obligate methylotroph was about 2.5 times higher than by the facultative isolate. A 50% reduction in the oxidation rate was observed at practically the same concentration. Oxidation was totally inhibited when concentration of methanol in the reaction mixture was raised up to 3.75 M (12%).

The ability to oxidize methanol was found to be associated with the ability to utilize C_1 compounds for growth. During selection of facultative methylotrophs starting from *Pseudomonas J26 Mut-1*, the generation time on media containing methanol as a sole source of carbon was shortened under the applied conditions from 7 h

Table 1

Oxidation of low-molecular alcohols, formaldehyde and formate by intact cells of methylotrophs

The methanol facultative *Pseudomonas J26 Mut-12* and methanol obligate *Methylomonas Pl1 Mut-11* were cultivated for 18 h. The cells were suspended in 0.1 M-phosphate buffer, pH 7.0. Oxidation of the substrates at concentration indicated was followed with Clark oxygen electrode and expressed in nmoles O_2/min/mg dry wt.

Substrate	*Pseudomonas J26 Mut-12*	*Methylomonas Pl1 Mut-11*
Methanol, 50 mM	88.2	147.0
12.5 mM	88.2	147.0
Ethanol, 50 mM	73.5	72.0
12.5 mM	49.0	88.2
Propanol, 12.5 mM	49.0	88.2
Formaldehyde, 25 mM	44.1	49.0
12.5 mM	63.7	73.5
Formate, 50 mM	34.3	88.2
12.5 mM	—	29.4

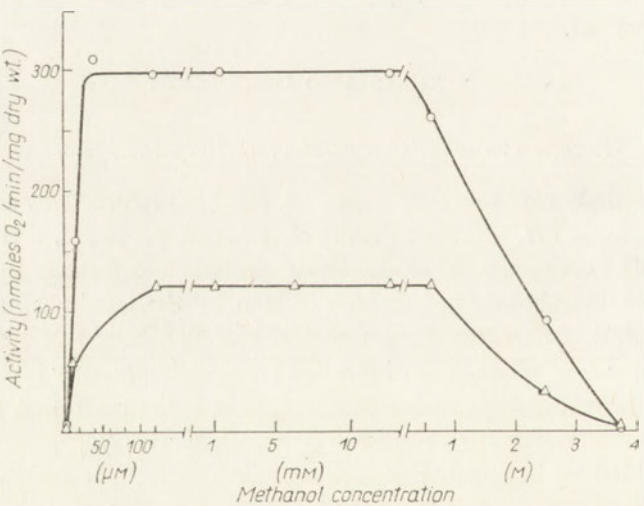

Fig. 1. Oxidation rate of methanol by the intact cells of *Pseudomonas J26 Mut-12* (△) and *Methylomonas Pl1 Mut-11* (○). The organisms were cultivated for 40 h, spun down and suspended in 0.1 M-phosphate buffer, pH 7.0. Oxidation was measured with Clark electrode at 30°C and expressed as nmoles O_2/min/mg dry weight.

to 3 h. The *J26 Mut-15* obtained in the four-step selection procedure, oxidized methanol and formaldehyde at a rate about 20-fold greater than the initial *J26* (Table 2). The oxidation rate of other substances was concomitantly several-fold greater. The strains obtained in the course of selection showed a gradually increasing

doubling rate and the ability to oxidize methanol. To get a better insight into the oxidative system of the isolated methylotrophs, oxidation of methanol, formaldehyde and formate was studied in the cell-free extracts.

Table 2

The oxidizing ability at consecutive steps of selection of Pseudomonas J26

To the intact cells suspended in 0.1 M-phosphate buffer, pH 7.0, methanol and formaldehyde were added at concentration of 12.5 mM, whereas formate, ethanol and propanol at concentration of 50 mM. Other conditions of the assay were as described in the legend to Table 1 and under Methods. The results are expressed as nmoles O_2/min/mg dry wt.

Substrate	*Pseudomonas J26*				
	Mut-1	*Mut-7*	*Mut-9*	*Mut-12*	*Mut-15*
Methanol	10.1	40.4	75.7	90.9	171.7
Form-aldehyde	5.0	30.3	50.5	75.5	90.9
Formate	traces	25.2	45.4	60.6	70.7
Ethanol	traces	25.2	50.5	65.6	75.7
Propanol	traces	10.0	15.1	40.4	60.6

Dehydrogenation of methanol and formaldehyde by the cell-free extracts

The results of preliminary experiments showed that neither NAD^+ nor $NADP^+$ participated in oxidation of methanol and formaldehyde by the cell-free extracts of both types of methylotrophs. Oxidation of these C_1 compounds was found to be PMS-dependent and could be assayed either by direct polarographic measurement in the presence of PMS with a Clark oxygen electrode or colorimetrically with dichlorophenolindophenol. These properties suggest the pteridine type of methanol dehydrogenases described previously in *Pseudomonas AM1* (Johnson & Quayle, 1964), *Pseudomonas M27* (Anthony & Zatman, 1967) and *Methylococcus capsulatus* (Patel & Hoare, 1971; Wadziński & Ribbons, 1975). Examination of the fluorescence spectrum of the protein-bound, water-soluble pigments produced by both our isolates revealed the presence of at least two components with fluorescence maxima at 410 - 430 and 520 - 530 nm (Raczyńska-Bojanowska *et al.*, 1977).

It seems that the 410 - 430 nm peak corresponds to the pteridine group of methanol dehydrogenase, as only the pigment of this fluorescence characteristics is released on heating the 10-fold purified enzyme for 10 min at 80°C, i.e. under conditions applied by Anthony & Zatman (1967) in the studies on methanol dehydrogenase of *Pseudomonas M27*. However, great lability of pteridine prosthetic group of methanol dehydrogenases (Wadziński & Ribbons, 1975), makes difficult comparison and chemical identification.

Effect of NH_4^+. The dehydrogenase activity in the extracts of both methylotrophs was stimulated by NH_4^+, as it was found previously with methanol dehydrogenases of other methylotrophs (Anthony & Zatman, 1964; Johnson & Quayle, 1964). The rate of oxidation of methanol and formaldehyde was lower by a factor of 8 in the

reaction mixture non-supplemented with NH_4^+. The quantitative response to NH_4^+ was practically the same for the enzymes of both methylotrophs. Data presented in Fig. 2 refer to the extracts of *Methylomonas Pl1 Mut-11*. In activation of methanol dehydrogenase, NH_4^+ could not be replaced by methylamine, which is inconsistent with the results obtained with *Methylococcus capsulatus* (Patel *et al.*, 1972).

As can be seen from the plot in Fig. 3, NH_4^+ ion raised V_{max} but did not influence the affinity of the enzyme of the two isolates toward methanol. The mechanism of this stimulation remains unclear; it might be connected with the oligomeric structure of methanol dehydrogenase reported by Patel *et al.* (1972) and association of subunits.

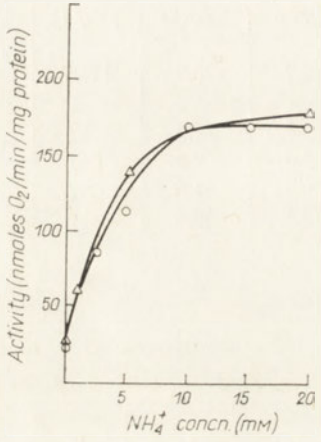

Fig. 2

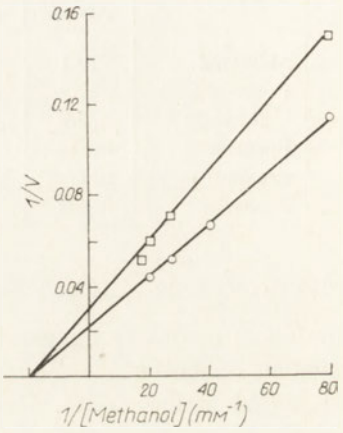

Fig. 3

Fig. 2. NH_4^+ dependence of dehydrogenation of methanol (○) and formaldehyde (△). Cell-free extracts of *Methylomonas Pl1 Mut-11* were prepared and oxygen consumption was measured as described under Methods.

Fig. 3. Effect of NH_4^+ on K_m and V_{max} values of methanol dehydrogenase. To the extracts of *Pseudomonas J26 Mut-12*, NH_4^+ was added at concentration of 3 mM (□) and 15 mM (○), and the activity was measured with Clark electrode.

Optimum pH and stability of the enzyme. The optimum pH for dehydrogenation of both methanol and formaldehyde in the two methylotrophs was 9.5; the activity at pH 9.0 was 60% and at pH 8.0 only about 10% of that found at pH 9.5 (Fig. 4). In the exponentially growing cells of *Pseudomonas J26 Mut-12*, besides the NH_4^+-stimulated formaldehyde dehydrogenase, another oxidizing activity with formaldehyde as a substrate was observed; it was not stimulated by NH_4^+, showed maximum activity at pH 7.0 (Fig. 5) and low substrate affinity (K_m about 10^{-2} M). This minor formaldehyde PMS-dependent dehydrogenase activity resembles the non-specific formaldehyde dehydrogenase of *Pseudomons AM*1 (Johnson & Quayle, 1964). Its occurrence in *Pseudomonas J26 Mut-12* was confined to the logarithmic phase of growth. The characteristics of the pH 9.5 methanol and form-

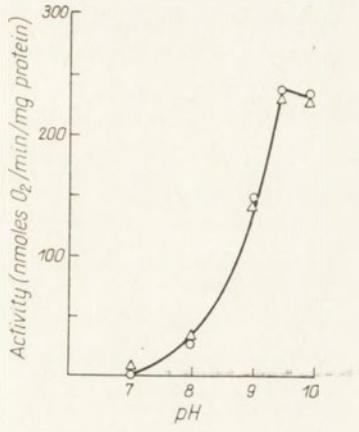

Fig. 4

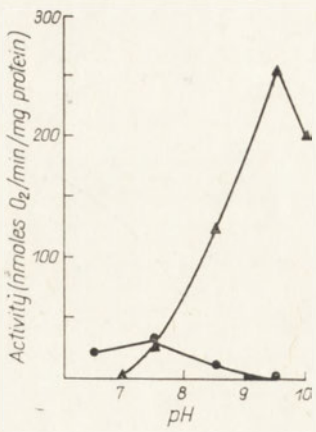

Fig. 5

Fig. 4. Optimum pH for dehydrogenation of methanol (○) and formaldehyde (△). Oxygen consumption by *Methylomonas Pll Mut-11* was measured in 0.1 M-Tris-HCl buffer with Clark electrode as described under Methods.

Fig. 5. Optimum pH for formaldehyde dehydrogenase activity in the exponentially growing *Pseudomonas J26 Mut-12*. The activity was measured with Clark electrode in 0.1 M-phosphate buffer (pH 6.0 - 8.5) and Tris-HCl buffer (pH 9.0 - 10), non-supplemented (●) and supplemented (▲) with 15 mM-NH_4^+.

aldehyde dehydrogenase did not depend on the growth phase and was very much like that of the enzyme in *Methylomonas Pll Mut-11*.

The pH 9.5 dehydrogenase activity with methanol and formaldehyde remained unchanged on storage in phosphate buffer, pH 7.0, at −15°C for 3 weeks. A 30 min incubation of the extract at pH 4.0 at room temperature followed by neutralization resulted in complete loss of the dehydrogenase activity. Under the same conditions, methanol dehydrogenase of *Pseudomonas M27* retained its full activity (Anthony & Zatman, 1967). Susceptibility of dehydrogenase of *Methylomonas* sp. to the elevated temperature was found to be the same with methanol and formaldehyde used as substrates (Fig. 6). A negligible decrease in the activity was noted upon elevation of temperature up to 55°C for 5 min, still about 40% of the activity remained when the reaction mixture was heated to 60°C, but at 65°C the enzymatic activity was undetectable. Inactivation was associated with the release of fluorescent pigments.

Half-life time of methanol dehydrogenase. The same response of the methanol and formaldehyde dehydrogenase activities to NH_4^+, storage conditions, pH and heating suggest that dehydrogenation of methanol and formaldehyde is catalysed by the same enzymatic protein. This was confirmed by determination of the half-life time of the dehydrogenase in the facultative methylotroph. Since methanol dehydrogenase is an inducible enzyme present exclusively in the cells growing on methanol as a sole source of carbon, one can measure duration of the dehydrogenase protein by monitoring the disappearance of the activity on transfer of the cells grown on methanol to the medium containing a non-C_1 compound. The possible induction

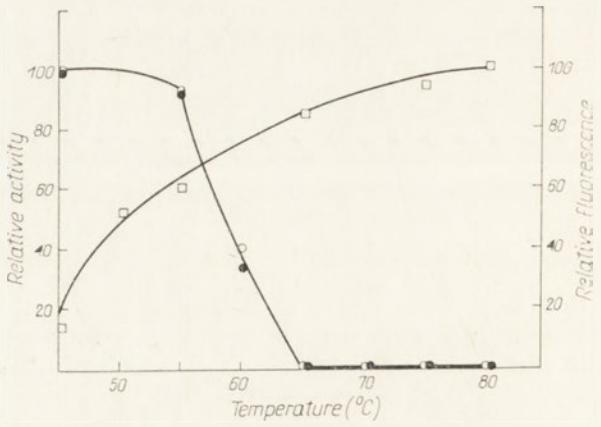

Fig. 6. Heat inactivation of methanol- (○) and formaldehyde-(●) dehydrogenase and the release of fluorescent pigment showing maximum fluorescence at 410 - 430 nm (□). The extracts of the exponentially growing cells of *Methylomonas Pl1 Mut-11* were heated for 5 min at the temperature indicated. The activity was measured at 30°C as described under Methods.

of a new mRNA by the intracellular non-metabolized methanol can be prevented by addition of actinomycin and translation of the existing mRNA by puromycin. In our experiment the cells cultivated on methanol (0.16 mM) as a sole carbon source for 18 h were harvested, washed twice with fresh medium at room temperature and transferred to the medium containing 0.2% citrate (2.5 g of wet cells/100 ml of the medium). In the two parallelly run experiments, actinomycin (5 μg/ml)+ puromycin (5 μg/ml) or puromycin (5 μg/ml) were added to the citrate medium. At 30 min intervals, samples were withdrawn and the dehydrogenase activity was measured with methanol and formaldehyde in the cellular extracts. During 120 min incubation in the citrate medium the absorbance remained constant, indicating that growth had not started under new nutritional conditions. The enzymatic activity with methanol and formaldehyde decayed parallelly (Fig. 7), and within 120 min dropped to a half of the initial values. In the presence of actinomycin and/or puromycin this drop was more rapid. This indicates that transcription and translation of genetic information for the synthesis of methanol dehydrogenase took place for about 30 min. On the arrestment of these two processes by inhibitors, the calculated half-life time of methanol dehydrogenase was about 90 min. This value may be biased since one cannot exclude some side-effects of antibiotics on cellular metabolism. Exactly the same results were obtained with formaldehyde as a substrate. This supports additionally the view on identity of methanol and formaldehyde dehydrogenases.

The double function of methanol dehydrogenase in the isolated by us obligate and facultative methylotrophs is consistent with the properties of methanol dehydrogenase from *Methylococcus capsulatus*. On about 20-fold purification of this enzyme the activity ratio with methanol and formaldehyde remained constant (Patel *et al.*, 1972).

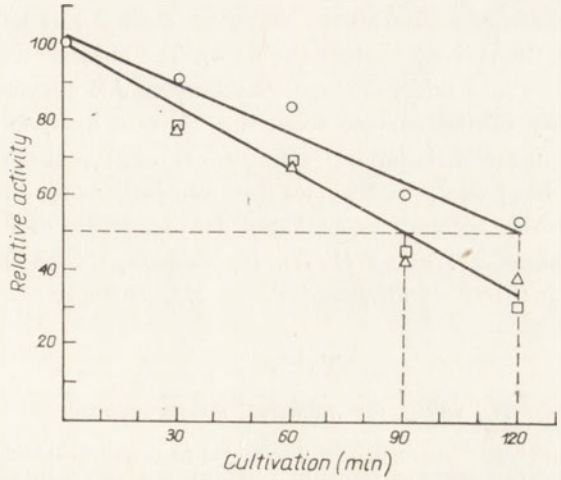

Fig. 7. Half-life time of methanol dehydrogenase. The cells of *Pseudomonas J26 Mut-12* growing in mineral medium of Tonge *et al.* (1972) containing 0.5% methanol, were harvested in the logarithmic phase of growth, washed with the medium devoid of carbon source and transferred to the medium in which methanol was replaced by 0.2% citrate. The cultures were incubated on rotatory shaker (300 rev./min) at 30°C, without inhibitors (○), with puromycin, 5 μg/ml (△) or puromycin+actinomycin D, each 5 μg/ml (□). The activity was measured in the cell-free extracts as described under Methods, with methanol as a substrate.

Substrate specificity. The cell-free extracts of both methylotrophs oxidized methanol, ethanol and propanol in accordance with oxidation of these three low-molecular alcohols by the intact cells (Table 1). Specific activity of the enzyme in the late logarithmic phase of growth was higher in the obligate *Methylomonas Pl1 Mut-11* than in the facultative *Pseudomonas* strain either with methanol or formaldehyde as the substrate (310 and 320 nmoles O_2/min/mg protein and 240 and 250 nmoles O_2/min/mg protein, respectively). The methanol/formaldehyde dehydrogenase activity ratio was approximately unity, and remained the same on 10-fold purification of the enzyme. Specific activity of the enzyme in both our isolates was somewhat higher than that found in *Methylococcus capsulatus* (221 nmoles/min/mg protein; Patel & Hoare, 1971), *Methylosinus trichosporium* (234 nmoles/min/mg protein; Weaver & Dugan, 1975) and *Pseudomonad C* (160 nmoles/min/mg protein; Stieglitz & Mateles, 1973).

The apparent Michaelis constants (Table 3) calculated from the Lineweaver-Burk plots were practically the same with methanol and ethanol in both methylotrophs ($5 - 7 \times 10^{-5}$ M). The respective values with propanol and butanol were also of the same order of magnitude for both isolates ($0.9\text{-}1.7 \times 10^{-4}$ M) and were 2 - 3 times higher than with methanol and ethanol. The divergence found between the oxidation rate of alcohols by intact cells and the substrate affinities of methanol dehydrogenase in the cell-free extract (Table 1 and 3) reflect probably differences in permeability. The K_m value with formaldehyde was within the same range as with methanol.

Effect of inhibitors. Methanol dehydrogenases from both methylotrophs did not respond to the action of K^+, Na^+, Mg^{2+} and Ca^{2+} at final concentration of 10 mM.

The effect of divalent ions could not be, however, excluded as EDTA at this concentration reduced the activity by half (Table 4). The enzymes from both isolates responded also to sodium azide, and the *Pseudomonas J26* enzyme — to bipyridyl. Higher susceptibility of the enzyme from the obligate methylotroph to sodium azide might be due not to chelation but to the reduction properties of this compound (You *et al.*, 1975). In general, the enzymes from our isolates were found to be more susceptible to chelating agents than methanol dehydrogenase of *Pseudomonas M27* described by Anthony & Zatman (1967). In addition, they responded to folate analogues, totally ineffective with *Pseudomonas M27* enzyme.

Table 3

K_m values for methanol dehydrogenase

The activity of methanol dehydrogenase was determined as described under Methods at 30°C in the presence of 15 mM-NH_4Cl. The Michaelis constants were calculated from the Lineweaver-Burk plots.

Substrate	*Pseudomonas J26 Mut-12* (M)	*Methylomonas Pl1 Mut-11* (M)
Methanol	5.0×10^{-5}	5.9×10^{-5}
Ethanol	7.3×10^{-5}	6.9×10^{-5}
Propanol	1.4×10^{-4}	1.4×10^{-4}
Butanol	1.7×10^{-4}	0.9×10^{-4}

Table 4

Effect of inhibitors on the activity of methanol dehydrogenase

The activity in *Pseudomonas J26 Mut-12* and *Methylomonas Pl1 Mut-11* was assayed as described under Methods, and expressed in nmoles O_2 consumed/min/mg protein.

Inhibitor	*Pseudomonas J26 Mut-12*	*Methylomonas Pl1 Mut-11*
None	100	100
Sodium azide, 10 mM	78	28
EDTA, 4 mM	62	66
EDTA, 10 mM	50	57
α,α′-Bipyridyl, 10 mM	69	105
Potassium cyanide, 20 mM	100	96
Aminopterin, 1 mM	73	77
Amethopterin, 0.1 mM	77	100

Localization. The total dehydrogenase activity with methanol and formaldehyde was recovered in supernatant obtained on 15 min centrifugation at 15 000 *g* of the sonicates, and remained in the soluble fraction upon centrifugation at 40 000 *g* for 20 min. Treatment of the pellet separated during centrifugation with Tween

80 (0.1%), Triton X-100 (0.01 - 0.03%) or incubation with phospholipase D under conditions recommended by Wadziński & Ribbons (1975) did not release any enzymatic protein showing dehydrogenase activity with methanol and formaldehyde. The enzyme was not sedimented from the 40 000 *g* supernatant on 60 min centrifugation at 109 000 *g*. Localization of methanol dehydrogenase in the soluble fraction is in agreement with the lack of the intracytoplasmic membrane systems in both methylotrophs (Raczyńska-Bojanowska *et al.*, 1977). Information on methanol dehydrogenase localization and ultrastructure of facultative methylotrophs are not available, whereas the intramembrane systems of two different types were found in the methane and methanol utilizing obligate methylotrophs such as *Methylococcus capsulatus* (Smith *et al.*, 1970) and *Methanomonas methanooxidans* (Smith & Ribbons, 1970). The identity of the enzymatic protein in the soluble fraction with the enzyme bound with membranes in *Methylosinus trichosporium* (Weaver & Dugan, 1975) suggested partial release of methanol dehydrogenase during preparation of the extract. Since the intracytoplasmic membranes were not detected in *Methylomonas* sp. showing obligatory requirement for methanol, one may anticipate that these structures are prerequisite for assimilation of C_1 exclusively in methane utilizers. This suggestion is supported by the absence of the complex membrane system in another obligate methylotroph *W1*, which grows on methanol and methylamine but not on methane, and was isolated by Dahl *et al.* (1972).

Dehydrogenation of formate by the cell-free extracts

Crude dialysed extracts of both methylotrophic isolates oxidize formate in the presence of NAD^+ at approximate rate of 5 - 10 nmoles/min/mg protein. These values are undoubtedly lowered due to the very active NADH oxidase (300 nmoles/min/mg protein) present in the crude extracts. Nevertheless, in other bacteria oxidizing C_1 units, e.g. *Pseudomonas AM1*, NAD-dependent oxidation of formate is almost twice that obtained with methanol, i.e. 136 nmoles/min/mg protein (Johnson & Quayle, 1964). In *Methylosinus trichosporium* the rate of formate oxidation was found to be 39 nmoles/min/mg protein (Weaver & Dugan, 1975).

Presence of the NAD-dependent formate dehydrogenase indicates generation of three ATP molecules in the third step of methanol oxidation. Since oxidation of methanol and formaldehyde is associated in *Methylomonas P11 Mut-11* with reduction of ubiquinone and cytochrome *b* and *c* (A. Drabikowska, personal communication) one may assume that in the obligate methylotroph oxidation of one molecule of methanol is accompanied by the production of 7 ATP molecules.

The technical assistance of Mr. Leszek Budohoski, Mrs. Krystyna Menke and Miss Izabella Menke is gratefully acknowledged.

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UTLENIANIE METANOLU PRZEZ WZGLĘDNE I BEZWZGLĘDNE METYLOTROFY

Streszczenie

1. Bezwzględny metylotrof *Methylomonas* i względny metylotrof *Pseudomonas* sp. utleniają metanol z niezmienną szybkością w zakresie stężeń od 0.1 do 600 mM; szybkość utleniania przez bezwzględny metylotrof jest około 2.5 razy wyższa (ok. 300 nmoli O_2/min/mg suchej masy). Niskocząsteczkowe alkohole, formaldehyd i mrówczan służą jako substraty w procesie oddychania komórek obu metylotrofów.

2. Dehydrogenazy metanolowe obu metylotrofów należy zaklasyfikować do grupy PMS-zależnych dehydrogenaz typu pterydynowego o podwójnej funkcji dehydrogenazy formaldehydowej i metanolowej. Są to rozpuszczalne enzymy o tej samej specyficzności i tym samym powinowactwie do metanolu i formaldehydu (ok. 5×10^{-5} M). Aktywności ich są zwiększone ok. 10-krotnie przez NH_4^+, co prowadzi do zwiększenia wartości V_{max}. Inaktywacji cieplnej 10-krotnie oczyszczonego enzymu towarzyszy uwalnianie rozpuszczalnego w wodzie, fluoryzującego barwnika o maksimum fluorescencji przy 420 - 430 nm.

3. Dehydrogenaza mrówczanowa zależna od NAD katalizuje trzeci etap utleniania metanolu u obu metylotrofów.

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RECENZJE KSIĄŻEK

HIGH PRESSURE LIQUID CHROMATOGRAPHY IN CLINICAL BIOCHEMISTRY. Edited by P. F. Dixon, C. H. Gray, C. K. Lim & M. S. Stoll; xvii+224 pp. Academic Press, London & New York, May 1976; price £ 4.80 or $ 12.25.

This volume presents the texts of the communications presented at an international conference on the subject organized in London in December 1975, and attended by 180 clinical chemists and biochemists from every continent.

The organization of this conference was very timely. The advent of gas liquid chromatography in the clinical laboratory about a decade ago provided the clinical biochemist with a potent tool which combined good resolution with sensitivity and reproducibility, and it is still in relatively wide use today. However, with the development in the past few years of column chromatography utilizing pressurized and metered solvent delivery systems, which provide both speed and reproducibility with dense packing materials to give good resolution, and with the use of integral detection and quantifying systems, high pressure liquid chromatography (or HPLC) is well on the way to supplanting gas chromatography in the clinic in many fields. The various articles in the present volume indicate why this is so, in part by presentations of the technical problems involved, and particularly by outlining in detail the applications to specific diagnostic techniques. These include such diversified fields as chromatography of plasma amino acids, analysis of porphyrins in various biological materials, drug levels in plasma, analysis of serum lipids and lipoproteins, determination of oestriol in human pregnancy urine, etc. The Introductory article, by John H. Knox of the University of Edinburgh on "High Pressure Liquid Chromatography — The Present Situation", which summarizes, with examples of specific applications, the present technical level of the subject, will be especially appreciated by the newcomer to the field.

The volume provides a list of all the participants, and not only the contributors, together with affiliations and addresses. It includes a short, but useful, Subject Index subdivided into three sections dealing with the biochemical compounds treated in the various presentations, the types of packing materials employed, and general matters such as techniques. There is also an Appendix, on pp. 218 - 219, of HPLC packing materials, their properties and applications, listed in the form of a table. It is to be regretted that the Discussion, following each presentation, was not included in the published text.

The utility of this small book is further enhanced by the fact that it is really up-to-date. The conference was held in December, 1975 and the volume was on sale in May, 1976. Its utility undoubtedly extends beyond the field of clinical biochemistry, and it should prove useful also to those carrying out research in such fields as biosynthesis of natural products, metabolic pathways, detection of polluants in the environment, drug metabolism, etc.

David Shugar

ENERGY TRANSFORMATION IN BIOLOGICAL SYSTEMS. Ciba Fundation Symposium 31 (new series). Elsevier - Excerpta Medica - North-Holland, Amsterdam, Oxford, New York 1975; pp. 416. price Dfl. 70.00, $ 29.25.

Ciba Foundation Symposium on "Energy Transformation in Biological Systems" was held on the 2nd - 4th July 1974 as a tribute to Fritz Lipmann on his 75th birthday. Proceedings of this Symposium contain full texts of all 19 lectures.

A review by F. Lipmann on the history of modern bioenergetics opens the book. It is followed by two articles on the most fundamental processes providing ATP in biological systems: oxidative phosphorylation and photophosphorylation, written by M. Klingenberg and F. R. Whatley, respectively. A unique system for the conversion of light energy into the energy of phosphate bond in ATP is present in the purple bacterium, *Halobacterium halobium*, dealt with in an article by D. Oesterhelt. Light-absorbing chromoprotein, bacterio-rhodopsin, present in the cell membrane of this species, can directly convert quantum energy into an electrochemical gradient across the membrane and thus builds up a high energy state of the membrane without the intervention of electron carriers.

Light is not only an energy source for photophosphorylation, but it also brings information to photosensitive organisms about the external world. Research on how light triggers electrical impulses in photoreceptors of the retina is described in the article by W. A. Hagins, W. E. Robinson and S. Yoshikami.

Production of light by some living organisms is reviewed by J. W. Hastings.

Energy-dependent transport processes are described in a series of articles. That by M. Klingenberg deals with ATP and ADP exchange transport in mitochondria. I. M. Glynn and S. J. D. Karlish describe the mechanism of sodium pump and P. J. F. Henderson and H. L. Kornberg the transport of carbohydrates in bacterial cell. Ionic channels in excitable membranes are discussed by R. D. Keynes. The phosphoenolpyruvate-sugar phosphotransferase system, described in the article by Saul Roseman, represents a bacterial active transport system whose molecular and enzymic mechanism has been recently elucidated.

Motility was certainly, besides heat production, the first energy-generating process recognized in the living world. Nevertheless, the mechanism by which chemical energy is transformed into the mechanical one is not yet fully understood. Four articles deal with this problem: A. F. Huxley — "The origin of force in skeletal muscle", C. W. F. McClare — "How does ATP act as an energy source?", D. R. Wilkie — "Muscle as a thermodynamic machine", and D. C. S. White and Madeleine M. K. Donaldson — "Mechanical and biochemical cycles in muscle contraction".

Enzyme-substrate interreactions and interconversions represent an important and intriguing problem not only for bioenergetics but also for metabolic regulation. This is described in two articles by (1) H. Gutfreund and D. R. Trentham and (2) Mildred Cohn.

Energetic aspects of metabolism regulation became a fascinating field of biochemical research since it was discovered that phosphorylation of certain enzymes produces a change in their activity. This is reviewed by E. G. Krebs and J. T. Stull. Other aspects of energy utilization for metabolic control are discussed by B. Hess.

All articles are extremely well written and, taken together, constitute an excellent review of contemporary bioenergetics. The value of the book is greatly increased by full reports of discussions which followed each lecture. Very careful editing of the volume should also be acknowledged.

The book can be highly recommended not only for anyone interested in bioenergetics but also for those working in other fields of biochemistry who want to extend their scientific horizons.

Lech Wojtczak

A. C. Neville, BIOLOGY OF THE ARTHROPOD CUTICLE. ZOOPHYSIOLOGY AND ECOLOGY (D. S. Farner, managing editor; W. S. Hoar, J. Jacobs, H. Langer & M. Lindauer, editors) vol. 4/5; Springer-Verlag, Berlin, Heidelberg, New York 1975; stron 448, cena 145.— DM.

Kutikula u stawonogów, pełniąc rolę szkieletu zewnętrznego, stanowi ważny czynnik w procesach fizjologicznych i stosunkach ekologicznych. Omawiana książka przedstawia obecny stan wiedzy o biologii kutikuli i daje bardzo starannie opracowany przegląd badań nad kutikulą w aspekcie biochemicznym, biofizycznym, fizjologicznym i ultrastruktury, głównie na przykładzie licznych gatunków owadów i skorupiaków. Autor, profesor uniwersytetu w Bristolu, czynny badacz i znawca przedmiotu, w sposób przejrzysty i krytyczny zgrupował w 10 rozdziałach wyniki ponad

1000 publikacji, ilustrując je 233 rycinami, z czego połowę stanowią zdjęcia preparatów kutikuli w mikroskopie elektronowym.

W dwóch pierwszych rozdziałach przedstawiono w skrócie rys historyczny rozwoju badań, omówiono rolę i znaczenie kutikuli w aspekcie morfologiczno-fizjologicznym, oraz podano szczegółowy opis warstwowej struktury okrywy ciała, głównie na przykładzie owadów.

Następny rozdział dotyczy składników kutikuli. Omówiono w nim konformację, biosyntezę i polimorfizm chityny, funkcję lipidów i ich pochodnych, skład aminokwasowy białek kutikularnych oraz udział enzymów w syntezie kutikuli u owadów i krabów. Podano także charakterystykę i skład chorionu osłonek jaj owadów i włókna jedwabiu.

W czwartym i piątym rozdziale opisano krzyżowe wiązania molekularne białek z niskocząsteczkowymi pochodnymi tyrozyny, jako czynnikami taninującymi w procesie melanizacji i sklerotyzacji kutikuli owadów, oraz udział w tym procesie oksydaz fenolowych. Omówiono także występowanie, biosyntezę i funkcję resiliny, elastycznego białka struktur nabłonkowych owadów. Przedyskutowano wyniki badań nad fibrylarną strukturą chityny i helikoidalnym uwarstwieniem kutikuli w niektórych narządach i odcinkach zewnętrznej okrywy owadów.

W kolejnym rozdziale przedstawiono fizjologiczną zależność między stałym wzrostem kutikuli a rozwojem osobniczym i wiekiem owadów. Wiele uwagi poświęcono oddziaływaniu hormonów, omówiono wpływ hormonu juwenilnego i jego analogów na elastyczność kutikuli u form larwalnych, działanie ekdysonu na inicjację procesu linienia i zmiany wywołane w strukturze kutikuli na skutek uruchomienia procesu melanizacji i sklerotyzacji. Przedstawiono model działania peptydowego hormonu bursikonu, produkowanego przez komórki neurosekrecyjne mózgu, wpływ kompleksu *corpora allata/corpora cardiaca* na sekrecję wosku, oraz hormonalną inicjację aktywności gruczołów kolateralnych.

W rozdziale siódmym, który jest kontynuacją szóstego, omówiono proces wapnienia kutikuli u skorupiaków i owadów.

W rozdziale ósmym, dotyczącym właściwości fizycznych, przedstawiono wyniki badań w nadfiolecie i podczerwieni, zdolność do absorpcji i fluorescencji. Obok wymienionych właściwości optycznych opisano właściwości mechaniczne kutikuli, jej twardość i wytrzymałość na ucisk oraz przepuszczalność poprzez system kanalikowy.

W rozdziale dziewiątym zgrupowano wyniki badań prowadzonych w aspekcie filogenetycznym nad strukturą kutikuli u różnych rzędów stawonogów.

Ostatni rozdział ma charakter dyskusji i poszukiwań możliwości powiązania niektórych problemów poruszonych we wcześniejszych rozdziałach tej książki.

Omawiana książka stanowi wartościową pozycję, wnosząc cenny wkład w uporządkowanie ogromnego materiału doświadczalnego, udostępnia wiele informacji dotąd nieopublikowanych, jest szczególnie użyteczna dla tych, którzy zajmują się fizjologią, biochemią i endokrynologią bezkręgowców, a szczególnie owadów.

Maria J. Piechowska

J. Tze-Fei Wong, KINETICS OF ENZYME MECHANISMS. Academic Press, London, New York, San Francisco 1975; str. 294+xiii, cena $ 7.80.

Książka Tze-Fei Wonga jest bardzo cenną pozycją zarówno dla studentów, jak i dla enzymologów i biologów interesujących się zagadnieniami kinetyki enzymów i kinetycznej interpretacji wyników oznaczeń enzymatycznych. Autor pisze we wstępie do książki, że zdając sobie sprawę z niebezpieczeństw wypływających ze zbyt wielkich uproszczeń w przedstawieniu kinetyki enzymów z jednej strony oraz zwykle dużej różnicy między dokładnością wyników doświadczalnych a złożonymi, precyzyjnymi opracowaniami matematycznymi z drugiej strony, zdecydował się omówić podstawy teorii kinetycznych, nie posługując się uproszczeniami oraz przedstawić w pełnej rozciągłości koncepcję i metody stosowane w interpretacji kinetycznych mechanizmów reakcji enzymatycznych. Jednocześnie jednak starał się, aby czytelnik nie zatracił ogólnego spojrzenia na kinetykę enzymów przez skupienie uwagi na szczegółach matematycznych. W wyniku takiego ujęcia ma-

teriał został podzielony na 12 rozdziałów. Pierwsze cztery dotyczą najczęściej spotykanych mechanizmów opartych na reakcji enzymu z jednym substratem; omówiono przy tym także metody graficzne ilustrujące zasadnicze typy współzależności między enzymem i substratem oraz mechanizmy inhibicji i aktywacji. W dalszej kolejności zostały przedstawione mechanizmy reakcji rozgałęzionych ze szczególnym uwzględnieniem dehydrogenazy alkoholowej wątroby oraz mechanizmy reakcji z dwoma substratami. W oparciu o przykłady przedstawiono współdziałanie kooperatywne i mechanizmy homotropowe oraz przedyskutowano zastosowanie metody wymiany izotopowej, oznaczeń stałych szybkości reakcji oraz podstawowe metody statystyczne. Książkę zamyka rozważanie na temat różnic w działaniu enzymu *in vitro* i *in vivo* oraz związanego z tym zagadnienia transportu przez osłony komórki.

Książka jest napisana w sposób bardzo przejrzysty i bardzo starannie opracowana pod względem edytorskim. Znaczna liczba odnośników do literatury i obszerne indeksy są pomocne w korzystaniu z przedstawionego materiału teoretycznego, a podane przykłady i zadania wraz z rozwiązaniami pozwalają na zrozumienie i opanowanie przedstawionych metod i interpretacji.

Konstancja Raczyńska-Bojanowska

GENETICS AND BIOCHEMISTRY OF PSEUDOMONAS, red.fP. H. Clark i M. H. Richmond. A Wiley - Interscience Publication. John Wiley and Sons, London, New York, Sydney, Toronto 1975; str. 366+IX, cena $ 25.—

Wydanie w formie monograficznej wyników badań genetycznych i biochemicznych nad bakteriami rodzaju Pseudomonas świadczy o rozległości i wadze informacji na temat tej grupy taksonomicznej bakterii. Grupa ta obejmuje wiele gatunków zarówno niepatogennych, jak i patogennych dla człowieka, zwierząt i roślin. Cechą znamienną bakterii z rodzaju Pseudomonas jest ogromna łatwość adaptacji do środowiska, wynikająca z niskiej specyficzności wymagań pokarmowych i zdolności wykorzystania jako źródła węgla i energii różnorodnych związków, takich jak gazowe i ciekłe węglowodory, metanol i związki aromatyczne. Dzięki tym właściwościom bakterie te odgrywają zasadniczą rolę w procesie biologicznego oczyszczania ścieków oraz są wykorzystywane jako t.zw. białko niekonwencjonalne (scp) w żywieniu zwierząt.

Redaktorem głównym monografii jest prof. Clarke, która sama od wielu lat pracuje nad genetyką *Pseudomonas putida* i zapewniła w monografii udział autorów znanych prac z dziedziny genetyki i biochemii Pseudomonas. Wprowadzeniem w złożone problemy klasyfikacji i taksonomii rodzaju Pseudomonas jest pierwszy artykuł Palleroniego. Autor wykazuje niedostateczność kryteriów fenotypowej klasyfikacji i proponuje oprzeć taksonomię w obrębie rodzaju na homologii RNA i DNA poszczególnych gatunków. Odrębny rozdział w monografii został poświęcony medycznym aspektom związanym z występowaniem *Pseudomonas aeruginosa*. Struktura ściany i membrany została omówiona również w odniesieniu do tego gatunku. Dane dotyczące innych gatunków zostały zamieszczone jedynie porównawczo. Plazmidy i przenoszenie materiału genetycznego jest tematem kolejnych trzech rozdziałów. Specjalną uwagę zwrócono przy tym na rolę plazmidów w biologii Pseudomonas i znaczenie fagów i bakteriocyn w epidemiologii. Zawarte w tych rozdziałach informacje na temat plazmidów i oporności na antybiotyki poparte odnośnikami literaturowymi stanowią cenny materiał dla badaczy posługujących się rekombinacją w analizie genetycznej gatunków Pseudomonas, jak również w konstruowaniu szczepów przydatnych dla przemysłu. Omówienie mechanizmów regulujących wykorzystanie różnych źródeł węgla przez Pseudomonas poprzedza dyskusja ogólnych mechanizmów kontrolujących utlenianie oraz przemiany anaboliczne i kataboliczne z uwzględnieniem roli cyklu Krebsa w obu tych przemianach. Monografię kończy interesujące rozważanie na temat perspektyw ewolucji gatunków rodzaju Pseudomonas.

Recenzowana książka jest nie tylko cenna dla biochemików, genetyków i epidemiologów zajmujących się bakteriami z rodzaju Pseudomonas, ale również jest pożyteczną lekturą dla wszystkich interesujących się regulacją genetyczną metabolizmu drobnoustrojów.

Konstancja Raczyńska-Bojanowska

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