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CONTENTS

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ON THE MECHANISM OF MALEATE ACTION ON RAT KIDNEY MITOCHONDRIA *

EFFECT ON SUBSTRATE-LEVEL PHOSPHORYLATION

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1. Maleate inhibits the substrate-level phosphorylation linked to anaerobic dismutation of 2-oxoglutarate in rat kidney mitochondria. 2. Phosphate and magnesium diminish the inhibitory effect of maleate. Arsenate also relieves the inhibition of 2-oxoglutarate consumption but only at low (1 mM) phosphate concentration; at higher concentrations, the action of phosphate and arsenate is competitive. 3. Acetoacetate, malonate and succinate, the substrates of CoA-transferase, relieve the inhibition of 2-oxoglutarate metabolism by maleate both in the respiring mitochondria and in the "anaerobic" system containing antimycin and rotenone. 4. The interference in succinyl-CoA metabolism by maleate is discussed as a possible mechanism of the inhibitory action of this compound.

In preceding papers it was demonstrated that inhibition of the tricarboxylic acid cycle by maleate at the stage of 2-oxoglutarate oxidation (Angielski & Rogulski, 1962; Rogulski *et al.*, 1974) was not due to the direct effect on oxoglutarate dehydrogenase. It was also proved that maleate inhibited phosphorylation accompanying oxidation of 2-oxoglutarate. This inhibition was distinctly relieved by arsenate, an uncoupler of substrate-level phosphorylation. It was suggested therefore that maleate interfered with the succinyl-CoA metabolism, and this in consequence resulted in inhibition of the oxidative metabolism of 2-oxoglutarate and the other CoA-dependent substrates (Rogulski *et al.*, 1974).

Additional data concerning the site and mechanism of maleate action presented in this paper, confirm the supposition that maleate does not affect any enzyme directly but forms maleyl-CoA, a slowly metabolized product, which acts as a trapping system for the intramitochondrial CoA.

MATERIALS AND METHODS

Mitochondria were prepared according to Hogeboom (1955) from kidneys and livers of rats starved for 12 h. Disintegration of mitochondria was accomplished by ultrasonic vibration as described in the preceding paper (Rogulski *et al.*, 1974). The preparation containing both the soluble and particulate fractions was used.

Anaerobic dismutation of 2-oxoglutarate and ammonia was assayed in the presence of antimycin and rotenone (Bruni & Azzone, 1964) in the incubation medium which contained in the final volume of 1 ml: 20 mM-Tris-HCl buffer (pH 7.4), 2.0 mM-potassium phosphate buffer (pH 7.4), 40 mM-KCl, 40 mM-NaF, 5 mM-2-oxoglutarate, 5 mM-NH$_4$Cl, 2.5 mM-MgSO$_4$, 1 mM-ADP, 30 mM-glucose, 0.5 mg of yeast hexokinase (Sigma, Type III), 0.1 mM-EDTA, 5 μg of antimycin, 1 μM-rotenone, 50 mM-sucrose and 3.5 - 5.0 mg of mitochondrial protein. The incubation mixture was shaken for 30 min at 30°C. The reaction was stopped by addition of trichloroacetic acid (final concentration, 0.43 M). 2-Oxoglutarate was measured by the procedure of Friedemann & Haugen (1943). Inorganic phosphate was measured by the method of Gomori (1953) or by the procedure of Berenblum & Chain (1938) as described by Lindberg & Ernster (1956). Protein was determined by the biuret method according to Gornall *et al.* (1949). Respiration was measured manometrically as described in the preceding paper (Rogulski *et al.*, 1974) except that the trapping system for phosphate was omitted.

Oligomycin, antimycin and rotenone were from Sigma Chem. Co. (St. Louis, Mo., U.S.A.), other reagents were from the sources described in the preceding paper (Rogulski *et al.*, 1974) or from Polskie Odczynniki Chemiczne (Gliwice, Poland).

RESULTS

Effect of maleate on the P/2e⁻ ratio and 2-oxoglutarate consumption by rat mitochondria. It was found that esterification of inorganic phosphate, consumption of 2-oxoglutarate and the *P/2e⁻* ratio were similar in kidney and liver mitochondria metabolizing 2-oxoglutarate under conditions when transport of electrons was arrested by antimycin and rotenone (Table 1). Under these conditions arsenate caused uncoupling of the substrate-level phosphorylation while dinitrophenol and

oligomycin had practically no effect. These results are in agreement with the earlier data of Bruni & Azzone (1964) and Danielson & Ernster (1963). In liver mitochondria the consumption of 2-oxoglutarate remained unchanged while in kidney mitochondria a significant (about 50%) stimulation of its uptake was observed.

Table 1

Effect of maleate on substrate-level phosphorylation linked to anaerobic dismutation of 2-oxoglutarate in rat kidney and liver mitochondria

Each tube contained 20 mM-Tris-HCl buffer, pH 7.4, 2.0 mM-potassium phosphate buffer, pH 7.4, 5 mM-2-oxoglutarate, 5 mM-NH$_4$Cl, 40 mM-NaF, 40 mM-KCl, 2.5 mM-MgSO$_4$, 1 mM-ADP, 30 mM-glucose, 0.5 mg of yeast hexokinase (Sigma, type III), 0.1 mM-EDTA, 5.0 µg of antimycin, 1 µM-rotenone, 50 mM-sucrose and 3.5 - 5.0 mg of mitochondrial protein. Maleate was added in concn. of 1 mM. Final volume 1.0 ml, time of incubation 30 min, temperature 30°C. DNP, oligomycin and arsenate were added in concentrations of 10^{-2} mM, 6×10^{-3} mM and 10 mM, respectively. The results are expressed as nmoles/mg protein/min.

Additions	Control			Maleate		
	phosphate uptake	substrate uptake	$P/2e^-$	phosphate uptake	substrate uptake	$P/2e^-$
Kidney mitochondria						
None	7.8	22.2	0.71	2.2	12.8	0.34
DNP	8.6	19.0	0.90	3.5	12.1	0.58
Oligomycin	7.0	22.3	0.63	1.7	11.5	0.30
Arsenate	1.8	34.0	0.11	0.2	13.8	0.03
Liver mitochondria						
None	9.2	29.8	0.62	10.5	32.5	0.65
DNP	9.9	27.4	0.72			
Oligomycin	7.6	27.3	0.56			
Arsenate	0.9	32.3	0.06			

Maleate at 1 mM concentration inhibited significantly the substrate-level phosphorylation linked to anaerobic dismutation of 2-oxoglutarate by kidney, but not by liver, mitochondria. In kidney, maleate decreased the consumption of phosphate by about 70% and that of 2-oxoglutarate by about 45%; consequently, the $P/2e^-$ ratio dropped by half. The action of maleate was not affected by oligomycin but DNP markedly lowered the inhibitory effect on esterification of phosphate. Arsenate at 10 mM concentration acted synergistically with maleate on esterification of phosphate but had no effect on 2-oxoglutarate consumption.

Figure 1 shows the relationship between the inhibition of substrate-level phosphorylation and maleate concentration. In these experiments, conducted under conditions specified in Table 1, concentration of inorganic phosphate in the medium was 2 - 3 mM. As can be seen, maleate at concentrations up to 0.5 mM had but a slight effect on the substrate-

-level phosphorylation. The $P/2e^-$ ratio remained practically unchanged. The consumption of 2-oxoglutarate and esterification of phosphate were inhibited to the same extent. At maleate concentrations above 0.5 mM, the uptake of phosphate was more inhibited than the consumption of 2-oxoglutarate; 2 mM-maleate inhibited the consumption of 2-oxoglutarate by about 80% whereas esterification of phosphate was completely inhibited. Sometimes there was a net production of phosphate.
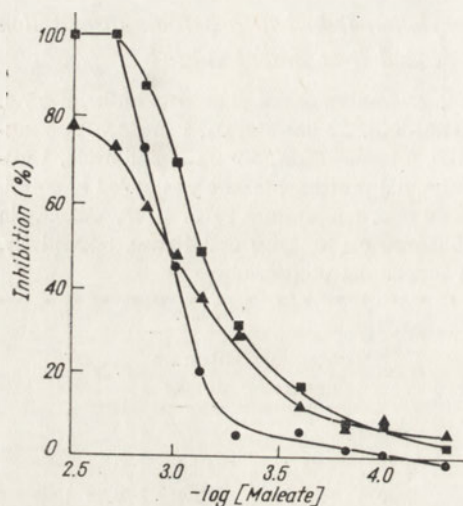
Fig. 1. Effect of maleate on substrate-level phosphorylation in kidney mitochondria. Experimental conditions as in Table 1. (■), Phosphate uptake; (▲), 2-oxoglutarate consumption; (●), $P/2e^-$ ratio.

Effect of inorganic phosphate, Mg^{2+} and arsenate. Inorganic phosphate and magnesium ions at high concentration had a significant influence on the inhibitory effect of maleate. In the system containing 2.5 mM-phosphate but no magnesium, 1 mM-maleate inhibited 2-oxoglutarate consumption by about 70% whereas esterification of phosphate was inhibited completely (Fig. 2). At increasing concentrations, magnesium gradually relieved the inhibition caused by maleate. Inhibition of phosphate esterification was more affected by Mg^{2+} than the consumption of 2-oxoglutarate. At Mg^{2+} concentration of 5 mM, inhibition of 2-oxoglutarate consumption and phosphate esterification ($P/2e^-$ ratio) approached the control values.

Similarly, an about 75% inhibition of 2-oxoglutarate consumption, with concomitant complete arrest of phosphate esterification, was observed in the system containing 5 mM-Mg^{2+} and devoid of exogenous phosphate (Fig. 3). The inhibition caused by maleate was relieved by increasing concentrations of exogenous phosphate; however, even at high phosphate concentrations, such as 14 mM, there was still a distinct difference in the degree of inhibition of 2-oxoglutarate consumption and phosphate esterification, as shown by lower $P/2e^-$ ratio (Fig. 3).

In the medium containing 2.5 mM-phosphate and the phosphate acceptor system, arsenate stimulated consumption of 2-oxoglutarate by
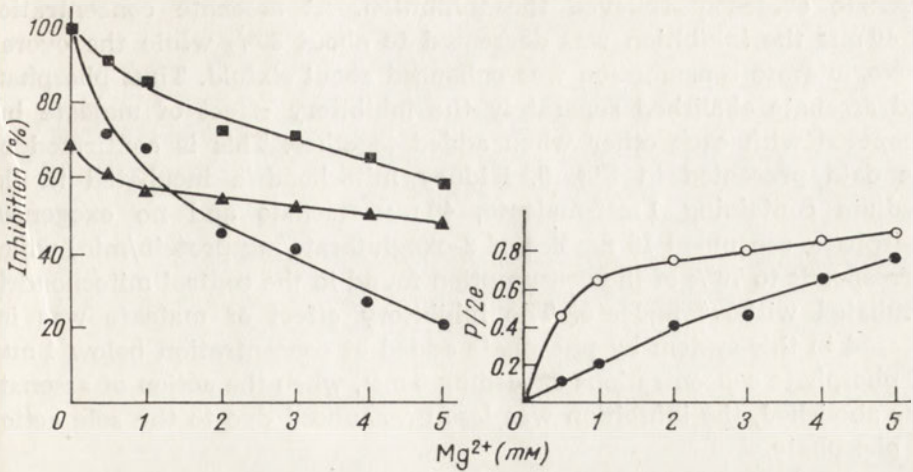
Fig. 2. Effect of Mg^{2+} on inhibition of substrate-level phosphorylation by 1 mM-maleate in kidney mitochondria. Experimental conditions as in Table 1 except that Mg^{2+} concentration varied. (■), Phosphate uptake; (▲), 2-oxoglutarate consumption; and $P/2e^-$ ratio: (●), with and (○), without maleate.
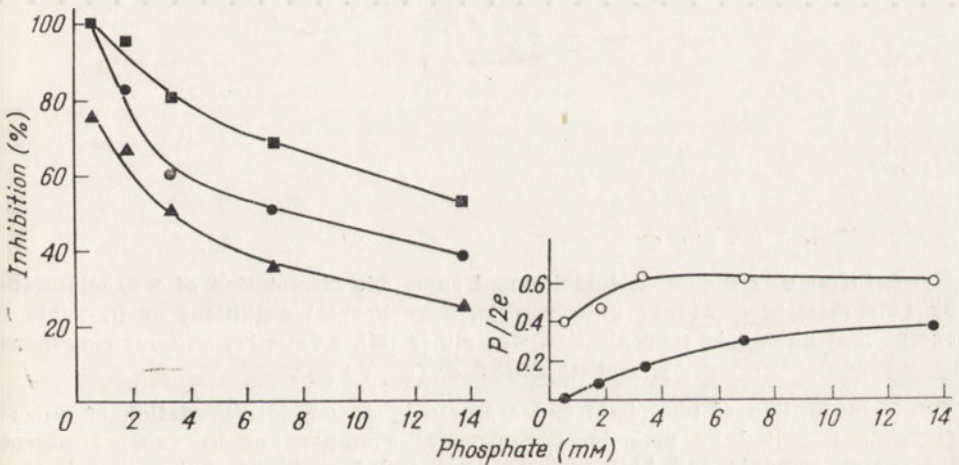
Fig. 3. Effect of phosphate on inhibition of substrate-level phosphorylation by 1 mM-maleate in kidney mitochondria. Experimental conditions as in Table 1 except that phosphate concentration varied. The endogenous phosphate content was about 120 nmoles/mg of mitochondrial protein, i.e. about 0.6 mM. (■), Phosphate uptake; (▲), 2-oxoglutarate consumption; and $P/2e^-$ ratio: (●), with and (○), without maleate.

kidney mitochondria but the inhibitory effect of maleate remained practically the same (Fig. 4). In the medium without exogenous phosphate, arsenate stimulated almost twofold the consumption of 2-oxoglutarate in the absence of maleate. In this medium, inhibition of 2-oxoglutarate consumption by maleate was distinctly higher (77%) and

arsenate evidently relieved the inhibition. At arsenate concentration
of 40 mM the inhibition was decreased to about 30% while the overall
2-oxoglutarate consumption was enhanced about sixfold. Thus phosphate
and arsenate abolished separately the inhibitory effect of maleate but
competed with each other when added together. This is confirmed by
the data presented in Fig. 5. Kidney mitochondria incubated in the
medium containing 1 mM-maleate, 40 mM-arsenate and no exogenous
phosphate, consumed 19 nmoles of 2-oxoglutarate/mg protein/min, which
corresponds to 70% of the consumption found in the control mitochondria
incubated without maleate. The inhibitory effect of maleate was in-
creased in this system by phosphate added at concentration below 1 mM.
At phosphate concentrations exceeding 5 mM, when the action of arsenate
was abolished, the inhibition was less pronounced due to the sole action
of phosphate.
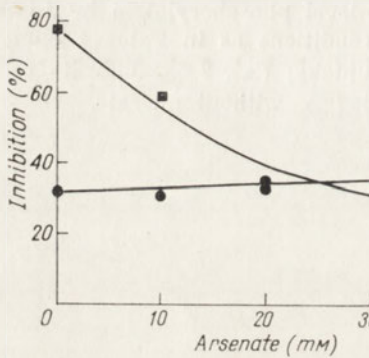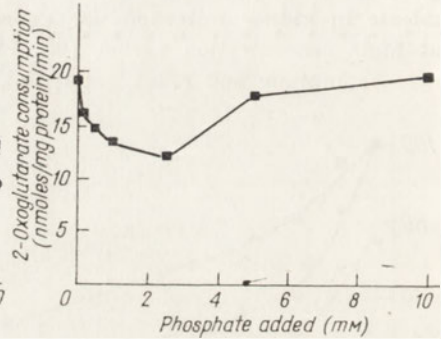
Fig. 4   Fig. 5

Fig. 4. Effect of arsenate on inhibition of anaerobic dismutation of 2-oxoglutarate
by 1 mM-maleate in kidney mitochondria. Experimental conditions as in Table 1
except that phosphate concentration was either (■), 0.6 mM i.e. without exogenous
phosphate added, or (●), 2.5 mM.

Fig. 5. Interaction of phosphate and arsenate in anaerobic dismutation of 2-oxo-
glutarate inhibited by maleate. Experimental conditions as in Table 1 except
that 40 mM-arsenate and 1 mM-maleate were added, and phosphate concentration
varied.

Effect of malonate, acetoacetate and succinate. All these three meta-
bolites are substrates of CoA-transferase catalysing the transacylation
reaction (Stern *et al.*, 1956; Menon & Stern, 1960). Data given in Fig. 6
and Table 2 show the effect of malonate and acetoacetate on oxidation
of 2-oxoglutarate and its anaerobic dismutation in the presence of
maleate. Malonate or acetoacetate added at concentration equimolar
with maleate, evidently decreased the inhibition of oxygen uptake and
substrate consumption, and at concentration two or three times higher
the inhibition was completely relieved (Fig. 6). The effect of these
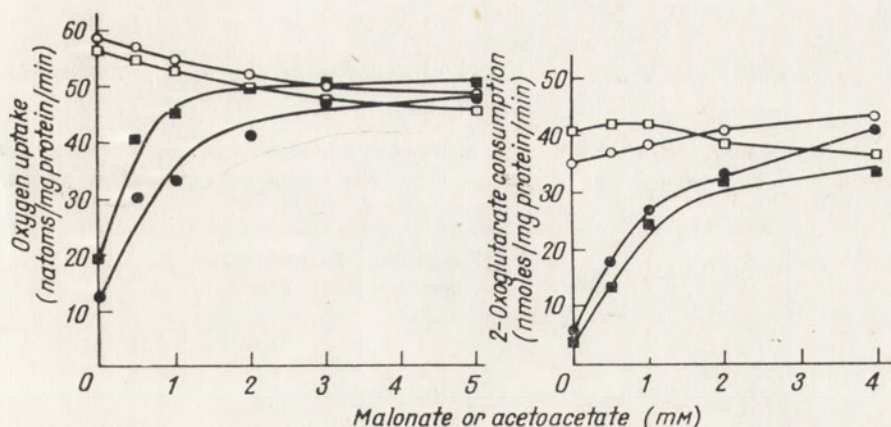
Fig. 6. Effect of malonate or acetoacetate on the inhibition by maleate of 2-oxo-glutarate oxidation in kidney mitochondria. 2-Oxoglutarate consumption and oxygen uptake in the presence of: (○), malonate; (●), malonate + maleate (1 mM); (□), acetoacetate; (■), acetoacetate + maleate (1 mM). Experimental conditions: each Warburg vessel contained 25 mM-Tris-HCl buffer, pH 7.4, 2.5 mM--phosphate buffer, pH 7.4, 25 mM-KCl, 5 mM-MgSO$_4$, 10 mM-2-oxoglutarate, 2 mM--ATP, 50 mM-sucrose, 0.1 mM-EDTA and 2.0 - 2.5 mg of mitochondrial protein. Final volume was 2.0 ml, time of incubation 30 min, gas-phase air, temperature 30°C.

compounds on the substrate-level phosphorylation (Table 2) was similar. Under the conditions when the transport of electrons was blocked by antibiotics, succinate also relieved the inhibitory effect of maleate. With the sonicated mitochondria (Table 3) the same results were obtained, which excludes interaction of membrane and proves that maleate does not affect 2-oxoglutarate transfer through the mitochondrial membrane.

Table 2

Effect of acetoacetate, malonate and succinate on the inhibition of substrate-level phosphorylation by maleate in rat kidney mitochondria

Experimental conditions as in Table 1; acetoacetate was added at concentration of 5 mM, and malonate, succinate and malate, of 2 mM. Maleate concentration was 1 mM. The results are expressed as nmoles/mg protein/min.

Additions	Control			Maleate		
	phosphate uptake	substrate uptake	$P/2e^-$	phosphate uptake	substrate uptake	$P/2e^-$
None	8.0	22.2	0.72	1.8	13.4	0.27
Acetoacetate	10.0	27.2	0.73	9.0	23.6	0.76
Malonate	8.4	24.7	0.68	5.4	17.8	0.61
Succinate	7.3	22.8	0.64	6.8	23.4	0.58
Malate	7.5	24.6	0.61	1.6	14.7	0.22

Table 3

Effect of maleate on substrate-level phosphorylation in sonicated mitochondria of rat kidney

Experimental conditions as in Table 1, except that sonicated mitochondria were used; acetoacetate concentration was 5 mM, and that of maleate, 1 mM. The results are expressed as nmoles/mg protein/min.

Additions	Phosphate uptake	2-Oxoglutarate uptake	$P/2e^-$
None	7.9	22.3	0.71
Maleate	2.4	13.2	0.36
Acetoacetate	7.7	21.4	0.72
Maleate + acetoacetate	6.8	17.4	0.78

DISCUSSION

The results previously reported (Rogulski *et al.*, 1974) and those presented in this paper suggest that integrity of the mitochondrial membrane is not essential for the inhibitory effect of maleate; the direct effect of maleate on oxidative decarboxylation of 2-oxoglutarate by oxoglutarate dehydrogenase or the reductive amination of 2-oxoglutarate by glutamate dehydrogenase, have also been excluded (Rogulski & Angielski, 1963; Angielski, 1963; Rogulski *et al.*, 1974). The results obtained suggest that maleate inhibits the consumption of 2-oxoglutarate linked with substrate-level phosphorylation and that the maleate action concerns succinyl-CoA synthetase or other reactions in which succinyl--CoA is involved. It has been demonstrated that Mg^{2+} and phosphate at high concentration counteract the inhibitory effect of maleate. Both these factors are involved in the succinyl-CoA synthetase reaction (Scheme 1). As can be seen from the diagram, all steps of substrate-level phosphorylation are reversible, thus phosphate at high concentration shifts the equilibrium of the reaction towards the formation of phosphorylated enzyme (E ∞ P complex). Mg^{2+} ions activate the two main steps of the reaction. The mechanism of uncoupling of phosphorylation by arsenate depends on the competitive action of arsenate and phosphate (Cha & Parks, 1964). Thus in the presence of arsenate one can expect decomposition of succinyl-CoA and reduced formation of E ∞ P complex. In agreement with this, arsenate relieved the inhibitory effect of maleate in the medium without added phosphate but did not influence the inhibitory effect of maleate in the medium containing exogenous phosphate.

The data presented suggest that the inhibitory effect of maleate depends to a large extent on the turnover of succinyl-CoA. In kidney, succinyl-CoA can be metabolized also by the non-specific CoA-trans-

ferase which catalyses the reversible transfer of CoA from succinyl-CoA to various acceptors (Burch *et al.*, 1964; Menon & Stern, 1960). It is postulated that maleate could be an acceptor of CoA in this reaction (Scheme 1). This would result in accumulation of the slowly metabolizing maleyl-CoA; consequently, in the tissues showing CoA-transferase activity, maleate would act as a trapping system removing free CoA from the metabolic pool. Liver is known to be the only tissue deficient in this enzyme (Stern *et al.*, 1956; Blair, 1969; Tilden & Sevdalian, 1972); therefore formation of maleyl-CoA would not be possible, and this explains insensitivity of liver mitochondria to the action of maleate.
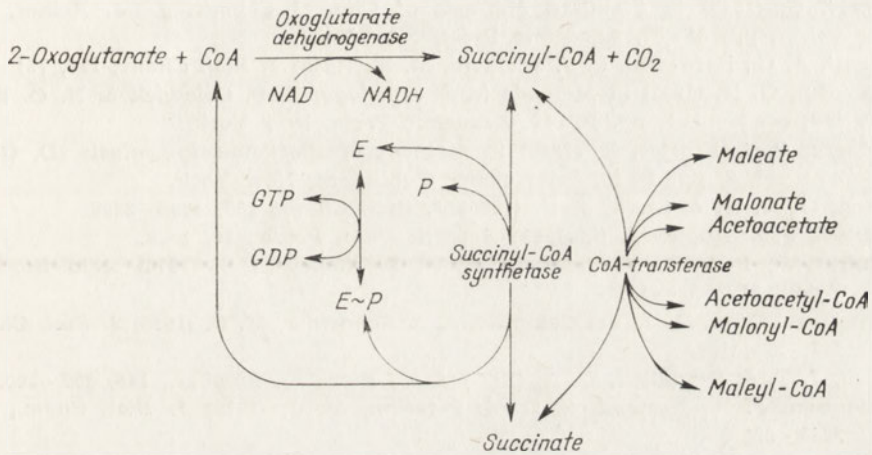
Scheme 1. The suggested mechanism of maleate action on 2-oxoglutarate metabolism.

The equilibrium of the CoA-transferase reaction is shifted strongly toward the succinyl-CoA formation (Stern *et al.*, 1956; Williamson *et al.*, 1971; Tilden & Sevdalian, 1972). Probably for this reason acetoacetate and malonate do not lower the efficiency of substrate-level phosphorylation but effectively interfere with the formation of maleyl-CoA. The release of the inhibitory effect of maleate by succinate may be explained in similar terms. In this respect it becomes obvious why succinate is the only substrate of Krebs cycle, oxidation of which is not inhibited by maleate.

The postulated mechanism of maleate action is similar, in many respects, to that of hypoglycine or 4-pentoic acid, which also bind CoA by forming slowly metabolized CoA derivatives. In this case, however, formation of acyl-CoA does not depend on the action of CoA-transferase as both these compounds are activated by the acyl-CoA synthetase system like typical fatty acids (Bressler *et al.*, 1969; Williamson *et al.*, 1970; Fukami & Williamson, 1971).

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O MECHANIZMIE DZIAŁANIA MALEINIANU W MITOCHONDRIACH NERKI SZCZURA

WPŁYW NA FOSFORYLACJĘ SUBSTRATOWĄ

Streszczenie

1. Maleinian hamuje fosforylację substratową, związaną z beztlenową przemianą 2-oksoglutaranu w mitochondriach nerek.

2. Fosforan i magnez w wysokich stężeniach zmniejszają hamujący wpływ maleinianu. Podobne działanie wykazuje arsenian przy niskim stężeniu fosforanu (1 mM). W wysokich stężeniach arsenian i fosforan działają w stosunku do siebie kompetycyjnie.

3. Acetooctan, malonian i bursztynian, substraty transferazy CoA, znoszą hamowanie wywołane maleinianem, zarówno w oddychających mitochondriach, jak i w układzie z zablokowanym łańcuchem oddechowym.

4. Przypuszczalny mechanizm działania maleinianu polega na jego oddziaływaniu na przemianę bursztynylo-CoA.

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PREPARATION AND CHARACTERISTICS OF THE STRUCTURE OF METHOXYAMINE-MODIFIED f2 RNA *

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Treatment of phage f2 RNA with [^{14}C]methoxyamine under non-de-
naturing conditions resulted in modification of exposed cytosines
only. On methoxyamine treatment in the presence of 6 M-guanidine,
all cytosines were modified. Under the conditions applied, no
modification of adenine base in RNA chain occurred. The structure
of modified f2 RNA preparations was studied by melting and
sedimentation analysis. The ratio between the modification products
(N-4-methoxycytosine and N-4-methoxy-6-methoxyamino-5,6-dihydro-
cytosine) was determined in RNA preparations modified under non-
-denaturing and denaturing conditions.

Phage f2 RNA is a polycistronic messenger active in synthesis of
three phage-specific polypeptides. These proteins are produced both
in vivo and in *E. coli* cell-free system in different amounts, coat protein
being always the predominant product synthesized (for review see Kozak
& Nathans, 1972). Several mechanisms were postulated to explain the
differences in translation of RNA cistrons. The mechanisms, which all
operate at the initiation step of polypeptide synthesis, include the
regulatory role of f2 RNA ordered structure (Lodish & Robertson, 1969)
and the existence of various cistron-specific components of initiation
factor IF3 (Berissi *et al.*, 1971; Lee-Huang & Ochoa, 1971). The latter
mechanism as well as the different ability of ribosomes from different
bacterial species to translate phage RNA (Lodish, 1970; Leffler & Szer,
1973) suggest that the initiation signal includes, along with the AUG
codon, some additional nucleotide sequence and/or secondary structure
features recognized specifically either by ribosomes or initiation factors.

Chemical modification of phage RNA could be helpful in elucidating the relationship between f2 RNA structure and its function as a template for synthesis of phage-specific polypeptides. Methoxyamine was chosen as a modifying agent mainly because of its high specificity and a relatively well known mechanism of its action.

It has been established by many authors that at pH 5.0 - 5.5 methoxyamine, similarly to hydroxylamine (for reviews see Phillips & Brown, 1967; Kochetkov & Budowsky, 1969), reacts with cytosine ring forming two stable products (cf. Scheme 1): *N*-4-methoxycytosine (I) and *N*-4--methoxy-6-methoxyamino-5,6-dihydrocytosine (II). In contrast to hydroxylamine, methoxyamine does not react with uracil residues (Kochetkov *et al.*, 1963). The fact that cytosine is the only base in RNA being modified by methoxyamine has an additional important advantage. The codons AUG and GUG at which synthesis of polypeptides is initiated (Steitz, 1969; Volckaert & Fiers, 1973) do not contain cytosine residues and are not modified by methoxyamine. The activity of a modified messenger to initiate synthesis of polypeptides can therefore directly indicate the involvement of other RNA structure features (beside AUG or GUG codons) in formation of initiation complexes.
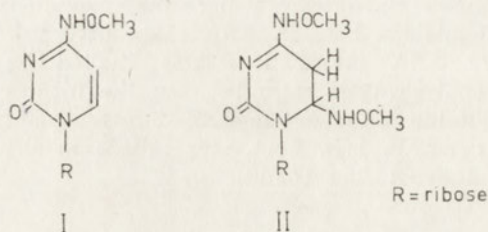
Scheme 1

Some preliminary data indicating the usefulness of modification with methoxyamine for studies of f2 RNA structure and function have been published (Filipowicz *et al.*, 1972). This paper describes the details of preparation and characteristics of f2 RNA molecules modified with methoxyamine under various conditions. Activity of these modified RNA molecules in initiation and elongation steps of protein synthesis in *E. coli* cell-free system is the subject of a separate paper (W. Filipowicz, A. Wodnar-Filipowicz and P. Szafrański, in preparation).

MATERIALS AND METHODS

Chemicals. O-Methylhydroxylamine·HCl (methoxyamine) was purchased from Eastman Organic Chemicals (Rochester, N.Y., U.S.A.), guanidine·HCl from Reachim, U.S.S.R. (twice crystallized and purified with charcoal) or from B.D.H. Chemicals Ltd (Poole, Dorset, England).

Tris, cytidine and adenosine were products of Calbiochem (Los Angeles, Calif., U.S.A.), PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-(5-phenyl-oxazol-2-yl)benzene] were from Packard Instrument Co. (Downers Grove, Ill., U.S.A.). Snake venom phosphodiesterase (EC 3.1.4.1) and *E. coli* alkaline phosphatase (EC 3.1.3.1) were obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Poly(AUG) was from Miles Laboratories (Elkhart, Ind., U.S.A.) and poly(GU) from Schwarz Bio-Research (Orangeburg, N.Y., U.S.A.). [^{14}C]-O-Methylhydroxylamine· ·HCl (spec. act. 0.05 mCi/1.7 mg) was from New England Nuclear Corp. (Boston, Mass., U.S.A.) or from Isotope (Leningrad, U.S.S.R.) (spec. act. 40 μCi/0.1 mg); [U-^{14}C]cytidine (spec. act. 223 mCi/mmole) and [U-^{14}C]a-denosine (28.4 mCi/mmole) were from the Radiochemical Centre (Amersham, Bucks., England).

Isolation of phage f2 RNA. Phage f2 RNA was isolated as described previously (Zagórski *et al.*, 1972) except that phage f2 used for RNA extraction was purified by two successive CsCl density-gradient centrifugations. RNA preparations contained 70 - 80% of material sedimenting at $s_{20·w} = 29$ S as judged from analytical centrifuge sedimentation profiles. RNA was stored in 1 mM-EDTA, pH 7.0, at -40°C.

RNA modification with methoxyamine. Methoxyamine·HCl solution was brought to pH 5.5 with conc. sodium hydroxide. RNA or synthetic polynucleotides were always modified at 37°C in 1 M-methoxyamine in the presence of either 10 mM-magnesium acetate, 6 M-guanidine·HCl or 6.7 M-urea. RNA or polynucleotide concentration was 1.5 - 3.0 mg/ml except when otherwise indicated. When guanidine or urea were used, the pH of the final incubation mixture was checked and, if necessary, adjusted to 5.5. Conditions of control incubations without methoxyamine and time of RNA modification are given in legends to Figures.

RNA modified in the presence of magnesium acetate was recovered by three precipitations with 66% ethanol from solutions to which potassium acetate, pH 5.5, was added to 0.2 M concentration. The final precipitate was additionally washed with ethanol, dissolved in 1 mM--EDTA, pH 7.0, and dialysed for 12 h against this solution. The RNA modified in the presence of guanidine was additionally diluted with 2 - 3 volumes of water prior to precipitation with ethanol, and dialysed against 10 mM-potassium acetate instead of EDTA. RNA preparations used for melting point determinations and for analytical sedimentation were additionally dialysed for 12 h against 0.1 mM-EDTA, pH 7.0. RNA solutions were stored at -40°C.

To estimate the content of N-4-methoxycytidine and N-4-methoxy--6-methoxyamino-5,6-dihydrocytidine, f2 RNA was modified by [^{14}C]methoxyamine (40 - 80 μCi/ml) for 7 days in the presence of either magnesium acetate or guanidine. The obtained labelled RNA preparations

had the specific activities of, respectively, 25 000 - 30 000 c.p.m./mg and 80 000 - 130 000 c.p.m./mg.

When the rates of reaction of [^{14}C]methoxyamine with f2 RNA or synthetic polynucleotides were measured (Fig. 1), 10 μl samples (containing usually 30 μg of the polymer) were withdrawn from the reaction mixtures at appropriate times. They were diluted with 250 μl of unlabelled 1 M-methoxyamine, pH 5.5, and after adding 50 μg of yeast RNA carrier precipitated with 1 volume of cold 10% trichloroacetic acid (TCA) containing 1 M-NH$_4$Cl. Samples were kept for 5 min at 0°C and then filtered through Whatman FG 83 paper discs (W. & R. Balston Ltd, Maidstone, England). Filters were washed 6 times with 5 ml portions of cold 5% TCA containing 1 M-NH$_4$Cl, transferred to the beaker and washed additionaly with 5% TCA containing 1 M-NH$_4$Cl, then with ethanol - ethyl ether (1:2, v/v) and ether. Filters were dried and radioactivity was measured in Packard Tri-Carb liquid scintillation counter using toluene-based scintillator.

Modification of nucleotides and identification of modified products. [^{14}C]Cytidine was modified in 1 M-methoxyamine - 10 mM-Mg-acetate, pH 5.5, for 3 days at 37°C. Cytidine concentration was 5 mg/ml and its specific activity, 0.07 μCi/mg. [^{14}C]Adenosine was modified in 3 M--methoxyamine, pH 5.0, for 7 days at 42°C. Adenosine concentration was 12.5 mg/ml and its specific activity, 0.04 μCi/mg. Methoxyamine was removed after the reaction by cyclohexanone and ether as described by Budowsky *et al.* (1971b).

Pure products of modification of cytidine (N-4-methoxycytidine and N-4-methoxy-6-methoxyamino-5,6-dihydrocytidine) and of adenosine (6--methoxyadenosine) were isolated by preparative paper chromatography in solvent A and B, respectively (see below) and extraction with water. Cytidine derivatives were identified by their characteristic absorption spectra at neutral pH, 6-methoxyadenosine by its spectrum at alkaline pH, 13.2 (Budowsky *et al.*, 1971a,b). Stability of 6-methoxyadenosine at alkaline pH was studied by measuring the u.v. spectrum at time intervals during the incubation at 37°C in 0.3 M-KOH. No differences in absorption spectrum were found after 18 h of incubation.

Estimation of the content of N-4-methoxycytidine (I) and N-4--methoxy-6-methoxyamino-5,6-dihydrocytidine (II) in modified f2 RNA. Hydrolysis of f2 RNA with snake venom phosphodiesterase was carried out in 0.1 M-Tris-HCl - 5 mM-Mg-acetate, pH 8.9. To 0.3 ml samples containing about 0.1 mg of the f2 RNA modified in the presence of guanidine or under non-denaturing conditions, the enzyme was added in portions of, respectively, 0.04 and 0.02 mg. The mixtures were incubated for 3 h at 37°C, new portions of enzyme added and incubation continued for another 3 h. This was repeated until the modified f2 RNA was completely digested into nucleotides, which was usually achieved

by adding the enzyme 10 times (for details see Fig. 4 and text). The resulting mononucleotides were dephosphorylated by the addition of 0.02 mg of *E. coli* alkaline phosphatase and incubation for 3 h at 37°C.

Alkaline hydrolysis of modified f2 RNA was performed in 0.3 M-KOH at 37°C for 18 h. After neutralization with $HClO_4$ and removing potassium perchlorate, the mononucleotides were dephosphorylated enzymically as described above.

Chromatography. The hydrolysed samples were chromatographed on Whatman no. 1 paper in 0.78% saturated ammonium sulphate containing 2% propan-2-ol (solvent *A*). The chromatogram was cut into 0.5 cm strips and radioactivity corresponding to ^{14}C-labelled *N*-4-methoxy-cytidine ($R_F = 0.44$) and *N*-4-methoxy-6-methoxyamino-5,6-dihydrocyti-dine ($R_F = 0.61$) was measured. Products of modification of [14C]cytidine with unlabelled methoxyamine were also separated by chromatography in solvent *A*.

It was found that adenosine and its modified derivative migrated together in solvent *A* with $R_F = 0.24$. They were separated by chromato-graphy in solvent *B* (saturated ammonium sulphate - 1 M-sodium acetate - - propan-2-ol, 80:18:2, by vol.). R_F values of the modified and unmodified nucleoside were 0.29 and 0.17, respectively.

Melting and sedimentation analysis of f2 RNA. Melting curves were determined in Unicam SP 500 spectrophotometer equipped with thermo-stated cuvette compartment. Sedimentation analysis of native and modified f2 RNA was carried out in 0.2 M-NaCl - 0.005 M-Tris-HCl, pH 7.2, using Spinco Model E analytical centrifuge. Sedimentation was made at 52 000 r.p.m. at 4°C and the sedimentation boundary registered at 4 min intervals. The sedimentation coefficient ($s_{20,w}$) was calculated according to a standard procedure (Schachman, 1959).

RESULTS

Reaction of [14C]methoxyamine with f2 RNA. Some authors reported that the use of labelled methoxyamine for this type of experiments with polynucleotides did not give reliable results. High sorption of unreacted methoxyamine to nucleic acids (Tikchonenko *et al.*, 1971, and references cited therein) or the possibility of existence of large-molecular labelled material in [14C]methoxyamine preparation (Fraenkel-Conrat & Singer, 1972) were pointed out as the possible reasons for the lack of reproducibility. The results presented in Fig. 1 show that [14C]meth-oxyamine can be used to study the reaction kinetics of that mutagen with RNA provided that a suitable washing procedure is applied to remove the unreacted radioactive material. The reaction of methoxy-amine at 1 M concentration with f2 RNA proceeded depending on the reaction conditions. In the presence of 10 mM-Mg-acetate, pH 5.5, the

reaction practically stopped after 5 - 7 days of incubation at 37°C at the value of about 400 methoxyamine residues reacted per molecule of f2 RNA. When the ordered structure of f2 RNA was loosened by adding a denaturing agent like urea or guanidine hydrochloride, the reactivity of RNA towards methoxyamine significantly increased. About 750 and 1250 moles of methoxyamine were found to be bound per mole of f2 RNA when modification proceeded for 7 days in the presence of urea and guanidine, respectively. The polynucleotides containing no cytosine bases, i.e. poly(GU) and poly(AUG), did not show any apparent reaction with [^{14}C]methoxyamine even under denaturing conditions. These results exclude the possibility that time-dependent sorption of methoxyamine to polynucleotides could contribute significantly to an increase in methoxyamine uptake during f2 RNA modification.
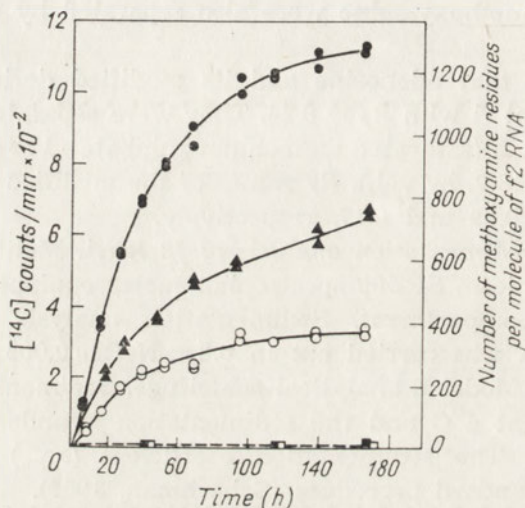
Fig. 1. Rates of reaction of [^{14}C]methoxyamine with f2 RNA and synthetic poly-nucleotides under different conditions. Reactions were carried out in stoppered tubes at 37°C in 1 M-methoxyamine, pH 5.5, containing in 1 ml 20 μCi of [^{14}C]methoxyamine and 3 mg of f2 RNA or synthetic polynucleotides: (○), f2 RNA and 10 mM-Mg-acetate; (●), f2 RNA and 6 M-guanidine; (▲), f2 RNA and 6.7 M-urea; (□), poly(AUG) and 6 M-guanidine; (■), poly(GU) and 10 mM-Mg-acetate. At appropriate time intervals, duplicate 10 μl samples were withdrawn and processed for counting as described in Methods. The values corresponding to 0 time of the reaction were subtracted. To calculate the uptake of methoxyamine per molecule of f2 RNA, the value of 1.1×10^6 (Gesteland & Boedtker, 1964) was taken as the molecular weight of RNA.

Changes in structure of f2 RNA caused by the medium in which the methoxyamination was performed, were studied by determining melting profiles under conditions resembling those used for modification of RNA (Fig. 2). The melting points in the presence of magnesium, urea,

and guanidine were, respectively, 74°, 60° and 39°C. Thus, the changes in f2 RNA ordered structure depending on the conditions applied for RNA modification were responsible for the different reactivity of f2 RNA towards methoxyamine.

Fig. 2. Melting profiles of f2 RNA under conditions resembling those used for RNA modification with methoxyamine (see Fig. 1). RNA was dissolved in: (○), 1 M-NaCl - 10 M-Mg-acetate - 10 mM-phosphate buffer, pH 5.5; (▲), 1 M-NaCl - 6.7 M-urea - 50 mM-phosphate buffer, pH 5.5; (●), 1 M-NaCl - 6 M-guanidine·HCl - 50 mM-phosphate buffer, pH 5.5.
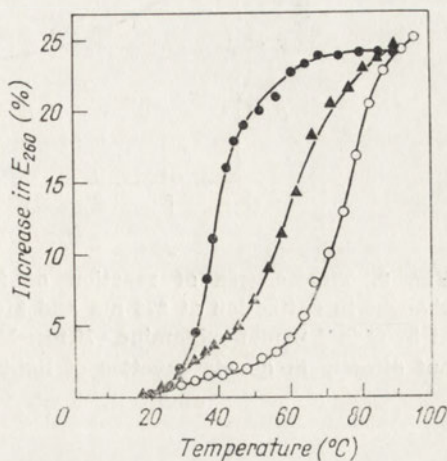
Estimation of modified bases in methoxyaminated f2 RNA. The appearance of the two types of cytosine modification products: *N*-4--methoxycytosine (I) and *N*-4-methoxy-6-methoxyamino-5,6-dihydrocytosine (II) during RNA treatment with methoxyamine can be followed directly by the spectrophotometric method. Saturation of C-5 - C-6 double bond of cytosine ring results in a decrease in absorption at about 270 nm. On the other hand, generation of *N*-4-methoxycytosine is characterized by an increase in absorption at 310 nm, i.e. in the region where absorption of cytosine and other bases is negligible (Kochetkov & Budowsky, 1969; Lawley, 1967).

The incubation of f2 RNA with methoxyamine in the presence of Mg^{2+} ions (under non-denaturing conditions) led to a marked decrease in absorption at 276 nm reflecting formation of *N*-4-methoxy-6-methoxyamino-5,6-dihydrocytosine (Fig. 3). Also the increase in absorption at 310 nm accompanying the formation of the second product, was observed. In the case of denaturing conditions for RNA modification it was not possible to follow the course of reaction spectrophotometrically. As it will become evident later, the reaction of RNA with methoxyamine in the presence of denaturing agent leads to a progressive unfolding of RNA ordered structure, which is accompanied by the change in absorption due to the loss of hypochromicity.

The relative amounts of the two modification products of cytosine base were determined in [^{14}C]methoxyamine-containing f2 RNA preparations modified extensively either in the presence of 10 mM-Mg^{2+} or 6-M-guanidine·HCl. An enzymic method was used for degradation of
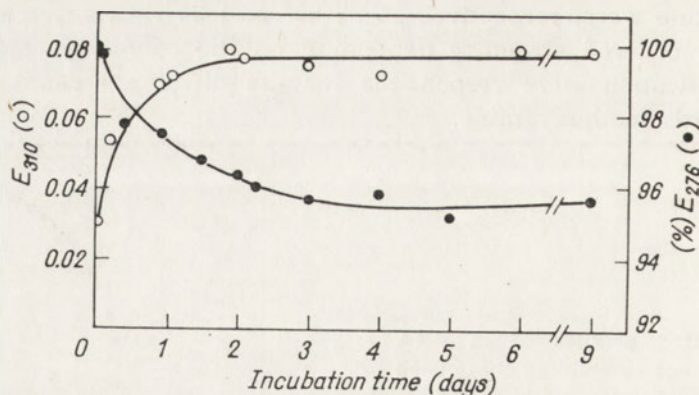
Fig. 3. Time-course of reaction of f2 RNA with methoxyamine, followed by changes in extinction at 276 nm and 310 nm. The f2 RNA, 28 μg/ml, was incubated at 37°C in 1 м-methoxyamine - 10 mм-Mg-acetate, pH 5.5. The reaction was carried out directly in quartz cuvettes of light path of: (●), 1 cm for E_{276} measurements, and (○), 4 cm for E_{310} measurements.

modified f2 RNA. The preparations were first digested with snake venom phosphodiesterase and the resulting nucleotides dephosphorylated using *E. coli* alkaline phosphatase. The obtained nucleosides labelled with [^{14}C]methoxyamine were separated by paper chromatography (Fig. 4a-d). It was found that in order to obtain a total degradation of modified f2 RNA by means of 5′-exonuclease it was necessary to use an excess of the enzyme. However, it is interesting to note that the same high radioactivity corresponding to N-4-methoxycytidine (I) was found after digestion even at a low enzyme/RNA ratio (Fig. 4a as compared with Fig. 4b). On further addition of the enzyme, the radioactivity on the chromatograms increased only in areas corresponding to N-4-methoxy-6-methoxyamino-5,6-dihydrocytosine (II). These results indicate that nucleotides containing the modified compound of type II are much more resistant to exonuclease cleavage than those possessing the modified base of type I.

Budowsky *et al.* (1971b) reported that adenosine base could also be modified with methoxyamine giving rise to 6-methoxyaminopurine. We confirmed these results and found that under conditions much more drastic than those used for f2 RNA modification, namely in 3 м-methoxyamine at pH 5.0, after 7 days at 42°C only 17% of adenosine was modified. The product could be readily separated from adenosine by chromatography in solvent *B* (see Methods and Fig. 4f). The isolated methoxyadenosine was identified by its characteristic u.v. spectrum at pH 13 (Budowsky *et al.*, 1971b).

It was important to see whether under conditions employed for modification of f2 RNA some of its adenine bases react with methoxy-
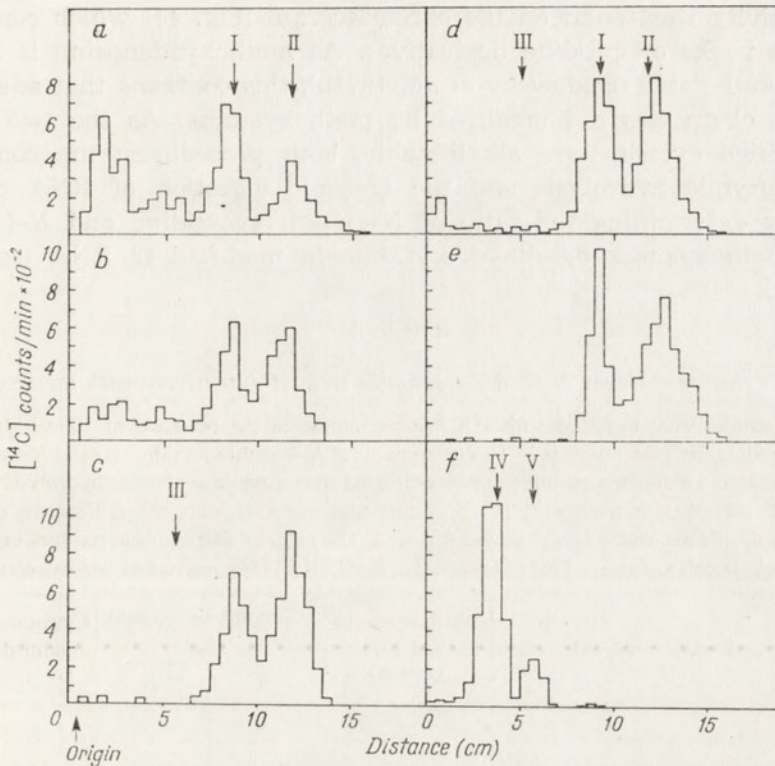
Fig. 4. Paper chromatography of hydrolysis products of f2 RNA modified with [¹⁴C]methoxyamine (a - e, solvent A), and chromatography of [¹⁴C]adenosine modified with 3 M-methoxyamine (f, solvent B). For details see Methods.

a, b, c - f2 RNA was modified under denaturing conditions, and submitted to enzymic hydrolysis and dephosphorylation. The final amount of snake venom phosphodiesterase added per sample was: a, 0.04 mg; b, 0.2 mg; c, 0.4 mg.

d, f2 RNA was modified under non-denaturing conditions, then submitted to enzymic hydrolysis and dephosphorylation. The final amount of the snake venom phosphodiesterase added was 0.2 mg/sample.

e, f2 RNA was modified under denaturing conditions, then submitted to alkaline hydrolysis followed by enzymic dephosphorylation.

f, [¹⁴C]Adenosine was treated extensively with 3 M-methoxyamine at pH 5.

I, II and III indicate the positions, respectively, of N-4-methoxycytidine, N-4-methoxy-6-methoxyamino-5,6-dihydrocytidine, and N-6-methoxyadenosine during chromatography in solvent A; IV and V indicate the positions of, respectively, adenosine and N-6-methoxyadenosine in solvent B.

amine. An apparent absence of [¹⁴C]methoxyamine label in poly(AUG) even after 7 days of incubation (Fig. 1), as well as the lack of radioactivity in position corresponding to 6-methoxyadenosine in chromatograms of enzymically digested modified f2 RNA (Fig. 4c and d), seem to disprove this possibility. Moreover, when the f2 RNA modified extensively under denaturing conditions was subjected to alkaline hydrolysis followed by enzymic dephosphorylation, no 6-methoxyadenosine

radioactivity was found on the chromatogram (Fig. 4e) which contained the two peaks of cytidine derivatives. As methoxyadenosine is known to be alkali-stable (Budowsky *et al.*, 1971b), this confirms that adenosine in RNA chain was not modified by methoxyamine. As the two forms of modified cytosine are alkali-stable, both procedures: the combined alkali-enzymic hydrolysis and the enzymic digestion of RNA can be used for determining the ratio of N-4-methoxycytidine and N-4-methoxy-6-methoxyamino-5,6-dihydrocytidine in modified f2 RNA (see also Table 1).

Table 1

Modified cytosine residues in f2 RNA samples treated extensively with methoxyamine

f2 RNA samples were modified with [^{14}C]methoxyamine in the presence of 10 mM-Mg-acetate or 6 M-guanidine·HCl for 7 days at 37°C. The amounts of N-4-methoxycytidine (I) and N-4-methoxy-6-methoxyamino-5,6-dihydrocytidine (II) were estimated after enzymic or alkaline hydrolysis followed by alkaline phosphatase treatment. [^{14}C]Cytidine was modified with non-radioactive methoxyamine. For details see Methods and legend to Fig. 4. The value of 880 cytosine residues in f2 RNA molecule (Loeb & Zinder, 1961; Gesteland & Boedtker, 1964) was taken for calculation.

Sample analysed	Methoxyamine reacted (moles/ /mole f2 RNA)[a]	Compounds formed (%)		Cytosines modified (%)
		I	II	
f2 RNA modified in 1 M-methoxyamine and 10 mM-Mg^{2+}, pH 5.5	390	63.7[b]	36.3[b]	32.5
f2 RNA modified in 1 M-methoxyamine and 6 M-guanidine, pH 5.5	1270	55.1[b] 55.0[c]	44.9[b] 45.0[c]	99.7
Cytidine modified in 1 M-methoxyamine and 10 mM-Mg^{2+}, pH 5.5	—	43.9	56.1	99

[a] Taken from Fig. 1.
[b] RNA hydrolysed with snake venom phosphodiesterase.
[c] RNA hydrolysed with alkali.

The results summarized in Table 1 indicate that on modification of f2 RNA under denaturing conditions, similarly as on treatment of free cytidine with 1 M-methoxyamine at pH 5.5, nearly 100% of cytosine bases were transformed to products I and II. On the other hand, when f2 RNA was treated with methoxyamine under non-denaturing conditions, only 32.5% of the cytosine bases were modified. Most probably the secondary and tertiary structure of f2 RNA did not allow the remaining cytosine residues to react with the modifying agent. Furthermore, the structure of RNA had apparently an important effect upon the ratio of products I/II. When RNA was modified by methoxyamine

under conditions which did not denature its ordered structure, 64% of product I and 36% of product II were formed. In RNA modified under denaturing conditions, the two-substituted derivative corresponded to 45% of modified bases while in the case of free cytidine modification, to 56%.

Estimation of ordered structure of modified f2 RNA preparations. Melting and sedimentation analysis was used to study the ordered structure of modified f2 RNA. The f2 RNA modified under non-denaturing conditions for 36 h or 5 days retained essentially unchanged melting properties, characteristic for native f2 RNA (Fig. 5A). On the other hand, melting curves of the RNA modified in the presence of guanidine for 30 h or 5 days became markedly flattened (Fig. 5B) indicating a progressive unfolding of RNA molecule. The melting properties of control RNA treated with 6 M-guanidine in the absence of methoxy-amine, were unchanged indicating that RNA molecule unfolded by guanidine action renatured after removal of the denaturing agent. The presence of methoxyamine with simultaneous unfolding of RNA chain led to modification of cytosine residues engaged in hydrogen bonding, and thus to an irreversible change in RNA higher structure.

Sedimentation profiles in analytical centrifuge of f2 RNA, native and modified for 5 days under non-denaturing conditions, were essent-
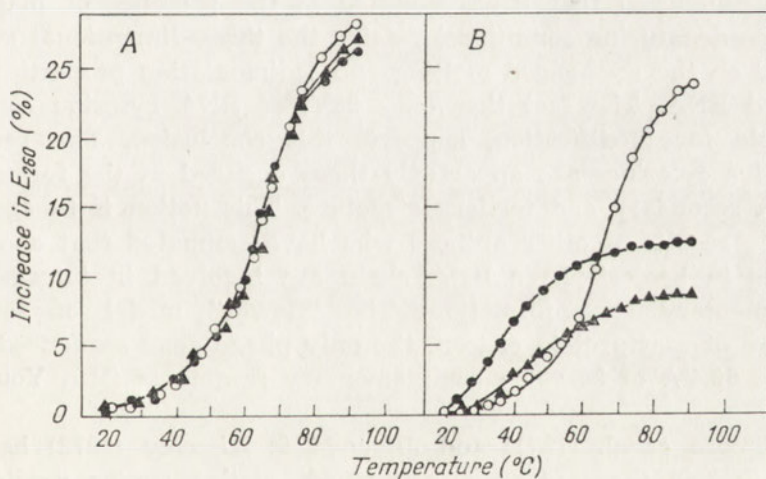
Fig. 5. Melting temperature profiles of f2 RNA preparations modified with meth-oxyamine: *A*, under non-denaturing conditions and *B*, under denaturing conditions. Melting was performed in 0.15 M-NaCl - 0.015 M-Na-citrate, pH 7.2.

A: (●), f2 RNA modified in 1 M-methoxyamine - 10 mM-Mg-acetate, pH 5.5, at 37°C for 36 h, and (▲), for 5 days; (○), control, non-modified f2 RNA incubated in 1 M-NaCl - 10 mM-phosphate buffer, pH 5.5, at 37°C for 36 h.

B: (●), f2 RNA modified in 1 M-methoxyamine - 6 M-guanidine·HCl, pH 5.5, at 37°C for 30 h, and (▲), for 5 days; (○), control f2 RNA incubated in 1 M-NaCl - 6 M-guanidine·HCl - 10 mM-phosphate buffer, pH 5.5, at 37°C for 5 days.

ially the same; the values $s_{20 \cdot w}$ were found to be 29.8 and 29.1, respectively. This indicates that treatment of f2 RNA with methoxyamine under non-denaturing conditions did not result in any degradation of RNA molecule, and the modified RNA retained its native compact three-dimensional structure. The RNA modified extensively with methoxyamine in the presence of guanidine sedimented much more slowly ($s_{20 \cdot w}$ about 10) indicating a strong unfolding of its ordered structure; however, the sedimentation boundary indicated that some degradation of RNA during modification or subsequent handling has occurred. This is not surprising as this type of unfolded RNA molecule is extremely susceptible to trace amounts of ribonuclease.

DISCUSSION

The presented results indicate that, depending upon the conditions used for f2 RNA treatment with methoxyamine, different well-defined types of modified RNA molecules can be obtained. Two modified RNA preparations studied in more detail were characterized. The one prepared under non-denaturing conditions retained the native ordered structure of f2 RNA and the other, obtained in the presence of 6 M-guanidine·HCl lost much of its secondary structure.

The conclusion that RNA modified in the presence of magnesium ions (non-denaturing conditions) retains the three-dimensional structure is based on the unchanged melting and sedimentation properties of the modified RNA. The fact that only 33% of RNA cytosine content is accessible for modification, supports this conclusion. The remaining unreactive cytosines are apparently those engaged in the formation of f2 RNA secondary and tertiary structure. This notion is in agreement with the results of other authors who have estimated that about 70% of bases in bacteriophage RNA chain are involved in intramolecular complementary base formation. The "flower" model of secondary structure of coat protein cistron, the only phage gene sequenced so far, contains 66.4% of bases in complementary fragments (Min You *et al.*, 1972).

Cashmore *et al.* (1971) and Jilyaeva & Kisselev (1972) have also shown that in the case of *E. coli* tRNA, methoxyamine reacts under suitable conditions only with exposed cytosine residues, proving to be a valuable tool in the studies of two- and three-dimensional structure of tRNA molecule.

Under denaturing conditions, the treatment of f2 RNA with methoxyamine led to a much greater modification of its cytosine content. Nearly all cytosines in f2 RNA were modified by methoxyamine in the presence of guanidine. The temperature profiles of this preparation indicate that the modification of the cytosines involved in hydrogen

bonding of intramolecular G-C pairs, results in irreversible unfolding of RNA ordered structure. This can be explained by the altered pairing properties of both modified cytosine derivatives (Budowsky *et al.*, 1971a,c). Under these conditions, removal of the denaturing agent after the reaction cannot restore the initial f2 RNA structure.

It should be stressed that the use of methoxyamine-guanidine treatment for obtaining RNA molecules devoid of highly organized structure, has certain advantages not shared by the commonly used formaldehyde treatment. Reaction of RNA with formaldehyde is much less specific; moreover, it results in formation of cross-links between RNA fragments (Freifelder & Davison, 1963; Grossman, 1968; Lodish, 1971).

The use of ^{14}C-labelled methoxyamine, together with application of an efficient procedure for removal of the unreacted reagent, permitted to study the kinetics of the reaction of methoxyamine with polynucleotides, and to estimate the amounts of both types of cytosine derivatives (I and II) in modified RNA preparations. It was found that the relative amounts of products I and II formed depend on the structure of polynucleotide chain, the product of type II predominating when modification proceeded under denaturing conditions. It seems that the destruction of RNA ordered structure, especially unstacking of the bases, favours formation of product II. It should be underlined that, when using the snake venom phosphodiesterase for digestion of modified RNA, attention must be paid to complete RNA hydrolysis as nucleotide residues containing N-4-methoxy-6-methoxyamino-5,6-dihydrocytosine are less susceptible to cleavage by this exonuclease than those containing N-4-methoxycytosine. This could possibly explain in part the very low amount of N-4-methoxy-6-methoxyamino-5,6-dihydrocytidine (II) found by Fraenkel-Conrat & Singer (1972) in methoxyamine-treated poly(C) and TMV RNA.

Although methoxyamine reacts also with free adenosine, its reaction with adenine present in poly(AUG) (Fig. 1) or in f2 RNA, if any, occurs at a rate by several orders of magnitude lower than that with cytosine.

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OTRZYMYWANIE I CHARAKTERYSTYKA STRUKTURY RNA FAGA f2 MODYFIKOWANEGO DZIAŁANIEM METOKSYAMINY

Streszczenie

Działanie [^{14}C]metoksyaminy na RNA faga f2 w warunkach niedenaturujących prowadzi do modyfikacji tylko tych cytozyn, które znajdują się w jednociowych odcinkach RNA. W obecności 6 M-guanidyny, która denaturuje RNA, metoksyamina modyfikuje prawie wszystkie cytozyny. W opisanych warunkach reakcji nie stwierdzono modyfikacji reszt adeniny. Strukturę zmodyfikowanych preparatów RNA badano za pomocą krzywych topnienia i stałych sedymentacji. Ustalono stosunek zmodyfikowanych produktów (*N*-4-metoksycytozyny i *N*-4-metoksy-6-metoksyamino-5,6-dwuhydrocytozyny) w preparatach RNA zmodyfikowanych w warunkach niedenaturujących i denaturujących.

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INTRACELLULAR LOCALIZATION AND SOME PROPERTIES OF UDPG : STEROL GLUCOSYLTRANSFERASE FROM *CALENDULA OFFICINALIS*

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UDPG : sterol glucosyltransferase is localized in the 2-week-old *C. offi-cinalis* seedling in the membrane structures, separated from chloro-plasts and mitochondria, and consisting probably of fragments of the Golgi apparatus. A minor part of the enzyme activity is associated with the microsomal fraction. A number of synthetic detergents stimulate the activity of the membrane-bound enzyme causing its solubilization. The enzyme preparation purified about 70-fold is strongly inhibited by $HgCl_2$ and *p*-chloromercuribenzoate; it is markedly stimulated by mercaptoethanol and dithiothreitol, and to a lesser extent by Mg^{2+} and Ca^{2+} as well as by some chelating and reducing agents. UMP stimulates and UDP and UTP markedly inhibit the enzyme activity. The enzyme does not act on 4-methylsterols although it utilizes a number of 4-demethylsterols. It seems that the presence of a double bond in ring B enhances the affinity of the substrate for the enzyme. Δ^5-Sterols are utilized at a higher rate than Δ^7-sterols. Saturated sterols and Δ^{25}-sterols are poor substrates.

Sterol glycosides (SG) and their 6′-acyl derivatives (ASG) are common constituents of higher plants (Lepage, 1968; Eichenberger & Grob, 1970; Méance & Dupéron, 1973) and until recently they have been regarded as transport or storage forms of sterols (Evans, 1972; Bush & Grunwald, 1972). However, the accumulating evidence (Eichenberger & Grob, 1970; Dupéron *et al.*, 1972; Wojciechowski, 1974) indicates that sterol glyco-sides are components of plant membrane structures, similarly as for a long time it has been assumed for free sterols. Recently a hypothesis has been advanced (Forsee *et al.*, 1974) that glycosylation of sterols incorporated into membranes results in a rapid modification of physico-

-chemical properties of membranes (e.g. of their permeability) due to changes in the interaction of polar groups of phospholipids with the hydroxyl groups of sterols. Enzymic glucosylation of sterols, with UDPG as the sugar donor, has been observed in homogenates and crude preparations of the cell organellae from several plants (Hou *et al.*, 1968; Kauss, 1968; Eichenberger & Newman, 1968; Péaud-Lenoel & Axelos, 1972; Wojciechowski, 1972; Forsee *et al.*, 1974). Data on localization of UDPG : sterol glucosyltransferase within the plant cell are widely divergent. According to Ongun & Mudd (1970), in spinach leaves this enzyme is associated with chloroplasts and in pea roots, with mitochondria. Kauss (1968) postulates that the structures involved in the synthesis of the cell wall polyglucans are the site of sterol glucosylation, while Lavirtmana & Cardini (1971) state that UDPG : sterol glucosyltransferase is one of the components of the enzyme complex bound to starch grains. Recently, Péaud-Lenoel & Axelos (1972) have obtained a partly purified preparation of this enzyme by solubilization of the microsomes from wheat roots. It is conceivable that the diversity of views on localization of this enzyme can — at least partly — be explained by insufficient purification of the subcellular fractions.

In our previous studies (Wojciechowski, 1972) it has been demonstrated that *Calendula officinalis* exhibits a very high activity of UDPG : sterol glucosyltransferase. The enzyme occurs in all organs of the plant during vegetation, attaining the highest activity in the young, developing tissues (Wojciechowski, 1974). This material has been used in our present studies on distribution of the enzyme and its properties, including substrate specificity.

MATERIALS AND METHODS

Chemicals. Tween 20 and Tween 60 were obtained from Schuchardt (Munich, G.F.R.). Tween 80, glucose-6-P (sodium salt), 2-mercaptoethanol, dithiothreitol and dipyridyl were from Koch-Light (Colnbrook, U.K.). Triton X-100, ATP, UMP, UDP, UTP, iodoacetic acid, o-phenanthroline, L-glutathione (reduced form), N-ethylmaleimide, L-cysteine· ·HCl, PCMB (p-hydroxymercuribenzoic acid, sodium salt) and INT [2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride] were from Serva (Heidelberg, G.F.R.). Lecithin (from marrow) was from Feinchemie (Berlin). The following sterols were used: cholesterol and 7-dehydrocholesterol from Calbiochem (Lucerne, Switzerland); stigmasterol, β-sitosterol, 5a-cholestanol and ergosterol from Koch-Light (Colnbrook, U.K.); lanosterol and dihydrolanosterol were obtained from a commercial preparation of lanosterol (Serva, Heidelberg, G.F.R.) by preparative t.l.c. separation using silica gel containing 10% AgNO$_3$ as an adsorbent and chloroform as a solvent. Cycloartenol, 24-ethylidenelo-

phenol and 7,22,25-stigmastatrienol were obtained from *Cucumis sativus* as described previously (Kintia & Wojciechowski, 1974). The purity of all sterols used was at least 96% as demonstrated by g.l.c. on SE-30 or OV-17 (Kintia & Wojciechowski, 1974). Only the commercial preparation of β-sitosterol contained about 12% of campesterol and traces of cholesterol. Uridine diphospho-D-[U-^{14}C]glucose, ammonium salt (spec. act. 261 mCi/mmole); uridine diphospho-D-[U-^{14}C]galactose, ammonium salt (spec. act. 260 mCi/mmole) and uridine diphospho-D-[^{14}C]glucuronic acid, ammonium salt (spec. act. 290 mCi/mmole) were purchased from the Radiochemical Centre (Amersham, U.K.).

Plant material. Seeds of *C. officinalis* var. Radio and of the other plants were germinated on filter paper moistened with distilled water, in a lumistat with 3000 lux illumination (16 h per day), at 24°C (day) and 16°C (night). Experiments were performed with the 2-week-old plants.

Preparation of subcellular fractions. The homogenization procedure of Janiszowska (1974) was used for preparation of subcellular fractions. The plant material (whole seedlings, embryo axes or cotyledons) was ground in a mortar for 20 sec at 0°C, in 0.1 M-Tris-HCl buffer (pH 8.5) containing 0.3 M-sucrose, with silica gel 30 - 70 mesh (Merck A. G., Darmstadt, G.F.R.). Per 1 g of the tissue, 0.5 g of silica gel and 3 ml of buffer were used. The homogenate was filtered through four layers of cheese-cloth, then it was successively centrifuged at 600 *g* (5 min), 3000 *g* (10 min), 15 000 *g* (20 min) and 105 000 *g* (1 h). The successive precipitated fractions were washed by resuspending in the initial volume of buffer and recentrifuged under the same conditions.

Centrifugation in sucrose-density gradient. The density gradients were prepared from sucrose solutions in the buffer appropriate for the particular enzymic assay: 0.1 M-Tris-HCl, pH 8.5, for UDPG : sterol glucosyltransferase; 0.1 M-phosphate, pH 7.4, for succinate dehydrogenase; and 0.1 M-maleate, pH 6.0, for glucose-6-phosphatase. In 5 ml centrifuge tubes, four 0.9 ml layers of sucrose solutions, differing in concentration by 0.3 M (0.9 - 1.8 M for the 15 000 *g* fraction of "mitochondria" and the 3000 *g* fraction of "chloroplasts", or 0.6 - 1.5 M for the 105 000 *g* fraction of "microsomes"), were successively layered. Gradients were allowed to set for 16 h before centrifugation. Immediately prior to centrifugation, 0.9 ml of the suspension of the respective fraction in 0.6 M-sucrose ("chloroplasts" and "mitochondria") or in 0.3 M-sucrose ("microsomes") were layered on the gradient. Samples were centrifuged at 105 000 *g* for 1 h using the Beckman Spinco ultracentrifuge. From the bottom of the tube nine fractions of 0.5 ml each were collected.

Enzyme solubilization. Subfractions 3 and 4 (from the bottom of the tube) obtained by the sucrose-density-gradient centrifugation of the 3000 - 15 000 *g* fraction were combined and diluted with 0.1 M-Tris-HCl

buffer, pH 8.5, to get a sucrose concentration of 0.3 M. To this suspension (0.112 mg protein per ml) Triton X-100 (10% aq. solution) was added to the final concentration of 0.3%. The mixture was kept in a Potter- -Elvehjem homogenizer with occasional stirring at 18°C, for 20 min. After centrifugation at 105 000 g (1 h) the pellet was discarded and the supernatant used for further experiments.

Determination of UDPG : sterol glucosyltransferase activity. The incubation mixture contained in a total volume of 2.2 ml: 2 ml of the suspension of the subcellular fraction (50 - 500 μg protein) in 0.1 M-Tris- -HCl buffer, pH 8.5, containing sucrose (0.3 M); 0.025 ml UDP-[^{14}C]glucose (0.9 nmole, 5.5×10^5 d.p.m.); 0.05 ml of sterol emulsion and other additions specified for each experiment. Incubation was carried out at 30°C for 10 - 30 min, depending on the activity of the preparation. The reaction was stopped by the addition of 3 ml of methanol and boiling for 3 min. Subsequently, 10 ml of *n*-butanol was added to the sample, and the butanolic extract was washed with water (5×5 ml). The extract was evaporated to dryness, dissolved in methanol and applied as 3 cm streaks to the plates coated with silica gel. Chromatograms were developed alternatively in *n*-propanol - ammonia - water (8:1:1, by vol.) or chloroform - methanol - water (62:7.5:2, by vol.) systems, dried and analysed by autoradiography, as previously described (Wojciechowski, 1972). The radioactive bands, with chromatographic mobility correspond- ing to that of the SG and ASG standards, were eluted with hot methanol, and their radioactivity was determined. Emulsions of sterols were prepared as described by Kaplan & Teng (1971) for triglycerides; 1 ml of the emulsion contained 62.5 mg of the detergent (Triton X-100 or Tween 20, 60 or 80), 20 mg of lecithin and 2.5 mg of sterol. If not indicated otherwise, β-sitosterol was emulsified with Triton X-100.

Radioactivity measurements. In the preliminary experiments on distribution of the enzyme in crude fractions, the radioactivity was measured with the aid of the thin-window G-M counter (efficiency about 6%), as previously described (Wojciechowski, 1972). In the sub- sequent experiments measurements were taken with the use of Packard scintillation spectrometer Tri-Carb (efficiency about 92%), using toluene containing 3% of PPO and 0.3% of POPOP as a scintillator.

Other methods. Protein was determined by the method of Lowry *et al.* (1951) and chlorophyll spectrophotometrically according to Arnon (1949). Succinate dehydrogenase, EC 1.3.99.1, used as a marker enzyme for mitochondria, was estimated according to Pennington (1961), as modified by Porteous & Clark (1965), and glucose-6-phosphatase, EC 3.1.3.9, a marker enzyme for microsomes, was determined as described by Hübscher & West (1965). EDTA and KF were included in the latter incubation mixture to inhibit non-specific phosphatases.

RESULTS AND DISCUSSION

Distribution of UDPG : sterol glucosyltransferase

In our experiments on subcellular localization of UDPG : sterol glucosyltransferase, the use was made of 2-week-old seedlings of *C. officinalis* which show high glucosyltransferase activity and at the same time, on account of the low content of fibrous structures, are a convenient source of intact cell organellae. Another advantage of this material consists in the relatively low concentration of endogenous sterols. In contrast to homogenates of mature leaves and flowers, in homogenates of the 2-week-old seedlings the rate of the synthesis of sterol [^{14}C]glucosides was about ten times higher in the presence of the added, emulsified β-sitosterol. Although the optimum pH for enzymic glucosylation is 7.6 (Wojciechowski, 1972), the enzyme was assayed at pH 8.5 to avoid additional incorporation of radioactivity (about 10%) into oleanolic acid glucosides as well as into mono- and digalactosyl-diglycerides, observed at pH 7.6. Formation of MGDG and DGDG undoubtedly resulted from the presence of UDPglucose 4-epimerase and of the appropriate galactosyltransferases in the homogenate. As it has been shown (Wojciechowski, 1972), sterol acylglucosides are secondary products obtained as a result of acylation of the previously formed sterol glucosides, endogenous acyl lipids being donors of the acyl moiety (Axelos & Péaud-Lenoel, 1971). Thus, radioactivity found in the *n*-butanolic extract of the samples incubated at pH 8.5 was a sufficiently accurate measure of the activity of UDPG : sterol glucosyl-transferase. This ruled out the need for using the time-consuming and inconvenient procedure for chromatographic isolation of SG and ASG from the butanolic extract and contributed to the accuracy of the enzymic assay.

Determination of glucosyltransferase activity of the crude subcellular fractions obtained by differential centrifugation of the homogenate demonstrated (Fig. 1) that both in the cotyledons and embryo axes most of the activity is localized in the "mitochondrial" fraction (3000 - - 15 000 *g*), less in the "chloroplast" fraction (600 - 3000 *g*), and still less in the "microsome" fraction (15 000 - 105 000 *g*). The supernatant obtained by centrifugation at 105 000 *g* showed only traces of the activity, which clearly indicates that the enzyme is exclusively bound to the membrane structures.

For comparative purposes, determination was made of the glucosyl-transferase activity of the subcellular fractions obtained by the same method from five other plants belonging to different families (Table 1). It was found that 2-week-old seedlings of all these plants exhibit rather high glucosyltransferase activity, and although it varied within
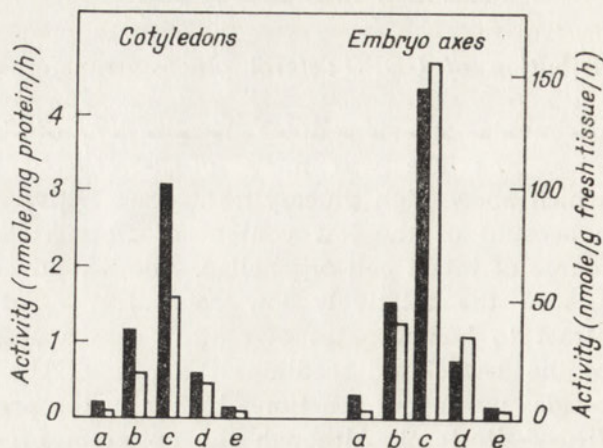
Fig. 1. UDPG : sterol glucosyltransferase activity in the crude subcellular fractions of the 2-week-old *C. officinalis* plants. Fractions sedimenting at: 600 *g* (*a*); 600 - 3000 *g* (*b*); 3000 - 15 000 *g* (*c*); 15 000 - 105 000 *g* (*d*), and the 105 000 *g* supernatant (*e*). The activity is expressed as nmoles of sterol glucosides formed: ■, per 1 mg protein/h, and □, per 1 g fresh tissue/h.

Table 1

Distribution of UDPG : sterol glucosyltransferase activity in the crude subcellular fractions from 2-week-old seedlings of various plants

The results are expressed as nmoles/mg protein/h.

Plant	Fraction sedimenting at (*g*)				Supernatant at 105 000 *g*
	600	600—3000	3000——15 000	15 000——105 000	
Helianthus annuus (Compositae)	0.01	0.17	0.42	0.13	0.01
Sinapis alba (Cruciferae)	0.03	0.19	0.92	0.22	0.01
Triticum aestivum (Graminae)	0.10	0.22	0.41	0.24	0.01
Beta vulgaris (Chenopodiaceae)	0.03	0.07	2.01	1.58	0.02
Pisum sativum (Papilionaceae)	0.19	0.82	1.25	1.47	0.02

a fairly large range, it was always found in the membrane fractions. In sunflower, white mustard, sugar beet and wheat the major part of the enzyme was associated, similarly as in *C. officinalis*, with the fractions sedimenting from 600 to 15 000 *g*. In pea somewhat higher

activity (calculated per mg of protein) was observed in the microsomal fraction. It is noteworthy that even in the absence of exogenous sterol the radioactivity in the butanolic extract of the homogenates incubated with UDP-[^{14}C]glucose was localized mainly in SG and ASG. In sunflower, wheat and white mustard virtually all the radioactivity was found, under the conditions applied, in sterol glucosides. In sugar beet and pea the overall radioactivity of SG and ASG accounted, respectively, for 77 and 82% of the total radioactivity; the remaining part of the radioactivity of the butanolic extract was associated with unidentified, more polar substances.

To locate the glucosyltransferase activity in a more unequivocal and precise manner, the fractions obtained were purified further by centrifugation in sucrose-density gradient and their homogeneity checked by determination of the marker enzymes. It is known that in plant cells distribution of succinate dehydrogenase is confined to mitochondria, and that of glucose-6-phosphatase to microsomes (Harwood & Stumpf, 1970). The results presented in Fig. 2 indicate that distribution of
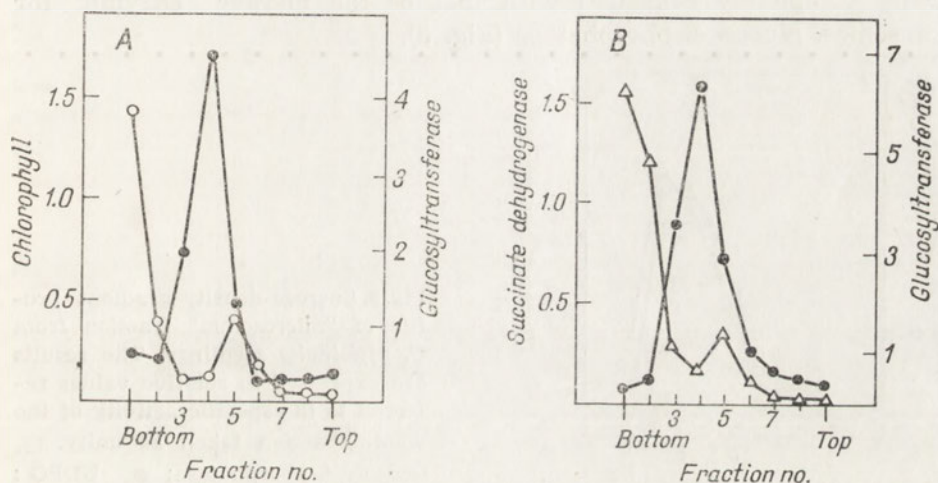
Fig. 2. Sucrose-density-gradient profiles of "chloroplast" (*A*) and "mitochondrial" (*B*) fractions from *C. officinalis* seedlings. The results are expressed in relative values referred to the specific activity of the whole fraction taken as unity. ○, Chlorophyll; ●, UDPG : sterol glucosyltransferase; △, succinate dehydrogenase.

UDPG : sterol glucosyltransferase activity in the subfractions obtained by centrifugation of the "chloroplast" and "mitochondrial" fractions is not consistent with the localization of chlorophyll and succinate dehydrogenase, respectively. The main peak of either chlorophyll or succinate dehydrogenase activity corresponded to subfraction *1,* i. e. to the whole chloroplasts or mitochondria. The other peak in both cases was associated with subfraction *5* which contained probably

smaller fragments of these structures. Subfractions 3 and 4 separated from mitochondria and chloroplasts on sucrose-gradient centrifugation showed the highest UDPG : sterol glucosyltransferase activity. These fractions did not contain fragments of the cell wall, since the 600 g fraction containing (according to microscopic examination) a substantial amount of the large cell wall fragments, exhibited only slight glucosyltransferase activity (Fig. 1). It might be, however, that the fractions obtained involved fragments of the Golgi apparatus which, as it has been recently suggested, participates in the syntheses of some complex saccharides by glycosyltransferases utilizing nucleotides as a source of sugar residues (Ray et al., 1969; Jilha et al., 1972). Our results are in agreement with the very recent observations of Forsee et al. (1974) who showed that UDPG : sterol glucosyltransferase of cotton seeds was confined to the structures involved in the synthesis of insoluble polyglucan. Our results showed, however, that in the C. officinalis seedlings a part of the enzymic activity is associated with the microsomal fraction. The peak of UDPG : sterol glucosyltransferase activity completely coincided with that of the marker enzyme for microsomes, glucose-6-phosphatase (Fig. 3).
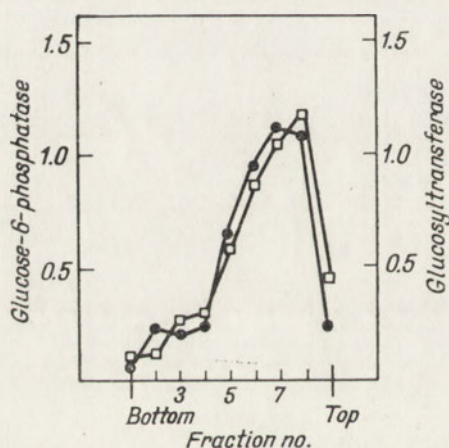
Fig. 3. Sucrose-density-gradient profile of "microsomal" fraction from C. officinalis seedlings. The results are expressed in relative values referred to the specific activity of the whole fraction taken as unity. □, Glucose-6-phosphatase; ●, UDPG : : sterol glucosyltransferase.

Isolation and purification of UDPG : sterol glucosyltransferase

Triton X-100, Tween 20 and Tween 80 stimulated at low concentration the enzymic activity in the particulate fraction sedimenting at 3000 - 15 000 g (Fig. 4), probably due to partial loosening of the membrane structures. Tween 60 was not effective. The solubilizing effect was the most pronounced with Triton X-100 (at concentration of 0.3%) and consequently this detergent was chosen in our attempts to liberate the enzyme from the membranes. On 20 min treatment of the 3000 - 15 000 g fraction with 0.3% Triton X-100 at 18°C, about 40%

Fig. 4. Effect of detergents on UDPG : sterol glucosyltransferase activity in the 15 000 g fraction from *C. officinalis* seedlings. The incubation mixtures contained 326 μg of protein; for other details see Materials and Methods.
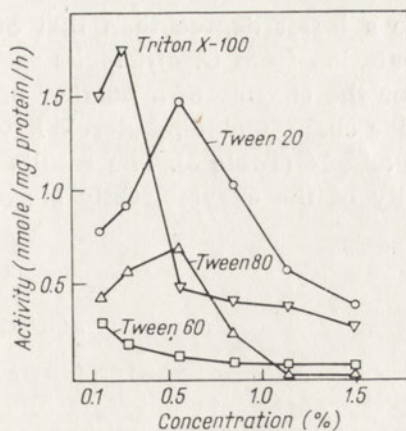
of the enzymic activity was recovered in the supernatant obtained upon centrifugation at 105 000 g for 1 h (Table 2). However, further attempts to fractionate the solubilized material with the use of cold acetone or ammonium sulphate resulted in nearly complete inactivation of the preparation. The procedure used, as shown in Table 2, enabled an about 70-fold purification of the enzyme with a yield of about 25%. The obtained soluble preparation exhibited only very low activity if no exogenous sterol was added; whereas in the presence of β-sitosterol added the rate of SG formation was about 40 times higher.

Table 2

Partial purification of UDPG : sterol glucosyltransferase activity from C. officinalis *seedlings*

The activity was determined in the presence of Triton X-100.

Fraction	Total protein (mg)	Total activity (nmole/h)	Specific activity (nmole/mg protein/h)
Homogenate	152	31.9	0.21
3000 - 15 000 *g* pellet	8	24.8	3.10
Subfractions *3 - 4* from sucrose-density gradient	1.3	16.8	12.96
105 000 *g* supernatant after treatment of subfractions *3 - 4* with 0.3% Triton X-100	0.6	8.3	13.88

Substrate specificity of UDPG : glucosyltransferase. Data on the substrate specificity of this glucosyltransferase are scarce. It has been reported by Péaud-Lenoel & Axelos (1972) that this enzyme from wheat roots catalysed glucosylation of stigmasterol and cholesterol although

to a lesser degree than that of sitosterol. Using a partly purified preparation from *C. officinalis* seedlings, we have investigated the affinity for the enzyme of a number of sterols differing in the structure of the side chain, including sterols having additional methyl groups at positions 4 and 14 (Table 3). The results obtained pointed to a rather low specificity of the enzyme. Only sterols containing one or two methyl groups

Table 3

Specificity of UDPG : sterol glucosyltransferase

Incubation mixtures contained the 15 000 *g* fraction (326 µg of protein) from *C. officinalis* seedlings. Sterols were added as emulsions with Triton X-100 and lecithin (see Materials and Methods). The control incubation mixture contained Triton X-100 and lecithin in the same concentration as in samples to which emulsified sterol was added.

Sterol added	Sterol glucoside formed (nmole/mg protein/h)
Control, without sterol	0.45
Lanosterol (C_{30}, $\Delta^{8,24}$)	0.47
Cycloartenol (C_{30}, Δ^{24})	0.46
24-Ethylidenelophenol (C_{30}, $\Delta^{7,24\,(28)}$)	0.46
Dihydrolanosterol (C_{30}, Δ^{8})	0.45
5α-Cholestanol (C_{27})	0.70
Δ^{7}-Cholestanol (C_{27}, Δ^{7})	1.02
Cholesterol (C_{27}, Δ^{5})	2.21
7-Dehydrocholesterol (C_{27}, $\Delta^{5,7}$)	0.89
Ergosterol (C_{28}, $\Delta^{5,7,22}$)	2.42
Brassicasterol (C_{28}, $\Delta^{5,22}$)	1.35
Stigmastanol (C_{29})	0.73
β-Sitosterol (C_{29}, Δ^{5})	3.30
Stigmasterol (C_{29}, $\Delta^{5,22}$)	3.19
α-Spinasterol (C_{29}, $\Delta^{7,22}$)	3.00
7,22,25-Stigmastatrienol (C_{29}, $\Delta^{7,22,25}$)	0.75

at position 4 (i. e. lanosterol, cycloartenol, 24-ethylidenelophenol and dihydrolanosterol) were not glucosylated. The rate of glycosylation was the highest with the sterols having a double bond in ring B, moreover Δ^{5}-sterols showed higher affinity for the enzyme than Δ^{7}-sterols. The saturated sterols (stanols) were glucosylated at a low rate. Among sterols having the same ring systems but differing in the number of carbon atoms in the side chain (e.g. cholesterol — β-sitosterol; 7-dehydrocholesterol — ergosterol), the sterols containing an additional alkyl group at position 24 were glucosylated much more effectively. The presence of a double bond at position 22 (β-sitosterol — stigmasterol) seemed to attenuate the affinity for the enzyme, whereas the double bond at position 25 exerted a pronounced adverse effect (α-spinasterol

— $\Delta^{7,22,25}$-stigmastatrienol). It is noteworthy that among the investigated sterols β-sitosterol and stigmasterol were glucosylated the most readily. These two most common plant sterols seem to prevail in the sterol glucoside fractions even in those plants in which other sterols predominate in the free sterol fraction (Kintia & Wojciechowski, 1974).

It was demonstrated by means of chromatography and autoradiography that the compounds exhibiting chromatographic properties of sterol glucuronosides were not synthesized on incubation of the purified enzyme preparation with β-sitosterol in the presence of UDP-[^{14}C]glucuronate. In the presence of UDP-[^{14}C]galactose, only negligible incorporation of ^{14}C into the sterol glycoside fraction was observed (about 2%, as compared with UDP-[^{14}C]glucose). It was found, however, by paper chromatography that even in this case glucose was the only radioactive product obtained upon acid hydrolysis of the sterol glycoside. This indicates that utilization of UDPgalactose is preceded by epimerization of UDPgalactose to UDPglucose by UDPgalactose 4-epimerase present in the enzyme preparation.

The effect of metal ions on UDPG : sterol glucosyltransferase activity is illustrated in Fig. 5A. It is evident that Mg^{2+} and Ca^{2+} within the concentration range of 10^{-4} - 10^{-2} M slightly enhanced this activity,

Fig. 5. Effect of A, metal ions, and B, nucleotides, on the activity of UDPG : sterol glucosyltransferase preparation, partially solubilized with Triton X-100. All metals were added as chlorides. The content of enzyme protein was 31 μg in A, and 42 μg in B.

Co^{2+} exerted a slight inhibitory effect, whereas Hg^{2+} and Zn^{2+} strongly inhibited the enzyme. The effect of other compounds on UDPG : sterol glucosyltransferase is presented in Table 4. PCMB, which is known to bind with SH groups, was a strong inhibitor. The requirement for SH groups was confirmed by the marked stimulation of the activity of glucosyltransferase by 2-mercaptoethanol and dithiothreitol. Inconsistent

with this is but a slight inhibitory effect of iodoacetic acid, a reagent alkylating the SH groups. Other reducing and chelating agents listed in Table 4 were practically ineffective.

Table 4

Effect of reducing and chelating compounds on UDPG : sterol glucosyltransferase

Incubation mixtures contained partially solubilized enzyme preparation (31 μg of protein).

Compound added	Concentration (M)	Stimulation (+) or inhibition (−) (%)
p-Chloromercuribenzoate	10^{-4}	−88
	10^{-3}	−94
Iodoacetic acid	10^{-4}	0
	10^{-3}	−3
N-Ethylmaleimide	10^{-4}	−3
	10^{-3}	−21
Dithiothreitol	10^{-3}	+32
	10^{-2}	+30
2-Mercaptoethanol	10^{-3}	+95
	10^{-2}	+70
Glutathione	10^{-3}	+5
	10^{-2}	+2
Ascorbic acid	10^{-3}	+5
	10^{-2}	+12
Cysteine·HCl	10^{-3}	+7
	10^{-2}	−3
Sodium hydrosulphite	10^{-3}	+7
	10^{-2}	+19
Dipyridyl	10^{-3}	+21
EDTA	10^{-3}	+12
o-Phenanthroline	10^{-3}	+8

UTP and UDP strongly inhibited the synthesis of sterol glucosides at concentrations from 10^{-5} to 10^{-3} M (Fig. 5B). The inhibitory effect of UDP is understandable since UDP is one of the products of the reaction catalysed by glucosyltransferase. As concerns UTP, is was demonstrated that its effect was not due to extensive contamination of the commercial UTP preparation with UDP since the concentration of the latter did not exceed 4%.

UMP exerted a pronounced stimulatory effect within the concentration range of 10^{-5} - 10^{-4} M (Fig. 5B). It is possible that the effect observed may be of importance for the regulation of the enzyme activity *in vivo;* however, more information is required to elucidate the mechanism underlying the effect of uridine nucleotides. The slight inhibitory effect of ATP observed over the whole concentration range studied, is at variance with the previous reports on its stimulatory effect on the

activity of UDPG : sterol glucosyltransferase in crude enzymic fractions from tobacco plants (Bush & Grunwald, 1974), soya-bean seeds (Hou *et al.*, 1968) and homogenates of *C. officinalis* leaves (Wojciechowski, 1972). These apparently contradictory results obtained with crude preparations are probably not due to activation of the glucosyltransferase but rather to protection against hydrolysis of UDP-[^{14}C]glucose, or to enzymic resynthesis of UDP-[^{14}C]glucose degraded by the enzymes present in the crude preparations.

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WEWNĄTRZKOMÓRKOWA LOKALIZACJA I NIEKTÓRE WŁASNOŚCI GLUKOZYLOTRANSFERAZY UDPG : STEROL Z *CALENDULA OFFICINALIS*

Streszczenie

Glukozylotransferaza UDPG : sterol jest zlokalizowana w dwutygodniowych siewkach *C. officinalis* w strukturach błonowych, różnych od chloroplastów i mitochondriów, a zawierających prawdopodobnie fragmenty aparatu Golgi'ego. Mniejsza część aktywności enzymu związana jest z frakcją mikrosomów. Szereg syntetycznych detergentów stymuluje aktywność związanego z błonami enzymu, powodując jego częściowe upłynnienie. Preparat enzymatyczny oczyszczony około 70-krotnie jest silnie hamowany przez $HgCl_2$ i *p*-chlorortęciobenzoesan, jest natomiast wyraźnie stymulowany przez merkaptoetanol i dwutiotreitol, jak również, w mniejszym stopniu, przez jony Mg^{2+} i Ca^{2+} oraz niektóre odczynniki chelatujące i redukujące. UMP stymuluje, natomiast UDP i UTP silnie hamują aktywność enzymu. Enzym nie działa na 4-metylosterole, jednak wykorzystuje szereg 4-demetylosteroli. Wydaje się, że obecność podwójnego wiązania w pierścieniu B podnosi powinowactwo substratu do enzymu. Δ^5-sterole wykorzystywane są z większą szybkością niż Δ^7-sterole. Sterole nasycone i Δ^{25}-sterole wykorzystywane są w nieznacznym stopniu.

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THE EFFECT OF CHLORIDE AND PERCHLORATE ANIONS ON THE ACTIVITY OF NATIVE AND ACETYLATED RIBONUCLEASE A

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The highly chaotropic ClO_4^- ion at increasing concentration stimulated the activity of native RNase A, and inhibited the acetylated enzyme preparation. Cl^- ion had a similar but much smaller effect. The difference in the effects of these two ions on the native and modified RNase is discussed.

Chaotropic properties of certain ions are related to their ability to disrupt the relatively weak hydrophobic and hydrogen bonds of macromolecules. The strong effect of chaotropic anions, and especially of perchlorate, on the enzymic activity has been demonstrated in the case of pyrophosphate phosphatase from *Vibrio alginolyticus* (Unemoto *et al.*, 1973).

The aim of the present work was to study the effect on ribonuclease A of NaCl and $NaClO_4$ which are known to differ widely in their chaotropic properties. The activity of RNase A is largely dependent on ionic strength (Dickman & Ring, 1958; Kalnitsky *et al.*, 1959); low ionic strength stimulates, and high ionic strength inhibits the enzyme activity. The maximum effect of ionic strength is pH-dependent.

So far, the influence of ionic strength on the activity of acetylated RNase has not been examined in detail. The presented experiments were carried out on the assumption that since acetylation is known to expose hydrophobic groups and lower the positive charge on the modified protein (Leach & Boyd, 1973), the non-chaotropic chloride ion, the chaotropic perchlorate ion, and ionic strength would influence in different ways the activity of native and acetylated RNase.

MATERIALS AND METHODS

Reagents. The reagents used were from the following sources: Ox pancreas ribonuclease A, Worthington Biochem. Corp. (Freehold, N. J., U.S.A.); yeast RNA, sodium salt, acrylamide, and 2,4,6-trinitrobenzene-sulphonic acid, B. D. H. Ltd (Poole, Dorset, England); Amido Black 10 B, Merck (Darmstadt, G. F. R.); *N,N'*-methylenediacrylamide, Koch-Light Lab. (Colnbrook, Bucks., England); *N,N,N',N'*-tetramethyleneethylenedi-amine, Eastman Kodak (New York, N. Y., U.S.A.); uranyl acetate, Chemapol (Prague, Czechoslovakia); gelatin, Difco (Detroit, Mich., U.S.A.). Other reagents were analytical grade products from P.O.Ch. (Gliwice, Poland).

Acetylation of RNase was performed by the method described by Morris *et al.* (1971), in methanolic solution of acetic acid anhydride at $4°C$. Under these conditions only free basic amino groups are acetylated, whereas hydroxyl groups of serine, threonine and tyrosine do not undergo acetylation. The degree of acetylation was controlled by changing the concentration of acetic anhydride and the reaction time. The decrease in free amino groups was measured by the method of Habeeb (1966).

Polyacrylamide-gel electrophoresis was performed according to Panyim & Chalkley (1969) using 15% gel.

Ribonuclease activity was determined according to Anfinsen *et al.* (1954) at pH 5.0 (0.04 M-acetate buffer) and at pH 7.8 (0.028 M-Tris-HCl buffer). The reaction was carried out for 10 min at $37°C$. When RNase activity was determined at pH 7.8, the enzyme was protected with dilute gelatin solution. One unit of RNase activity is defined as that amount of the enzyme which gave an increase in extinction at 260 nm of 0.1 per 10 min at $37°C$. The ratio of RNase activity at pH 7.8 and pH 5.0 is further referred to as the pH 7.8 : pH 5.0 activity ratio, and the ratio of activity in the presence of chloride and perchlorate ions, as the $Cl^- : ClO_4^-$ activity ratio.

Protein was determined at 278 nm a Carl Zeiss (Jena, G. D. R.) VSU-2P spectrophotometer with a 1 cm light-path.

RESULTS

Ribonuclease A acetylated in 70% retained about 20% of the initial activity measured at pH 5.0 and 0.04 ionic strength. On polyacrylamide-gel electrophoresis at pH 2.7 this preparation, unlike native RNase which gave a single band, separated into three distinct and two faint bands, which all migrated much slower than the native RNase. The RNase acetylated in 90%, which possessed only trace activity, on poly-acrylamide gel gave one distinct and three faint bands. Neither of the

preparations of acetylated RNase gave even a faint band with the mobility corresponding to that of native RNase (Fig. 1).

The activity of native RNase increased with ionic strength, irrespective whether ClO_4^- or Cl^- was used, the effect of perchlorate at pH 5.0 being somewhat higher (Fig. 2A). The activity of RNase at pH 7.8 was much higher than at pH 5.0. The pH 7.8 : pH 5.0 activity ratio (Table 1) decreased with increasing ionic strength both in the presence of Cl^- and ClO_4^-.
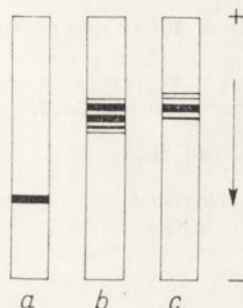

Fig. 1. Polyacrylamide-gel electrophoresis of ribonuclease: *a*, native; *b*, acetylated in 70%; *c*, acetylated in 90%. To the gel 20 μg of native RNase, or 70 μg of acetylated RNase, was applied.
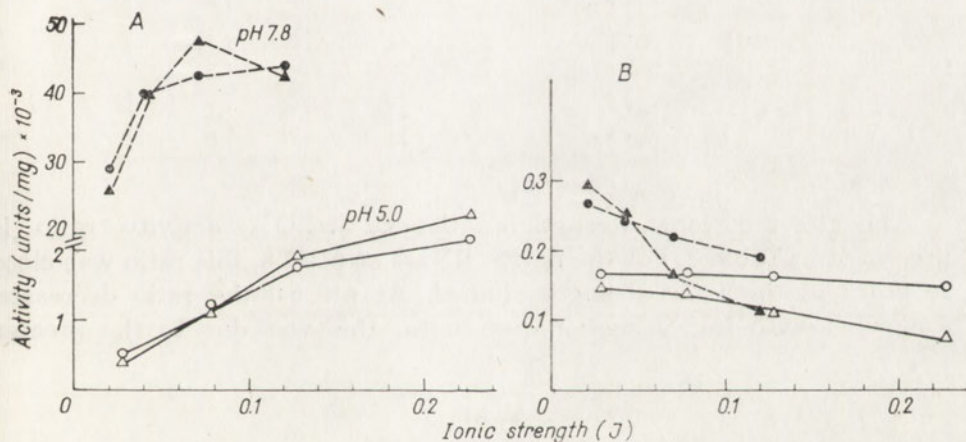

Fig. 2. The effect of: ○, ●, NaCl and △, ▲, $NaClO_4$ concentration on the activity of: *A*, native RNase, and *B*, RNase acetylated in 70%. Filled-in symbols, activity at pH 7.8; outlined symbols, activity at pH 5.0. The ionic strength values were calculated taking into account also the ionic strength of the buffer.

The effect of sodium chloride and sodium perchlorate on the activity of the modified enzyme was studied using the preparation acetylated in 70%, which showed rather high activity despite the high degree of acetylation. The effect of ionic strength on the activity at pH 7.8 and 5.0 is presented in Fig. 2B. At low ionic strength, the activity at these two pH values differed but little, and became almost equal at higher

ionic strength. At pH 7.8 the increase in ionic strength of either salt inhibited the enzyme activity; at pH 5.0 the activity did not change with changes in NaCl concentration, but was somewhat inhibited by higher concentration of $NaClO_4$. At 0.03 ionic strength, the pH 7.8 : pH 5.0 activity ratio was 1.6 with sodium chloride and 1.9 with sodium perchlorate; at higher ionic strength in both cases this ratio decreased approaching unity (Table 1).

Table 1

Effect of ionic strength of NaCl *and* $NaClO_4$ *on the activity of native and acetylated ribonuclease A, at* pH *7.8 and 5.0*

The activity ratios were calculated from the diagrams presented in Fig. 2A,B.

Ionic strength (*I*)		pH 7.8:pH 5.0 activity ratio	
		native RNase	acetylated RNase
0.03	NaCl	63	1.6
	$NaClO_4$	74	1.9
0.05	NaCl	51	1.4
	$NaClO_4$	59	1.6
0.07	NaCl	41	1.3
	$NaClO_4$	46	1.2
0.12	NaCl	27	1.1
	$NaClO_4$	24	1.0

The effect of ionic strength on the $Cl^- : ClO_4^-$ activity ratio is presented in Table 2. For the native RNase at pH 7.8, this ratio was close to unity at the ionic strength studied. At pH 5.0 the ratio decreased somewhat with increasing ionic strength; this was due to the greater

Table 2

Effect of ionic strength on the activity of native and acetylated ribonuclease A

The activity ratios were calculated from the diagrams presented in Fig. 2A,B.

Ionic strength (*I*)	$Cl^- : ClO_4^-$ activity ratio			
	native RNase		acetylated RNase	
	at pH 7.8	at pH 5.0	at pH 7.8	at pH 5.0
0.03	1.1	1.3	0.9	1.1
0.05	1.0	1.1	1.0	1.2
0.07	0.9	1.0	1.3	1.2
0.12	1.0	0.9	1.7	1.4
0.22	—	0.9	—	2.0

stimulating effect of perchlorate at this pH value. The anionic activity ratio for the modified RNase was doubled, both at pH 7.8 and 5.0, at higher ionic strength; this was due to greater inhibition by perchlorate than by chloride.

DISCUSSION

The presented experiments showed that higher concentrations of perchlorate exert a stronger influence on RNase activity than the corresponding concentrations of chloride. In the case of native RNase, perchlorate has a somewhat enhancing effect, whereas it inhibits distinctly the acetylated enzyme. The inhibition of modified RNase is probably due to the change in the spacial arrangement of hydrophobic groups of the enzyme molecule, which may be considerably more exposed to the action of chaotropic anions that the hydrophobic groups of native RNase. It seems that the effects of chaotropic ions and ionic strength are additive, as higher concentrations of NaCl also have an inhibitory, although less pronounced, effect. The observed different effect of ionic strength on native and acetylated RNase could be also due to a change in the enzyme charge, which in consequence would lead to a considerable weakening of electrostatic interactions within the enzyme-substrate or enzyme-product complex.

The effect of ionic strength on ribonuclease activity was assumed by Dickman & Ring (1958) to be due to the "increased dissociation of certain acidic groups of RNA". Our results on native and modified RNase indicate that the effect of ionic strength concerns rather the enzyme molecule and its electrostatic interaction with the polyanionic substrate.

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WPŁYW ANIONÓW CHLORKOWYCH I NADCHLORANOWYCH NA AKTYWNOŚĆ RYBONUKLEAZY A PRZED I PO ACETYLACJI

Streszczenie

Wzrastające stężenia silnie chaotropowego anionu ClO_4^- podwyższają aktywność natywnej RNazy i obniżają aktywność acetylowanego enzymu. Anion Cl^- działa podobnie ale wyraźnie słabiej. Przedyskutowano różnice w działaniu tych dwóch anionów na natywny i modyfikowany enzym.

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AN *IN VIVO* AND *IN VITRO* PHOSPHORYLATION OF YEAST RIBOSOMAL PROTEINS *

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Phosphorylation of yeast ribosomal proteins has been demonstrated *in vivo* and *in vitro*. ^{32}P-labelled product represents an ester-linked class of phosphoprotein. Acrylamide-gel electrophoresis has shown that in both types of experiments radioactive proteins migrate similarly; this might indicate that closely related groups of proteins become phosphorylated *in vivo* and *in vitro*. In the presence of [^{32}P] ATP the amount of covalently bound phosphate was 1.0 - 1.2 moles/mole of ribosome. The phosphorylation of ribosomal proteins did not appreciably affect the activity of ribosomes in a cell-free protein-synthesizing system containing poly(U) and elongation factors.

Mammalian ribosomal proteins are phosphorylated *in vivo* and *in vitro* by protein kinases bound to ribosomes or free in the cytosol (Loeb & Blat, 1970; Kabat, 1970, 1971; Eil & Wool, 1971; Bitte & Kabat, 1972; Walton & Gill, 1973). Most of the kinases require cyclic AMP for activation. The enzymes transfer the γ-phosphoryl group of ATP to serine and threonine residues of ribosomal proteins. It has been shown recently that an enzyme present in rabbit reticulocyte supernatant (Traugh *et al.*, 1973) and rat liver cytosol (Ventimiglia & Wool, 1974) uses GTP as a phosphate donor to phosphorylate several ribosomal proteins.

The number of individual proteins which can be phosphorylated *in vitro* varies with the degree of purification of ribosomes (Stahl *et al.*, 1972; Traugh *et al.*, 1973). It also depends on the compactness of ribo-

some structure (Kabat, 1972; Stahl *et al.*, 1972). The intact purified ribo-
somes are phosphorylated to a lesser extent than unfolded ribosomes
or isolated ribosomal proteins (Stahl *et al.*, 1972). Different phosphoryla-
tion patterns have also been observed *in vivo* for polysomes, monosomes
and native ribosomal subunits (Kabat, 1970, 1972).

The aim of this paper was to study the *in vivo* and *in vitro* phospho-
rylation of yeast ribosomal proteins and to test to what extent the
phosphorylation affects the activity of ribosomes in a cell-free protein-
-synthesizing system.

MATERIAL AND METHODS

Saccharomyces cerevisiae cultures. Low-phosphate medium of Retel
& Planta (1967) containing [^{32}P]orthophosphate (100 μCi of ^{32}P/ml) was
used for *in vivo* labelling of ribosomal proteins. For all other ex-
periments yeasts were cultivated in a medium described by Cooper *et
al.* (1962). Yeast cells grown aerobically were harvested at log phase
of growth, centrifuged and washed twice with a standard buffer solution
(50 mM-Tris-HCl, pH 7.5, 10 mM-KCl, 10 mM-Mg^{2+} and 6 mM-mercapto-
ethanol). The cells were used immediately or stored frozen at −15°C
before use.

Ribosomal preparations. Cell-free yeast extracts and crude ribosomes
were prepared as described by Grankowski *et al.* (1974). The Triton
X-100 treated ribosomes were prepared in the following way: Ribosomal
pellets were suspended in the standard buffer and gently homogenized
in a teflon-glass homogenizer. Any insoluble material was removed by
low-speed centrifugation and to the resulting supernatant Triton X-100
was added with continuous stirring to the final concentration of 1%.
Then, ribosomes were sedimented by 2 h centrifugation at 150 000 *g*.
These ribosomes retained almost whole protein kinase activity and
served as starting material for the isolation of this enzyme; they were
also used for *in vitro* phosphorylation. They are further referred to as
Triton-treated ribosomes.

Purified ribosomes were prepared by twice washing the Triton-X-100
treated ribosomal preparation with buffered KCl solution (50 mM-Tris-
-HCl, pH 7.0, 0.5 M-KCl, 50 mM-Mg^{2+} and 6 mM-mercaptoethanol)
followed by ultracentrifugation. This preparation is further referred
to as purified ribosomes.

Purity of ribosomes was tested by the enzymic method of Gąsior &
Moldave (1965); it showed that they contained no elongation factors,
but were highly active at 10 mM-Mg^{2+} in the presence of partly purified
transferases. The lack of activity of purified ribosomes at low Mg^{2+}
(2 - 3 mM) in the presence of elongation factors indicated that such
preparations did not contain polypeptide chain initiation activity.

Isolation of protein kinase. Triton X-100 treated ribosomes were extracted twice with 0.5 M-KCl in Tris-HCl, pH 7.5. Supernatants were collected and processed as described by Grankowski *et al.* (1974); the hydroxylapatite fraction was used as source of the enzyme.

Isolation of elongation factors. From a ribosome-free supernatant, a partly purified fraction containing both elongation factors was isolated by the procedure of Ayuso & Heredia (1967).

Phosphorylation of ribosomal proteins in vitro. The incubation mixture contained: 20 mM-Tris-HCl, pH 7.5, 10 mM-Mg^{2+}, 6 mM-mercaptoethanol, 0.1 - 0.5 mg of Triton X-100 treated ribosomes containing endogenous protein kinase, or 0.1 - 0.2 mg of purified ribosomes, 5 - - 10 μg of protein kinase where indicated and 20 nmoles of [γ-^{32}P]ATP (spec. act. 3 - 12 × 10^4 c.p.m./nmole). The samples in the total volume of 0.5 ml were incubated at 30°C for 10 min unless otherwise stated.

Analysis of phosphoproteins. The suspension of ribosomes labelled *in vivo* or the whole incubation mixture after the *in vitro* phosphorylation was adjusted to 10% trichloroacetic acid (TCA) at 0°C to precipitate macromolecules. The samples were then heated at 90 - 95°C for 20 min and filtered through Millipore filter (0.45 μ), the precipitates were thoroughly washed with 5% TCA and then with a mixture of methanol - - chloroform (3:1, v/v; Kurek *et al.*, 1972b). Filters were dried and counted on a window G-M counter. Radioactive amino acids were identified by paper electrophoresis of acid hydrolysate of the protein sample previously freed of nucleic acid and phospholipid contaminants. Hydrolysis was carried out for 10 h with 2 M-HCl in sealed ampoules at 105°C. Approximately 200 μg of protein hydrolysate was applied onto Whatman 3 *MM* paper. High-voltage electrophoresis was run at 40 - 50 V/cm and a current of 25 mA, with an electrode buffer essentially as described by Kabat (1970). Molybdate test was used for the detection of the orthophosphate and phosphate-containing compounds. Phosphoserine was detected by ninhydrin test. Radioactivity of the electrophoretogram strips was scanned on a G-M counter.

The phosphoproteins were also identified using alkaline phosphatase which is known to hydrolyse the ester-linked phosphate.

Isolation of ribosomal proteins. Proteins were isolated from both the *in vivo* and *in vitro* labelled ribosomes according to the procedure of Spitnik-Elson (1965), using 4 M-LiCl and 8 M-urea mixture for protein extraction. Isolated proteins were freed of nucleic acid contamination as described by Kurek *et al.* (1972a).

Acrylamide-gel electrophoresis. Ribosomal proteins were separated by electrophoresis in 7.5% polyacrylamide gel according to the method of Leboy *et al.* (1964). Electrophoresis was run in glass tubes (5 × 70 mm) at 4 mA/tube. To the gel 100 μg of protein was applied and stained with 1% Amido Black in 7.5% acetic acid. For counting of radioactivity,

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gels were sliced into 1 mm transverse sections, these were dried and counted on a window G-M counter.

Assay of the in vitro *protein synthesis.* The incubation mixture contained in the final volume of 0.25 ml: 100 mM-Tris-acetate buffer, pH 6.5, 60 mM-NH_4Cl, 0.4 mM-GTP, approximately 200 μg of purified, phosphorylated or control ribosomes, 25 μg of poly(U), 20 - 30 μg of [^{14}C]phenylalanyl-tRNA (8000 - 12 000 c.p.m./sample), 50 - 80 μg of elongation factors protein, and Mg^{2+} as indicated. The samples were incubated for 20 min at 30°C and then processed for counting as described by Gąsior & Moldave (1965). A mixture of 4 g of PPO and 50 mg of POPOP per 1 litre toluene was used for counting in Packard scintillation spectrometer.

Other methods. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard, and RNA was measured spectrophotometrically.

Reagents. PPO (2,5-diphenyloxazole), POPOP [1,4-bis(5-phenylo-xazolyl)benzene], alkaline phosphatase from calf intestinal mucosa and Tris were purchased from Koch-Light Lab. (Colnbrook, Bucks, England); acrylamide and bis-acrylamide from British Drug Houses (Poole, Dorset, England) and hydroxylapatite from Bio-Rad Lab. (Richmond, Calif., U.S.A.); N,N,N',N'-tetramethylethylenediamine from Fluka A.G. (Buchs, Switzerland). Mercaptoethanol, Triton X-100 were the products of Serva Feinbiochemica (Heidelberg, G.F.R.). [γ-^{32}P]ATP (spec. act. 13 - - 16 Ci/mmole) was purchased from the Radiochemical Centre (Amersham, England), [^{14}C]phenylalanine (spec. act. 270 mCi/mmole) was supplied by the Institute for Research, Production and Application of Radioisotopes (Prague, Czechoslovakia), and [^{32}P]orthophosphate was from Nuclear Research Institute, Warsaw, Poland. GTP was from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

RESULTS

[^{32}P]*Phosphate incorporation into ribosomal proteins*

Yeast ribosomal proteins, like ribosomal proteins of animal tissues, were phosphorylated both *in vivo* and *in vitro* (Table 1). The isolated crude ribosomal preparations contained some ^{32}P-radioactive non--ribosomal contaminants which could be removed by washing with buffered 0.5 M-KCl solution. The resulting purified particles contained approximately 80% (expt. *in vivo*) and 76% (expt. *in vitro*) of the total radioactivity found in Triton X-100 treated ribosomal preparations. On the basis of the widely accepted criteria of the ribosome purity (Eil & Wool, 1973a) we can consider the ^{32}P-labelled proteins isolated from purified ribosomes as the structural proteins of the ribosome.

Table 1

Radioactivity of proteins isolated from yeast ribosome preparations labelled in vivo *and* in vitro

For the *in vitro* phosphorylation, Triton X-100 treated ribosomes, containing endogenous protein kinase activity, were used. The ribosomal preparation and ribosomal proteins were isolated as described under Materials and Methods. Spec. act. of [^{32}P]orthophosphate used for *in vivo* labelling was several-fold lower than the spec. act. of [γ-^{32}P]ATP used for *in vitro* phosphorylation.

Ribosome preparation	^{32}P incorporated (c.p.m./mg protein)	
	in vivo	*in vitro*
Crude	905	—
Triton X-100 treated	650	5920
Triton-treated, washed once with 0.5 M-KCl	550	—
Triton-treated, washed twice with 0.5 M-KCl	525	4420

Quantitative analysis of the *in vitro* phosphorylation has shown that using Triton X-100 treated ribosomes, which contain both the protein to be phosphorylated and the kinase, approximately 1.0 - 1.2 pmoles of phosphate was incorporated per 1 pmole of purified ribosomes (Table 2). However, the purified ribosomes in the presence of added kinase preparation incorporated only 0.5 pmole of [^{32}P]phosphate per 1 pmole of ribosomes. It is difficult to explain these differences; one can assume that purified ribosomes have a more compact structure than Triton--treated ribosomes due to the high concentration of K$^+$ and Mg^{2+} used during the purification procedure. The compact ribosome structure can make some serine and threonine residues less accessible to the kinase

Table 2

Stoichiometry of phosphorylation reaction in vitro

The values represent the amount of [^{32}P]phosphate incorporated into structural ribosomal proteins. In the case of Triton X-100 treated ribosomes used for phosphorylation, after the reaction had been stopped they were purified by twice washing with 0.5 M-KCl. All details as under Material and Methods. The molecular weight of 80S yeast ribosomes was assumed after Petermann (1964) as 4×10^6.

Preparation of ribosomes used for phosphorylation	Kinase	^{32}P incorporated (pmole/pmole of ribosome)
Triton X-100 treated	Ribosome-bound	1.0 - 1.2
Purified	Hydroxylapatite fraction	0.4 - 0.6

action. As it was shown recently by Grankowski *et al.* (1974), addition of cyclic AMP or its dibutyryl derivative did not enhance the activity either of the ribosome-bound kinase or the isolated enzyme preparation.

Polyacrylamide-gel electrophoresis patterns of proteins isolated from ribosome preparations phosphorylated *in vivo* and *in vitro* were very similar (Figs. 1A and B). This might mean that a closely related, if not the same individual proteins are phosphorylated either *in vivo* or *in vitro*. Our results are consistent with the findings of Kabat (1970) who demonstrated that the same proteins of reticulocyte ribosomes undergo phosphorylation *in vivo* and *in vitro*.
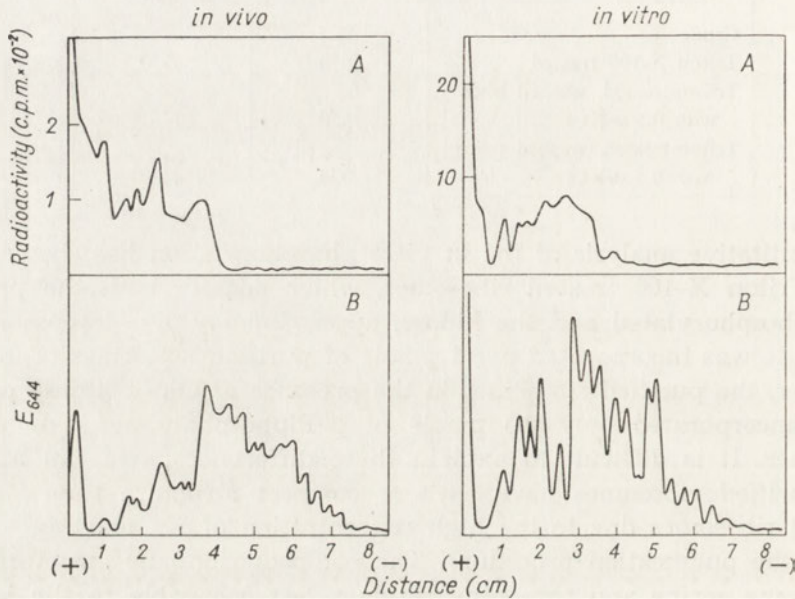

Fig. 1. Polyacrylamide-gel electrophoresis of proteins from ribosomes phosphorylated *in vivo* and *in vitro*. To the gel, 100 μg of protein was applied. *A*, Radioactivity; *B*, protein stained with Amido Black.

For identification of phosphorylated product, the labelled *in vivo* proteins from twice washed ribosomes were isolated and subjected to acid hydrolysis to identify the phosphorylated amino acids. On high-voltage paper electrophoresis of protein hydrolysate (Fig. 2), the radioactivity of ^{32}P corresponded to the spot of phosphoserine indicating that the phosphoproteins contained an ester-linked phosphate. The partial decomposition of phosphorylated amino acids occurring during hydrolysis was responsible for a highly active spot of orthophosphate. Further evidence for the presence of ester-bound phosphate was obtained by treatment with alkaline phosphatase (Table 3). Almost 100% of ^{32}P-label was removed from proteins as a result of the enzyme action.
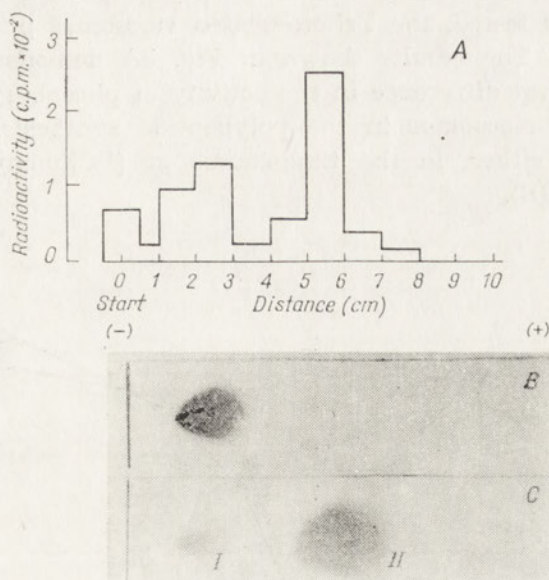
Fig. 2. Paper electrophoresis of acid hydrolysate of phosphorylated ribosomal protein. The hydrolysate of protein isolated from ribosomes labelled with ^{32}P *in vitro*, was subjected to electrophoresis on Whatman 3 *MM* paper, together with standard orthophosphate (*I*) and phosphoserine (*II*). *A*, ^{32}P-radioactivity; *B*, ninhydrin test for phosphoserine: *C*, molybdate test for phosphoserine and inorganic phosphate.

Table 3

The effect of alkaline phosphatase on ribosomal phosphoproteins

Two samples of Triton X-100 treated ribosomes (100 µg protein each) were incubated with 10 nmoles of [γ-^{32}P]ATP for 5 min. Alkaline phosphatase (100 µg/ml) was then added to one sample, and both were incubated for another 30 min. The reaction was stopped by adding trichloroacetic acid to a concentration of 10%. In the isolated protein, radioactivity was measured. For details see Methods.

Treatment	Radioactivity (c.p.m./sample)
None	2250
Alkaline phosphatase	150

Activity of phosphorylated ribosomes

For studying the activity of phosphorylated ribosomes in the cell--free polyphenylalanine-synthesizing system, highly phosphorylated ribosomes were used, that is the Triton-treated ribosomes phosphorylated *in vitro*. Furthermore, it was ascertained that the partly purified elongation factors contained no protein phosphatase activity.

In the experiment in which the activity of different Mg^{2+} ion concentrations was tested, the Triton-treated ribosomes purified by washing were used. The results shown in Fig. 3A demonstrate that there was no significant difference in the activity of phosphorylated and non-phosphorylated ribosomes in the polypeptide synthesis. No difference was observed, either, in the time-course of [^{14}C]phenylalanine incorporation (Fig. 3B).
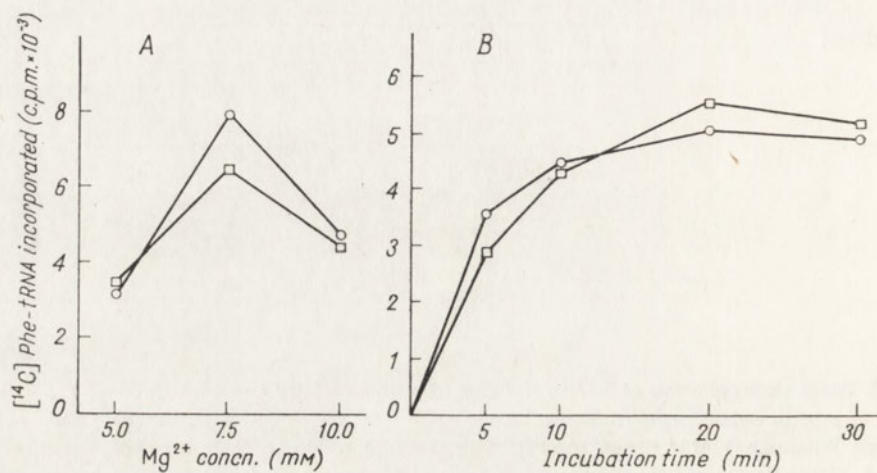

Fig. 3. Incorporation of [^{14}C]phenylalanyl-tRNA in the presence of a saturating amount of elongation factors, in the cell-free protein-synthesizing system, containing: ○, phosphorylated *in vitro*, purified ribosomes, and □, non-phosphorylated purified (control) ribosomes. *A*, Effect of Mg^{2+} concentration; *B*, time-course of the reaction in the presence of 10 mM-Mg^{2+}.

In another experiment phosphorylated Triton-treated ribosomes which were not purified by washing, were used. Such a preparation contained not only phosphorylated ribosomal proteins but also phosphorylated proteins loosely bound to ribosomes. It contained also initiation factors and a limited amount of elongation enzymes. Thus this preparation, due to the presence of almost all necessary factors, seemed to be a more adequate material for the study of the effect of phosphorylation on the ribosome. As it is shown in Fig. 4, also this system, when elongation factors were supplemented, did not exhibit a significant difference in the activity of phosphorylated and non-phosphorylated ribosomes; however, at concentrations of Mg^{2+} up to 7.5 mM, the activity of phosphorylated ribosomes was always somewhat lower as compared with control ones. This decrease in activity was more pronounced when the polymerization reaction was carried out in the presence of a limited amount of elongation factors (Table 4). At 5.0 mM-Mg^{2+}, the activity of phosphorylated ribosomal preparation in phenylalanine incorporation attained approximately 50% of the control value.

Fig. 4. Incorporation of [^{14}C]phenylalanyl-tRNA at different Mg^{2+} concentrations, in the presence of saturating amount of elongation factors in the cell-free protein-synthesizing system containing Triton-treated, non purified ribosomes: ○, phosphorylated *in vitro*; □, non-phosphorylated (control).
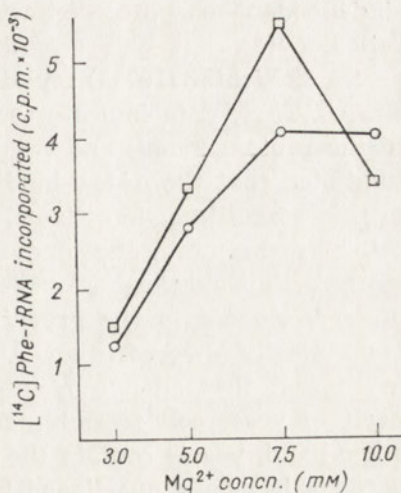
Table 4

Activity of phosphorylated ribosomes in the presence of a limited amount of elongation factors

The Triton X-100 treated ribosomes phosphorylated *in vitro* and not purified by washing, were incubated in a cell-free protein-synthesizing system not supplemented with elongation factors. For details see Materials and Methods.

Mg^{2+} concn. (mM)	[^{14}C]Phe-tRNA incorporated (c.p.m./sample)		Decrease in activity after phosphorylation (%)
	Control ribosomes	Phosphorylated ribosomes	
5.0	860	490	43
7.5	1230	780	37
10.0	1550	1170	25

DISCUSSION

The results presented in this paper show that ribosomal proteins of yeast, a primitive eukaryotic organism, are phosphorylated like ribosomal proteins from animal tissues. Polyacrylamide-gel electrophoresis of ribosomal proteins ^{32}P-labelled *in vivo* and *in vitro* demonstrated that similar groups of high-molecular-weight proteins are phosphorylated. We have shown recently (Grankowski *et al.*, 1974) that yeast cells contain two kinds of protein kinase; one of them is insensitive to cAMP and seems to be a protein specific for ribosome. The second, present in a high-speed supernatant, phosphorylates preferentially histones. The ribosome-bound protein kinase has been also found in rabbit reticulocyte ribosomes (Traugh *et al.*, 1973) and characterized as a cAMP-

-independent enzyme, distinct from other protein kinases present in reticulocytes.

Sy & Richter (1972a) found a cAMP-binding protein in various yeast strains. Their data indicate that this protein is not a regulatory subunit of the protein kinase and cannot be related to its function. It has been suggested that the cAMP-binding proteins might play a regulatory role in the catabolite gene activation process as it has been shown for *E. coli* cells. Furthermore, the amount of adenyl cyclase is strongly affected by growth conditions of yeast culture. For instance, in the presence of 10% glucose in the growth medium the activity of adenyl cyclase is drastically lowered (Sy & Richter, 1972b). All those observations seem to suggest that adenyl cyclase, the cAMP-binding protein and cAMP itself of yeast cell resemble functionally those of prokaryotes. On the other hand, yeasts contain the protein kinases the presence of which in bacteria has been questioned by Rahmsdorf *et al.* (1974).

The amount of phosphate incorporated into ribosomal proteins varies in experiments of different authors. This is mainly due to the differences in the procedures of isolation of purified ribosomal preparations, the methods of isolation of protein kinases, the presence or absence of inhibitors of phosphorylation reaction etc. Kabat (1972) has shown that during *in vivo* labelling approximately 5 moles of phosphate was incorporated into proteins per 1 mole of rat liver ribosomes. From *in vitro* experiments, the following values of ^{32}P incorporation per 1 mole of ribosomes were reported: for bovine anterior pituitary gland ribosomes, less than 0.1 mole (Barden & Labrie, 1973), for reticulocyte ribosomes in the presence of GTP as a phosphate donor, 0.17 mole (Ventimiglia & Wool, 1974), for 40S subunit of rat liver (Eil & Wool, 1973a) and adrenal cortex ribosomes (Walton & Gill, 1973) as much as 2.0 - 3.5 moles, and for 60S ribosomes from the same sources, even about 10 moles. Usually phosphorylation *in vitro* of ribosomal subunits is several-fold higher than that of undissociated ribosomes (Eil & Wool, 1973a). Our experiments have shown that phosphorylation of 80S yeast ribosomes *in vitro* in the presence of a ribosome-bound kinase, amounted to 1.0 - 1.2 mole of phosphate per mole of ribosome and on prolonged incubation (data not shown) this value could be doubled.

It may be assumed that chemical modification of ribosome as a result of phosphorylation of its protein component might affect the activity of this particle. Using a purified protein-synthesizing system, however, we have not been able to find any appreciable difference in the activity of phosphorylated and non-phosphorylated purified ribosomes. During the course of this work an excellent paper of Eil & Wool (1973b), related to the function of phosphorylated rat liver ribosomes, was published. Also those authors were unable to demonstrate a significant difference in the activity of modified and un-

modified ribosomal subunits either in the presence of poly(U) or natural, encephalomyocarditis virus RNA.

There are several possible explanations for the failure to detect the change in the activity of phosphorylated ribosomes:

1. The *in vitro* systems could be depleted of some essential factor(s) which were lost during the purification procedures.

2. The phosphorylation reaction might change the ribosome activity concerning other steps of translation process than those tested in the present experiments and by Eil & Wool (1973b).

3. We cannot exclude *a priori* the possibility that concomitant phosphorylation of ribosomal proteins and proteins transiently associated with ribosomes (e.g. initiation and elongation factors) affects the activity of protein-synthesizing machinery.

Our results on the activity of Triton X-100 treated ribosome preparation demonstrated an almost $50^0/o$ decrease of the polymerization activity of phosphorylated ribosome preparation at low Mg^{2+} and at limited concentration of transferases. This new experimental approach seems to be valuable for a study on the effect of phosphorylation.

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FOSFORYLACJA BIAŁEK RYBOSOMOWYCH DROŻDŻY *IN VIVO* i *IN VITRO*

Streszczenie

Stwierdzono, że białka rybosomowe drożdży zarówno *in vivo* jak i *in vitro* ulegają fosforylacji. Wbudowany fosforan znaleziono w estrach hydroksyaminokwasów. Rozmieszczenie radioaktywności w białkach rybosomowych rozdzielonych na poliakrylamidzie z obu typów doświadczeń było podobne, co świadczy, że te same białka, bądź białka o zbliżonych właściwościach fizyko-chemicznych, ulegają fosforylacji *in vivo* i *in vitro*. Ilość inkorporowanego fosforanu w doświadczeniach z [^{32}P]ATP osiągała poziom 1,0 - 1,2 mola/mol rybosomów. W układzie bezkomórkowym drożdży w obecności poli(U) rybosomy ufosforylowane i kontrolne wykazały podobną aktywność.

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MOLECULAR PROPERTIES OF THE INDUCIBLE
LUPANINE HYDROXYLASE FROM GROWING CULTURES OF
PSEUDOMONAS LUPANINI *

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An improved method for isolation of lupanine hydroxylase, giving a 450-fold purification, is presented. The molecular weight of the enzyme is about 72 000, and the sedimentation coefficient $s_{20,w}$ 5.05. The enzyme contains a component similar to Pseudomonas cytochromes c. Its oxidation-reduction potential was found to be below +45 mV.

Toczko *et al.* (1963) demonstrated that in the cells of soil bacteria *Pseudomonas lupanini* an enzyme catalysing 17-hydroxylation of lupanine is induced by D-lupanine. Toczko (1970) studied the molecular properties of this lupanine hydroxylase and suggested that haem and flavin were present in the enzyme preparation.

In the present work, a more efficient purification procedure was used and some molecular properties of the enzyme were investigated.

MATERIALS AND METHODS

Chemicals. Brilliant Cresyl Blue and thionine (Merck, Darmstadt, G.F.R.); calcium phosphate gel was prepared as described by Toczko (1970); DEAE-cellulose (Serva, Heidelberg, G.F.R.); DL-6,7-thioctamide and ubiquinone-10 (Koch-Light Lab. Ltd, Colnbrook, Bucks., England); N-ethylmaleimide (NEM), p-chloromercuric benzoate (PCMB) from Sigma (St. Louis, Mo., U.S.A.); 2-mercaptoethanol (Loba-Chemie, Wien-

-Fischamend, Austria); riboflavin (S. A. Hoffman La-Roche, Basle, Switzerland); Sephadex G-100 and G-200 fine (Pharmacia, Uppsala, Sweden); Whatman column Chromedia CF-11 (Balston Ltd, Maidstone, Kent, England). D-Lupanine was isolated by the method of Toczko (1970) from lupin seeds as perchlorate (melting temp. 212 - 213°C). Other reagents were of Polish origin.

The biological material. *Pseudomonas lupanini* cells were cultured on the mineral basic medium containing per 100 ml: 60 mg of KH_2PO_4, 60 mg of K_2HPO_4, 100 mg of $MgSO_4$, 100 mg of NaCl, 0.3 mg of $FeCl_3$, and supplemented with 0.1% of lupanine as the sole carbon and nitrogen source. The bacteria were six times passaged on liquid medium and incubated at 30°C. After harvesting of the cells, the medium contained traces of lupanine. The cells were collected by centrifugation (about 30 g wet wt. from 20 litres of the medium), washed several times with cold acetone, and dried over solid NaOH in a vacuum desiccator. The obtained acetone-dried material could be stored for several months at -15°C without loss of the lupanine hydroxylase activity.

The assay of lupanine hydroxylase activity. The activity was determined spectrophotometrically by measuring the reduction of 2,6- -dichlorophenolindophenol (DCIP) at 600 nm. The incubation mixture contained 10 μmoles of 0.1 M-phosphate buffer, pH 6.8, 10 μmoles of lupanine, 0.16 μmole of DCIP and the enzyme preparation in a total volume of 0.4 ml. The control contained no lupanine. The samples were incubated at 30°C up to 20 min, the reaction rate being linear during this time. The enzyme activity is expressed in units (1 μmole of reduced DCIP/min at 30°C).

Analytical methods. Protein was determined according to Lowry *et al.* (1951); total flavin after Burch *et al.* (1948) and the peptide-bound flavin after Singer *et al.* (1962). Total and non-haem iron was determined after Doeg & Zingler (1962).

The amino acid composition of lupanine hydroxylase was analysed after Spackman *et al.* (1958), using an amino acid analyser of Beckman Unichrom type, following 24 h hydrolysis in 6 M-HCl at 105°C.

Polyacrylamide-gel electrophoresis was performed on 9% polyacrylamide gel (0.5 $\times$ 5 cm) at pH 6.8 according to Raymond & Wang (1960). About 10 μg of the protein in 20% sucrose was layered directly on the top of gel and electrophoresis was carried out for 120 min at 3 mV per gel. Protein was stained with 0.6% Amido Black, and the enzyme activity located by immersing the gel for 5 min into 30 ml of a mixture containing 0.2 M-phosphate buffer, pH 6.8, 0.25 mM-DCIP and 0.15 mM- -lupanine; decolorized areas corresponded to the position of enzyme activity.

Molecular weight was estimated by sedimentation equilibrium in OV-102 type ultracentrifuge (Hungary), equipped with Philpot-Svenson

optics, and by molecular gel filtration. The gel filtration was performed on a column (1.5 × 80 cm) containing a mixture of Sephadex G-100 and G-200 (2:1, w/w). The proteins used as standards: egg albumin, soluble, bovine blood albumin, chymotrypsin A, cytochrome c, γ-globulin, chymotrypsin, and myoglobin were all from Reanal (Budapest, Hungary). The proteins were eluted with 25 mM-sodium phosphate buffer, pH 6.8, containing 80 mM-NaCl at a flow rate of 1 ml/7 min.

Isolation of lupanine hydroxylase. The acetone-dried bacteria (2 g) were suspended in 72 ml of 0.1 M-Na-phosphate buffer, pH 6.8, and disintegrated by three cycles of freezing and thawing; then 17.2 ml of 4% 2-ethoxy-6,9-diaminoacridine (rivanol) solution was added and the mixture stirred for 20 min at 0°C. The extract was separated by centrifugation at 4500 r.p.m. for 10 min.

In some experiments, n-butanol was additionally used for extraction. The suspension of acetone-dried bacteria disintegrated by freezing and thawing, was treated with 1/6 its volume of n-butanol for 1 h at 0°C, then rivanol was added and the mixture centrifuged as above. The preparation obtained in this way is further referred to as the butanol--treated preparation.

In either case, from the obtained extract (about 60 ml) the protein was precipitated at 0.35 - 0.85 ammonium sulphate saturation, suspended in 0.5 ml of 0.1 M-phosphate buffer, and centrifuged. The sediment, free of the excess of ammonium sulphate, was dissolved in 3 ml of buffer and dialysed for 14 h at 4°C against 3 litres of 5 mM-phosphate buffer containing 0.25 mM-aluminium chloride and 0.1 μM-2-mercapto-ethanol. The solution was clarified by centrifugation and subjected to column chromatography. The column (2 × 15 cm) contained a mixture of: 10% suspension of Whatman cellulose, calcium phosphate gel (70 - 80 mg of dry gel in 1 ml) and water (80:8:12, by vol.) equilibrated with 5 mM--phosphate buffer. The elution was made with 5 mM- and 45 mM--phosphate buffer of pH 6.8 (Fig. 1). The active fractions eluted with the 45 mM-buffer were pooled, diluted threefold (to a buffer concentration of 15 mM) and applied to a DEAE-cellulose column (1 × 10 cm, equivalent to 1.3 g of dry powder) equilibrated with 15 mM-phosphate buffer. The column was eluted first with 24 ml of the above buffer, and then with the same volume of the buffer containing 80 mM-sodium chloride. Lupanine hydroxylase was eluted with the latter eluent (Fig. 2).

RESULTS AND DISCUSSION

The modification of the method of Toczko (1966, 1970), consisting in application of acetone and rivanol, and successive chromatography on calcium phosphate gel and DEAE-cellulose columns, permitted to
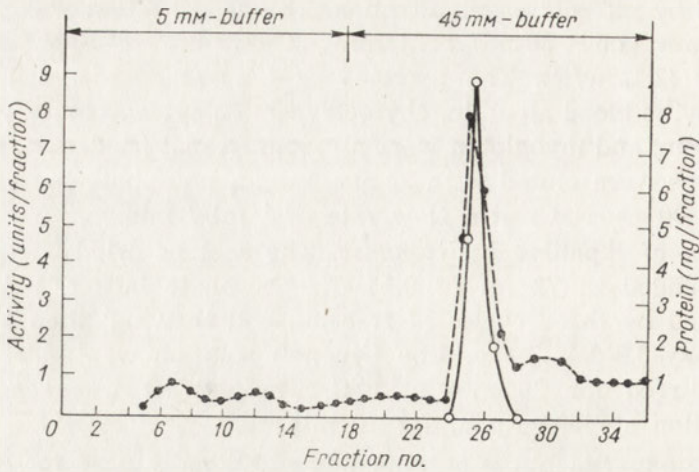
Fig. 1. Calcium-phosphate-gel chromatography of the dialysed ammonium sulphate precipitate. To the gel, 67.7 mg of protein was applied, and eluted with 5 mM- and 45 mM-phosphate buffer, pH 6.8. Fractions of 5 ml were collected at a flow rate of 1.5 ml/min. (○), Enzyme activity; (●), protein.
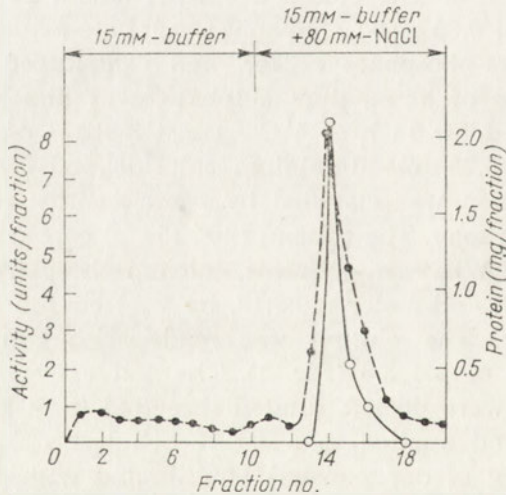

Fig. 2. DEAE-cellulose-column chromatography of the active fractions from calcium-phosphate-gel chromatography (Fig. 1). To the column, 15.5 mg of protein was applied and eluted successively with 15 mM-phosphate buffer, pH 6.8, and the buffer containing 80 mM-NaCl. Fractions of 3 ml were collected at a flow rate of 1.5 ml/min. (○), Enzyme activity; (●), protein.

obtain from *Pseudomonas lupanini* a preparation of lupanine hydroxylase of higher purity and with a much better yield. The course of lupanine hydroxylase isolation is presented in Table 1.

Table 1

Course of purification of lupanine hydroxylase from Ps. lupanini *cells grown in lupanine-containing medium*

The acetone-dried cells were used for purification.

Procedure	Total protein (mg)	Total activity (unit)	Spec. act. (units/mg protein)	Purification factor	Yield* (%)
Crude phosphate extract	1253.0	10.3	0.0082	—	—
Rivanol extract	614.0	13.0	0.021	2.5	—
Ppt. at 0.35 - 0.85 $(NH_4)_2SO_4$ sat.	71.3	15.0	0.210	25	100
After dialysis	67.7	14.6	0.216	26	97
Calcium phosphate gel fractions	16.6	15.2	0.916	111	101
DEAE-cellulose fraction no. 14	2.3	8.4	3.7	453	56

* The yield was calculated in relation to the total activity of the ammonium sulphate fraction, taken as 100.

The purified preparation (DEAE-cellulose fraction no. 14) contained 56% of the activity present in the ammonium sulphate precipitate; its specific activity was 3.7 units/mg protein, and the degree of purification about 450-fold. The earlier procedure gave a preparation with spec. act. of 1.95 units, the yield being 21% and purification 40-fold. In comparison with the extract prepared in the absence of butanol, the corresponding values for the butanol-treated preparation were somewhat lower: spec. act. 3.17 units, yield 53% and purification 350-fold; losses of the activity occurred during dialysis and DEAE-cellulose column chromatography.

The purified lupanine hydroxylase preparation was homogeneous on ultracentrifugation (Fig. 3), and the butanol-treated preparation gave an identical pattern.
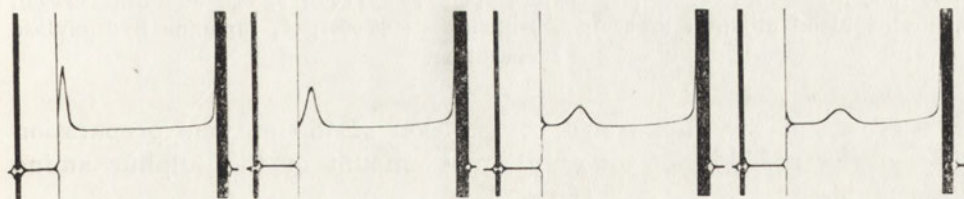

Fig. 3. Analytical ultracentrifugation pattern for the purified lupanine hydroxylase preparation (4 mg of protein) dissolved in 15 mM-phosphate buffer, pH 6.8, containing 80 mM-NaCl. The photographs were taken, successively, after 3, 6, 12 and 15 min at 55 000 r.p.m. and 20°C; sedimentation of protein from left to right.

On polyacrylamide-gel electrophoresis, the preparation separated into four well-defined bands, and the butanol-treated preparation, into three bands (Fig. 4a,b). Two main protein bands of each preparation showed enzymic activity (Fig. 4c).
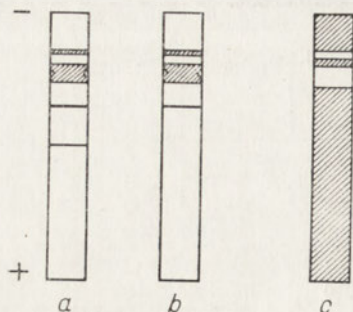

Fig. 4. Polyacrylamide-gel electrophoresis of the lupanine hydroxylase preparation; *a* and *b*, protein fractions, respectively, of the preparation untreated and treated with *n*-butanol; *c*, enzyme activity of the untreated preparation (decolorized sites); for the butanol-treated preparation the same pattern was obtained.

The molecular weight of the preparation estimated by gel filtration was 67 000 (Fig. 5) and by sedimentation equilibrium 76 300 ($s_{20 \cdot w} = 5.05$). The mean value, 72 000, was used for further calculations.
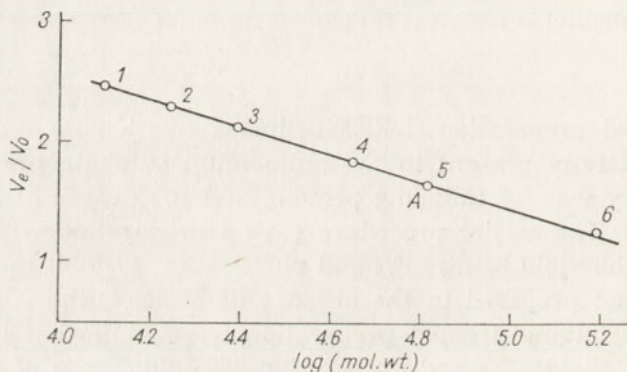

Fig. 5. Molecular weight determination of lupanine hydroxylase preparation by gel filtration on Sephadex. The column (1.5 × 80 cm) containing a mixture of Sephadex G-100 and G-200 (2:1, w/w), was equilibrated with 25 mM-phosphate buffer, pH 6.8, containing 80 mM-NaCl, and eluted with the same buffer. The proteins used (5 mg each) were dissolved in the same buffer. *1*, Cytochrome *c* (mol. wt. 12 400); *2*, myoglobin (17 800); *3*, chymotrypsin A (25 000); *4*, egg albumin (45 000); *5*, bovine blood albumin (67 000); *6*, γ-globulin (160 000); *A*, lupanine hydroxylase preparation.

Analysis of the amino acid composition of the enzyme preparation showed the presence of an only small amount of the sulphur amino acids (Table 2).

Two thiol-group specific reagents, NEM and PCMB, even at 1 mM concentration, did not affect the enzyme activity. The inhibition by mercury ions observed by Toczko (1970) and Niedzielska & Rogoziński (1973) may be regarded as a non-specific action.

Table 2

Amino acid composition of the purified lupanine hydroxylase preparation

The enzyme preparation was hydrolysed in 6 M-HCl at 105°C and analysed in amino acid analyser of Beckman Unichrom type.

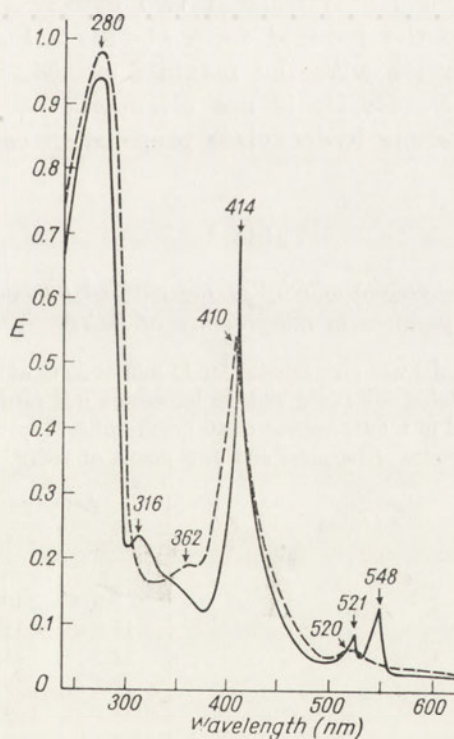| Amino acid	Content (g/100 g)	Amino acid	Content (g/100 g)
Non-identified	3.71	Valine	6.51
Aspartic acid	10.77	Methionine	0.12
Threonine	5.42	Isoleucine	3.63
Serine	5.26	Leucine	7.17
Glutamic acid	8.47	Tyrosine	2.30
Proline	3.77	Phenylalanine	3.52
Glycine	9.73	Lysine	6.42
Alanine	10.46	Histidine	1.23
Cystine	trace	NH_3	9.10
		Arginine	2.41

Fig. 6. Absorption spectra of the purified lupanine hydroxylase preparation in 25 mM-phosphate buffer, pH 6.8, containing 80 mM-NaCl. ——, Reduced form obtained in the presence of 7 μM-$Na_2S_2O_4$ or 0.7 μM-lupanine; — — —, oxidized form obtained in the presence of 0.2 mM-$K_3Fe(CN)_6$.

The presence of flavin suggested by Toczko (1970) was not confirmed. No riboflavin was detected in the enzyme preparation by the method of Burch *et al.* (1948). However, about 0.08 μmole of peptide-bound flavin per 1 μmole of lupanine hydroxylase was detected by the method of Singer *et al.* (1962), pointing to a contamination of the enzyme preparation by some other flavin-containing protein.

Toczko (1970) on the basis of the decrease of enzyme activity by cyanide suggested the presence of haem in lupanine hydroxylase. This was now confirmed by the absorption spectra of the reduced and oxidized forms of lupanine hydroxylase (Fig. 6) and by the presence of 1 atom of haem-bound iron per 1 μmole of the enzyme (no other form of iron was found in the enzyme preparation). It should be noted that the attempts at separating the haem component from the enzyme resulted in the loss of activity.

The absorption spectra of the lupanine hydroxylase preparation at pH 6.8 (Fig. 6) indicate the presence of cytochrome c. In the reduced form, the absorption maxima were at 548, 521 and 414 nm.

From *Ps. aeruginosa* Horio (1958a,b) and Horio *et al.* (1960), and from *Ps. fluorescens* Ambler (1963) isolated two cytochromes c, one with absorption maxima in the reduced form at 551, 521 and 416 (P-cyto-chrome 551) and another with the maxima at 554, 525 and 416 nm (P-cytochrome 554). So far, it is not clear whether the cytochrome component of the lupanine hydroxylase preparation corresponds to one

Table 3

Effect of some coenzymes on the activity of the purified lupanine hydroxylase preparation in the presence of DCIP

The purified enzyme (0.2 nmole) was preincubated for 15 min at 30°C with 2.5 μmoles of the indicated compound, then lupanine was added and the incubation was carried out for 20 min. The incubation mixture contained in a total volume of 10 μmoles of 0.1 M-phosphate buffer, pH 6.8, 10 μmoles of lupanine and 0.16 μmole of DCIP.

Addition	Activity	
	units	%
None, control	8.6	100
NAD$^+$	13.6	158
NADP$^+$	12.5	145
FMN	8.2	95
FAD	7.8	91
Ubiquinone-10	8.1	94
n-Butanol (2.5%)	5.9	68
n-Butanol (2.5%)+thioctamide	6.5	75
n-Butanol (12.5%)	2.0	24
n-Butanol (12.5%)+thioctamide	3.8	45

of these cytochromes or differs from both and forms a distinct type of P-cytochrome.

NAD^+ and $NADP^+$ could not replace DCIP as electron acceptor in the reaction of lupanine hydroxylase. However, in the presence of DCIP and at high concentrations (12.5 mmoles per 1 μmole of the enzyme), they distinctly stimulated the reduction of DCIP (Table 3).

The oxidation-reduction potential of lupanine hydroxylase was estimated using different electron acceptors (Table 4). The E'_0 value of the enzyme at 30°C and pH 6.8 was found to be below +45 mV.

Table 4

The activity of lupanine hydroxylase in the presence of different electron acceptors

The sample contained 0.2 nmole of the enzyme preparation, 10 μmoles of D-lupanine; and 0.1 μmole of electron acceptor; the time of incubation was 10 min (except where indicated), temperature 30°C, pH 6.8. The values of E'_0 were taken from Singer *et al.* (1957).

Electron acceptor	The carried electron number	E'_0, pH 7 (V)	Reduced acceptor (μmoles)	Activity (%)
DCIP	2	+0.217	88	100
Potassium ferricyanide	1	+0.360	192	219
Cytochrome *c*	1	+0.262	58	67
Phenazine methosulphate	2	+0.080	0	0
Thionine (60 min)	2	+0.062	0.34	0.4
Brilliant Cresyl Blue (60 min)	2	+0.045	0.13	0.15
Gallocyanine (60 min)	2	+0.021	0	0
Methylene Blue (60 min)	2	+0.011	0	0

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WŁAŚCIWOŚCI MOLEKULARNE HYDROKSYLAZY LUPANINOWEJ
INDUKOWANEJ W BAKTERIACH *PS. LUPANINI*
W FAZIE WZROSTU

Streszczenie

1. Opracowano ulepszoną metodę oczyszczania hydroksylazy lupaninowej
i uzyskano preparat oczyszczony ok. 450-krotnie.

2. Masa cząsteczkowa enzymu wynosi ok. 72 000, stała sedymentacji $s_{20,w} =$
$= 5.05$. W preparacie enzymu stwierdzono obecność składnika o właściwościach
zbliżonych do cytochromów c izolowanych z bakterii Pseudomonas. Potencjał
oksydo-redukcyjny enzymu znajduje się poniżej $+45$ mV.

W. JANKOWSKI, T. MAŃKOWSKI and T. CHOJNACKI

UNDECAPRENOL PHOSPHATE, THE ENDOGENOUS ACCEPTOR OF GLUCOSE FROM UDPGLUCOSE IN
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The evidence is presented for the presence of undecaprenol phosphate in *Sh. flexneri* 2a.

The involvement of undecaprenol phosphate glucose in the type--specific glucosylation of *O*-antigen in *S. typhimurium* has been demonstrated by Nikaido *et al.* (1971) and Wright (1971). A similar phenomenon was observed in *Sh. flexneri* by Jankowski *et al.* (1972) with chemically phosphorylated plant polyprenol (ficaprenol), the enzyme synthesizing ficaprenol monophosphate glucose being found only in these strains in which glucosylated lipopolysaccharide was present. The chemical nature of endogenous lipid acceptor of glucose in *Sh. flexneri* is not known, though from the fact that, on using various long-chain polyprenol phosphates, the highest rates of formation of lipid-linked glucose were obtained with ficaprenol phosphate (Jankowski *et al.*, 1974), the undecaprenoid structure could have been postulated, similarly as found in *Salmonella* producing the glucosylated *O*-antigen (Nikaido & Nikaido, 1971; Wright, 1971).

It is well established that undecaprenol phosphate- and pyrophosphate sugars are intermediates in the formation of bacterial sugar polymers. As shown by the results of mass spectrometry, the C_{55}-polyisoprenoids occur commonly in the majority of studied bacteria (cf.

Umbreit *et al.*, 1972). Nevertheless, polyprenols of different structure have been found in *Mycobacteria* (Takayama *et al.*, 1973).

The aim of the present paper was to demonstrate the presence and to establish the structure of the endogenous lipid acceptor in *Sh. flexneri* that might be involved in the glucosylation of *O*-antigen. In addition, the solubilization by detergents of the UDPglucose : polyprenol monophosphate glucosyltransferase is reported.

MATERIALS AND METHODS

Sh. flexneri 2a strain was obtained from the Shigella Laboratory of the Department of Bacteriology, State Institute of Hygiene, Warsaw. It was grown as described previously (Jankowski & Chojnacki, 1972) and collected from cultures in the exponential phase.

Ficaprenol (a mixture of C_{50}-, C_{55}- and C_{60}-polyprenols) was the same as in the previous paper (Jankowski & Chojnacki, 1972).

The C_{55}-polyprenol was isolated from the ficaprenol mixture by column chromatography on paraffin-impregnated mixture of kieselguhr and cellulose powder. Kieselguhr, 50g, was mixed with 50 g of cellulose powder and suspended in 500 ml of 7.5% solution of liquid paraffin in light petroleum (b.p. 60 - 80°C). The volatile solvent was evaporated on a rotary evaporator, and the dry kieselguhr-cellulose mass impregnated with paraffin was packed tightly in the column (4×30 cm). The ficaprenol mixture, 100 mg, was mixed thoroughly with about 5 g of kieselguhr-cellulose impregnated with paraffin using a glass rod; the uniform dry material was placed on the top of the column, and the solvent, acetone - water (95:5, v/v) saturated with paraffin was allowed to soak down the column (the time of soaking to the bottom being usually 2 - 3 h) and to run through it. The course of elution of polyprenols from the column was followed by reversed-phase thin-layer chromatography. The first 5 - 10 ml of the effluent contained an orange-coloured substance. Polyprenols were eluted between 50 and 150 ml. This part of effluent was collected as 2 ml fractions. The fractions containing pure C_{55}-polyprenol formed the middle part. They were preceded by fractions containing lower prenologues and followed by fractions containing higher prenologues [1]. The yield of C_{55}-polyprenol after removing the paraffin from the preparation (Wellburn *et al.*, 1967) was 20 mg.

The phosphorylation of C_{55}-polyprenol and of ficaprenol mixture was performed by the method of Cramer & Böhm (1959) as described in the previous paper (Jankowski & Chojnacki, 1972).

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[1] The separation of prenologues can be improved by using hydroxyalkoxypropyl Sephadex for column chromatography.

UDP-[U-^{14}C]glucose (248 Ci/mole) was from The Radiochemical Centre (Amersham, Bucks., England), Na deoxycholate (DOC) from POCh (Gliwice, Poland); Triton X-100 from B.D.H. (Poole, Dorset, England); Triton X-114 from Rohm and Haas Ltd (West Hill, Ont., Canada), and Poly-Tergent S 305LF from Olin Chemicals (Stamford, Conn., U.S.A.). Cellulose powder for column chromatography "B" was from Whatman Biochemicals Ltd. (Maidstone, Kent, England); kieselguhr G was from Merck (Darmstadt, G.F.R.); DEAE-cellulose "SS" was from Serva (Heidelberg, G.F.R.); cellulose sheets for thin-layer chromatography were from Eastman-Kodak (Rochester, N.Y., U.S.A.).

Reversed-phase thin-layer chromatography of polyprenols in acetone - - water (23:2, v/v) saturated with liquid paraffin (Wellburn *et al.*, 1967) was performed on cellulose sheets impregnated with 10% solution of liquid paraffin in light petroleum. The spots of polyprenols were detected with iodine.

Gas-liquid chromatography of polyprenol phosphates was performed on Pye 104 gas chromatograph fitted with flame ionization detector using glass columns (30 × 0.4 cm) of 1% OV-17 (methyl-phenyl-silicone) on Gas-Chrom Q (150 - 200 μ) with glass-to-metal sealed outlets; the temperature was 295°C and carrier gas, argon.

Mass spectrometry of standard C_{55}-polyprenol monophosphate and lipid fraction from *Sh. flexneri* obtained by column chromatography on DEAE-cellulose was performed using LKB-9000 apparatus (ionization energy, 70 eV; source temperature, 160°C; probe temperature, 290°C).

Alkali-stable lipid fraction from *Sh. flexneri* was prepared from 10 g of wet bacterial mass following the procedure of Osborn *et al.* (1972) up to the column chromatography on DEAE-cellulose; this step was performed according to Jankowski & Chojnacki (1972) using concentration gradient of ammonium formate in chloroform - methanol (2:1, v/v).

UDPglucose : polyprenol phosphate glucosyltransferase was solubilized from *Sh. flexneri* cell envelope fraction which was prepared by sonication of bacteria and subsequent differential centrifuging as described by Jankowski *et al.* (1972).

The enzyme was assayed in the reaction mixture containing in a final volume of 0.05 ml: 0.2 M-glycylglycine buffer, pH 7.5; 0.1 M-mercaptoethanol; 0.4 mM-UDP-[U-^{14}C]glucose (12 500 c.p.m.); 0.6% Triton X-100; 0.01 M-Mg_2EDTA; 1 mM-ficaprenol phosphate, and 30 μl of enzyme preparation. The mixture was incubated at 37°C for 30 min with constant shaking, and the lipid-linked [^{14}C]glucose was estimated as described by Jankowski & Chojnacki (1972).

The polyprenol monophosphate in lipid fractions from *Sh. flexneri* was enzymically detected using the soluble enzyme preparation obtained

by treatment with Triton X-100 (Table 1). To detect the presence of lipid acceptor of glucose, bacterial lipids were added to the enzyme assay instead of ficaprenol phosphate.

Protein was assayed according to Lowry *et al.* (1951) and phosphorus by the micromethod of Bartlett (1959).

RESULTS

The glucosyltransferase catalysing the formation of polyprenol monophosphate glucose can be solubilized at pH 8.5 by all four detergents studied (Table 1). No solubilization was observed with deoxycholate at pH 7.8, though this might have been due to the inactivation of the enzyme, since the activity remaining in the pellet was also low. The solubilizing effect of other detergents at pH 7.8 was slightly lower than at pH 8.5. Both with Triton X-100 and Triton X-114 the release of enzyme from the cell envelope fraction was almost complete and was accompanied by the release of more than 50% of protein material from the particulate fraction sedimenting at 105 000 *g*. Poly-Tergent at pH 8.5 resulted in a more selective release of glucosyltransferase, as a large amount of other proteins (76%) remained in the pellet. The concentrations of detergents used for solubilization were selected individually so as to obtain the highest glucosyltransferase activity (cf. also Jankowski *et al.*, 1974). The soluble enzyme preparation obtained by the action of Triton X-100 at pH 8.5 (1st supernatant, Table 1) was used for detecting the presence of endogenous lipid in alkali-stable lipid fractions from *Sh. flexneri*.

Table 1

Solubilization of UDPglucose : polyprenol phosphate glucosyltransferase

Cell envelope fraction from *Sh. flexneri* 2a (5 mg of protein) was treated twice with 1 ml portions of 20 mM-Tris-HCl, pH 7.8 or 8.5, containing the indicated detergents: DOC (10 mg), Triton X-100 (50 mg), Triton X-114 (50 mg) or Poly-Tergent (30 mg). The incubation time was 30 min at 37°C. The enzymic activity was estimated in 30 µl samples of two successive supernatants obtained after centrifuging the suspensions for 1 h at 105 000 *g*, and in a 30 µl sample of the final pellet suspended in 1 ml of the respective detergent-buffer solution. The figures represent the amount of lipid-linked [14C]glucose (c.p.m.) estimated as described in Materials and Methods.

Preparation	DOC		Triton X-100		Triton X-114		Poly-Tergent	
	pH 7.8	pH 8.5	pH 7.8	pH 8.5	pH 7.8	pH 8.5	pH 7.8	pH 8.5
1st supernatant	41	1026	4097	4792	4336	6757	1982	3912
2nd supernatant	24	21	172	113	174	164	756	566
Pellet	72	251	239	132	41	11	778	450
Insoluble protein remaining in pellet (% of total)	37	31	46	38	35	40	70	76

Figure 1 shows the DEAE-cellulose column chromatography pattern of alkali-stable lipids from *Sh. flexneri*. The lipids were separated into two phospholipid fractions by applying ammonium formate gradient. Fraction *II* was found to stimulate the formation of lipid-linked [^{14}C]glucose from labelled UDPglucose in the presence of soluble enzyme preparation. The elution curves of sugar-binding activity and of lipid phosphorus were not exactly parallel, thus implying that the phospholipid acceptor (presumably polyprenol phosphate) in Fraction *II* was not pure.
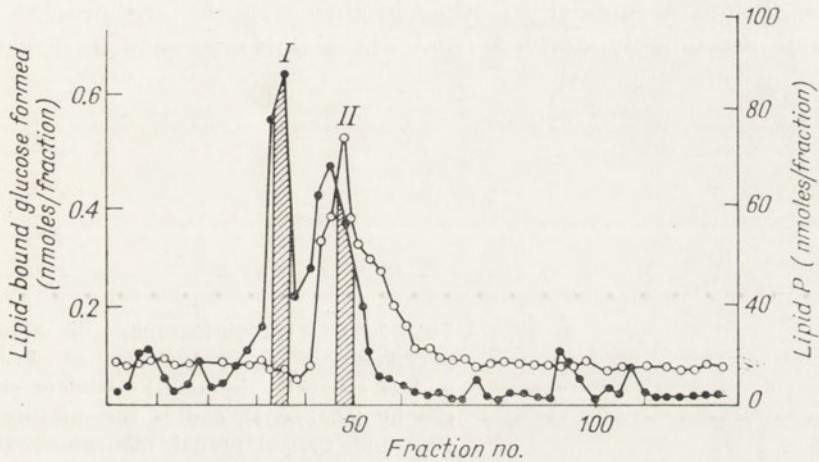
Fig. 1. Column chromatography on DEAE-cellulose of alkali-stable lipids from *Sh. flexneri* 2a. To the column (0.6 × 10 cm) of DEAE-cellulose in acetate form, lipids (0.1 mg P) obtained from 10 g of wet bacteria were applied in 5 ml of chloroform - methanol (2:1, v/v) and eluted with continuously increasing concentrations of ammonium formate (pH 4) in chloroform - methanol (2:1, v/v). The upper reservoir contained 60 mM-ammonium formate in chloroform - methanol solvent. Fractions of 2.5 ml were collected every 10 min. The test for the presence of lipid acceptor of glucose was performed as described in Materials and Methods using 30 μl of soluble glucosyltransferase and 50 μl of the tested lipid fraction. (●), lipid phosphorus; (○), sugar-binding activity. The shaded areas correspond to fractions used in further determinations.

The phospholipids eluted from DEAE-cellulose were subjected to gas-liquid chromatography (Fig. 2). Fraction *II* contained a substance that gave a record similar to that obtained with ficaprenol phosphate (a mixture of C_{50}-, C_{55}- and C_{60}-polyprenol phosphates, with the C_{55}-compound predominating) in that it contained the peaks corresponding to C_{55}-polyprenoid; it did not contain, however, C_{60}-polyprenoid. Thus it is evident that undecaprenol phosphate was present in the bacterial lipids. No similar substance arising from long-chain polyprenol

phosphates was detected in Fraction *I* which was inactive in the enzymic test with glucosyltransferase.

The presence of undecaprenol phosphate in the lipids of Fraction *II* from DEAE-cellulose was confirmed by mass spectrometry (Fig. 3a). A characteristic peak was obtained at m/e 748 corresponding to the hydrocarbon — undecaprenene. Figure 3b gives the mass spectrum of the monophosphate of the isolated plant C_{55}-polyprenol. Both spectra are similar in the region of $m/e > 600$. Characteristic groups of peaks in the mass spectrum of C_{55}-polyprenol phosphate standard in the region of $m/e < 600$ are also visible in the mass spectrum of the bacterial lipid, though a number of other groups of peaks are present in this region, presumably derived from other lipids present in Fraction *II*.
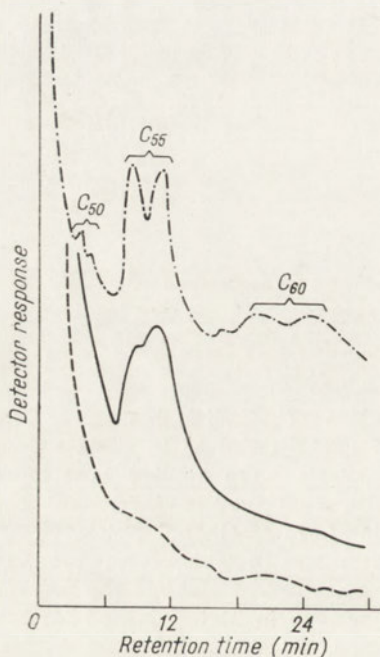

Fig. 2. Gas-chromatography of alkali-stable phospholipids of *Sh. flexneri* 2a, Fractions *I* and *II* separated by DEAE-cellulose chromatography (cf. Fig. 1), and of the mixture of C_{50}-, C_{55}- and C_{60}-polyprenol monophosphates. The column (30 × 0.4 cm, 1% OV-17 on Gas-Chrom Q, 150 - 200 μ) was operated isothermally at 295°C in a Pye 104 apparatus fitted with flame ionization detector using a dual column compensation system. The flow of carrier gas Ar was 40 ml/min. The amounts of phospholipids injected were: —·—, mixture of polyprenol monophosphates, 3 nmoles P; — — —, phospholipid Fraction *I*, and ——, phospholipid Fraction *II*, 30 nmoles P each. Attenuator, 1 × 10².

In the mass spectrum of bacterial lipids no peaks corresponding to higher prenologue were observed, which is in agreement with the results of gas chromatography (Fig. 2). It is evident from the mass spectrum of bacterial lipids that a lower prenologue, C_{50}-polyprenol phosphate, could be present only in low amount compared with undecaprenol phosphate. This conclusion, though not quantitative, can be drawn from the comparison of peaks at m/e 680 both in bacterial preparation and in the standard substance with those of undecaprenene at m/e 748. Usually it is possible to determine the amount of C_{50}-polyprenoid by gas chromatography as described in this paper, provided the solvent front is not as large as that observed in analyses given in Fig. 2.
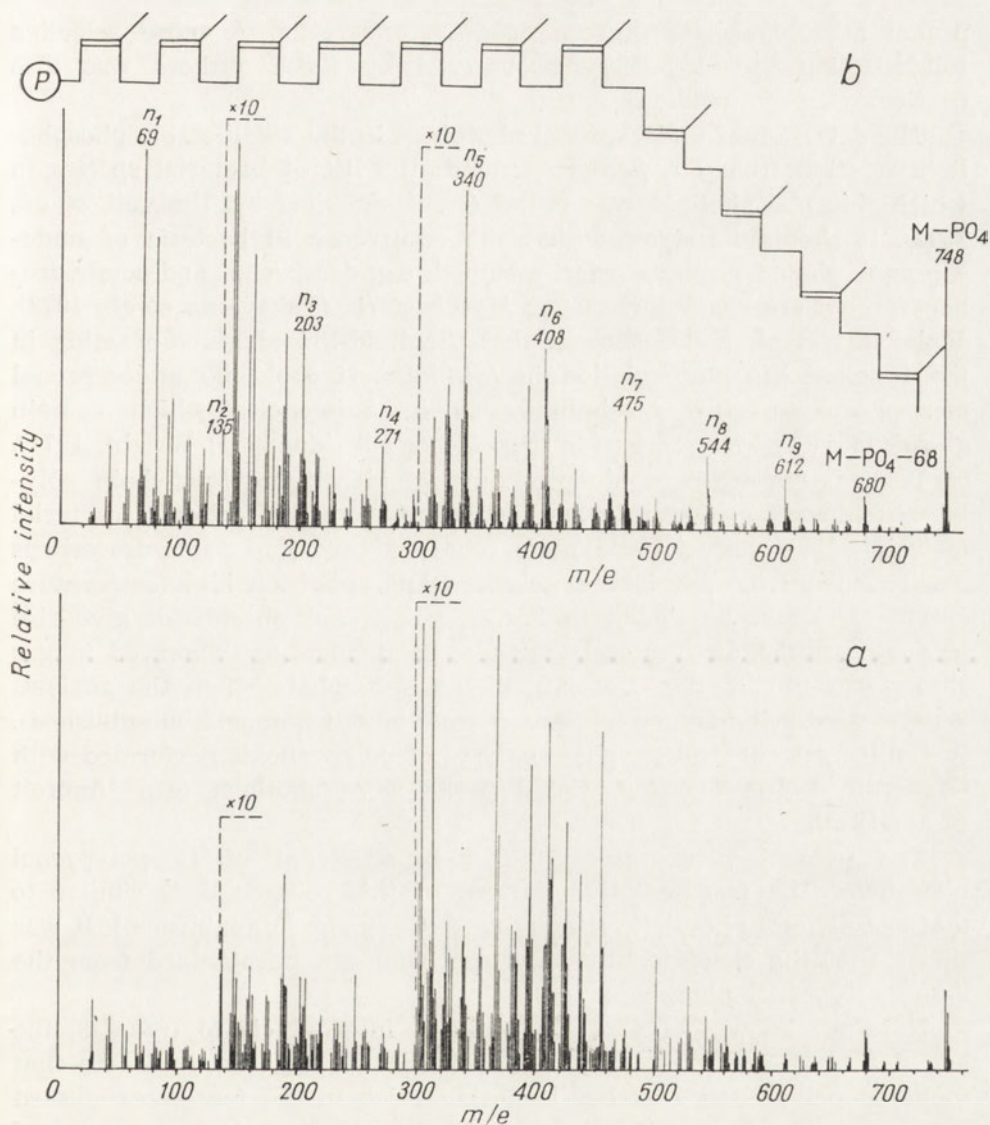
Fig. 3. Histogram of the mass spectrum of more prominent peaks of *a*, *Sh. flexneri* phospholipid Fraction *II* from DEAE-cellulose (Fig. 1), and *b*, C_{55}-polyprenol monophosphate. The structure of C_{55}-polyprenol monophosphate is superimposed in the top part of the figure over the appropriate fragmentation peaks. Samples were subjected to mass spectrometry by direct probe injection.

DISCUSSION

As shown above, the procedure of Osborn *et al.* (1972) with the use of detergents for solubilization of glycosyltransferases involved in the synthesis of polyprenol pyrophosphate galactosyl rhamnosyl mannose in *S. typhimurium*, is applicable for solubilizing the synthetase of poly-

prenol monophosphate glucose of *Sh. flexneri* 2a. A more selective solubilization by Poly-Tergent observed by these authors was also confirmed in our studies.

The detection of undecaprenol phosphate in the alkali-stable phospholipid fraction from *Sh. flexneri* extends the list of bacterial species in which this phospholipid was found (cf. the review by Umbreit *et al.*, 1972). It should be stressed that the occurrence in bacteria of undecaprenols should not be *a priori* assumed; e.g. decaprenol and octahydroheptaprenol are characteristic for *Mycobacteria* (Takayama *et al.*, 1973). Jankowski *et al.* (1972) showed that the lipid-linked glucose acting in the type-specific glucosylation in *Sh. flexneri* could be undecaprenol monophosphate glucose, similarly as in *Salmonella* which contain glucosylated lipopolysaccharide (Nikaido & Nikaido, 1971; Wright, 1971).

The gas chromatographic procedure of identifying long-chain polyisoprenoid compounds can be applied directly to lipid fraction of bacterial material. The characteristic peaks observed are due to hydrocarbons resulting from the pyrolysis of polyprenol phosphates in high temperature chromatography. As shown in Fig. 2, polyprenol phosphates give rise to double peaks. In fact at least three compounds are observed in gas chromatography of the C_{45}-solanesol monophosphate when the analysis is performed using longer columns (Hemming & Chojnacki, unpublished). In similar gas chromatographic analyses of polyprenoids performed with temperature programming, single peaks were obtained (e.g. Umbreit *et al.*, 1972).

The present paper gives the mass spectrum of C_{55}-polyprenol phosphate. The fragmentation pattern of this compound is similar to that of solanesol phosphate (Hemming & Chojnacki, unpublished). It was shown that the characteristic mass spectrum can be obtained from the intact polyprenol phosphate.

It is not known whether the ratio of internal *cis* to *trans* double bounds in undecaprenol phosphate from *Sh. flexneri* is similar to that found in polyprenols of other bacteria, which in the few cases studied was always 8:2 (e.g. Umbreit & Strominger, 1972). A larger amount of bacterial material will be necessary for performing the n.m.r. analysis in order to establish the structure of undecaprenol from *Sh. flexneri*.

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UNDEKAPRENOLOMONOFOSFORAN — ENDOGENNY AKCEPTOR GLUKOZY PRZENOSZONEJ Z UDPGLUKOZY U *SHIGELLA FLEXNERI*

Streszczenie

Z niezmydlalnych lipidów *Sh. flexneri* 2a wyizolowano na kolumnie z DEAE--celulozy undekaprenolomonofosforan zidentyfikowany na podstawie własności chromatograficznych oraz widma masowego. Związek ten funkcjonuje jako endogenny akceptor glukozy przenoszonej z UDPglukozy przez enzym zawarty we frakcji „cell envelope". Enzym ten otrzymano w formie rozpuszczalnej po ekstrakcji osadu „cell envelope" roztworami detergentów stymulujących syntezę poliprenolomonofosfoglukozy.

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PURIFICATION AND SOME PROPERTIES OF ARGINASE FROM HUMAN LUNG *

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Arginase from human lung has been isolated and purified about
100-fold. During the purification procedure the enzyme was stabilized
by Mn^{2+}. The molecular weight determined by Sephadex G-150 gel
filtration was found to be 120 000. The enzyme is highly specific
towards L-arginine. Incubation of the enzyme with EDTA for 60 min
at pH 7.5 and 37°C results in dissociation into inactive subunits of
mol. wt. 30 000. On addition of Mn^{2+} ion to the inactivated enzyme,
the subunits reassociate into the native form of the enzyme of mol.
wt. 120 000, and the activity is restored.

Arginases from various sources have been shown to differ in their
K_m values, charge and molecular weights. The occurrence of multiple
forms of arginase (isoenzymes) within a given tissue of the animal
studied, has been reported (for ref. see Porembska, 1973).

To extend the scope of comparative investigations, in the present
work some properties of partially purified arginase from human lung
were studied.

MATERIAL AND METHODS

Material. Lungs were taken within 10 - 15 h after death of persons
20 - 40 years old, killed in traffic accidents, in whom on autopsy no
pathological changes were observed.

Chemicals. L-Arginine hydrochloride, β-guanidinopropionic acid and
α-isonitrosopropiophenone were from Sigma Chem. Co. (St. Louis, Mo.,

U.S.A.), L-α-amino-β-guanidinopropionic acid, 4-guanidinobutyric acid, L-α-amino-γ-guanidinobutyric acid · HCl, D-arginine hydrochloride, L-canavanine sulphate, L-homoarginine hydrochloride and Aquacid II were from Calbiochem (Los Angeles, Calif., U.S.A.), ninhydrin was from Reanal (Budapest, Hungary), ethylenediaminetetraacetic acid, disodium salt from Ciech (Gliwice, Poland), Whatman DEAE-cellulose DE 11 and Whatman Carboxymethyl CM-cellulose CM 11 were from Whatman Biochemicals (Maidstone, Kent, England), Sephadex G-150 and Blue Dextran 2000 were from Pharmacia (Uppsala, Sweden).

Marker proteins: bovine serum albumin, chicken ovalbumin, bovine γ-globulin (Cohn fraction II) and horse myoglobin were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Arginase assay. The activity was assayed by measuring the production of ornithine from arginine in barbitone buffer of pH 9.2. The previously used glycine buffer was found to interfere with ornithine determination. The incubation mixture (1 ml) contained 100 mM-sodium barbitone buffer, pH 9.2, 5 mM-$MnCl_2$, 20 mM-arginine and appropriate amount of enzyme preparation. After 15 min at 37°C, the mixture was deproteinized by adding 1 ml of trichloroacetic acid. To the supernatant 2 ml of conc. acetic acid and 1 ml of acid ninhydrin reagent were added, the mixture heated for 1 h in a boiling-water bath and the extinction at 515 nm determined (Gąsiorowska *et al.*, 1970).

When as substrate guanidine derivatives were used, urea was determined in the deproteinized solution after Ratner (1955).

The enzyme activity is given in units (1 μmole of product formed per min at 37°C).

Protein determination was done by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard, or spectrophotometrically by the method of Warburg as described by Layne (1957).

Polyacrylamide-gel electrophoresis. This was performed after Davis (1964) in a 7.5⁰/₀ acrylamide gel at pH 8.9, at 3 mA per gel during 1 h.

Molecular weight was estimated by gel filtration on Sephadex G-150 column (40 × 2 cm) equilibrated with 100 mM-KCl in 50 mM-Tris buffer, pH 7.5. On the column, marker proteins (5 mg each) and 4 - 6 mg of arginase preparation were applied and eluted with the equilibration buffer. The void volume of the column, as determined by Blue Dextran 2000, was 30 ml.

Enzyme purification. Unless otherwise stated, all steps in the purification procedure were carried out at 4°C.

About 24 g of human lung freed from trachea and main bronchial tubes, was washed three times with saline, minced, and homogenized (1 min at 1900 rev./min) in 5 vol. of a solution containing 5 mM-$MnCl_2$, 100 mM-KCl and 10 mM-Tris-HCl buffer, pH 7.5. The homogenate was left for 30 min with gentle stirring, and stored at -10°C for 12 h. After

thawing, the homogenate was centrifuged at 6000 rev./min and to the supernatant ammonium sulphate was added to 0.9 saturation. After 1 h the sediment was collected by 20 min centrifugation at 9000 rev./min, and suspended in 60 ml of 5 mM-MnCl$_2$ - 1 mM-mercaptoethanol - 5 mM--Tris-HCl buffer, pH 8.3. The dialysed preparation was clarified by centrifugation, applied to columns (18 × 1 cm) of DEAE-cellulose equilibrated with 5 mM-Tris-HCl buffer, pH 8.3, and eluted with the same buffer. The arginase activity was not adsorbed on the column and emerged with the buffer front (Fig. 1A). The most active fractions were pooled and concentrated using Aquacid in the presence of MnCl$_2$ to a final MnCl$_2$ concentration of about 5 mM.
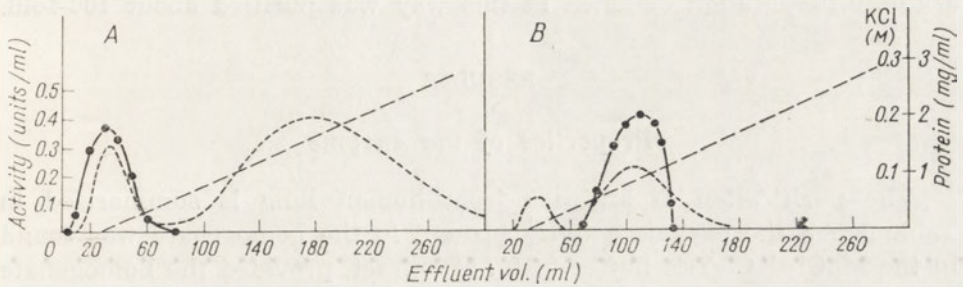

Fig. 1. Elution profile of arginase preparations on successive chromatography on A, DEAE-cellulose column and B, CM-cellulose column. A, About 400 mg of dialysed protein precipitated at 0.9 (NH$_4$)$_2$SO$_4$ sat. was applied to the DEAE--cellulose column (18 × 1 cm) equilibrated with 5 mM-Tris-HCl buffer, pH 8.3, and eluted with the same buffer and KCl concentration gradient. Fractions of 5 ml were collected. B, The active fractions from DEAE-cellulose chromatography were pooled, concentrated, dialysed and applied (38 mg protein) to a CM-cellulose column (18×1 cm) equilibrated with 5 mM-Tris-HCl buffer, pH 7.5, and eluted with the same buffer and KCl concentration gradient. Fractions of 5 ml were collected. ●, Arginase activity; - - -, protein; — — —, KCl gradient.
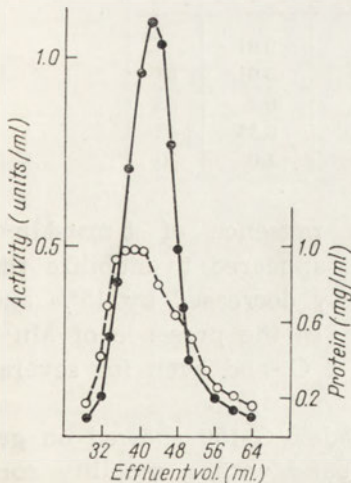

Fig. 2. Sephadex G-150 gel filtration of arginase preparation from CM-cellulose column (Fig. 1B). The active fractions (22 mg protein) were concentrated, dialysed and applied to a Sephadex G-150 column (40×2 cm) equilibrated with 100 mM-KCl - 50 mM-Tris-HCl buffer, pH 7.5, and eluted with the same buffer solution. Fractions of 2 ml were collected at a flow rate of 25 ml/h. ●, Arginase activity; ○, protein.

The concentrated arginase preparation was dialysed against 5 litres of 5 mM-$MnCl_2$ - 1 mM-mercaptoethanol - 5 mM-Tris-HCl buffer, pH 7.5, then applied to a CM-cellulose column (18 × 1 cm) equilibrated with 5 mM-Tris-HCl buffer, pH 7.5, and eluted with a KCl concentration gradient in the same buffer. The arginase activity emerged at 0.06 - - 0.1 M-KCl (Fig. 1B). The active fractions were pooled, concentrated as above, and dialysed against 2 litres of 5 mM-$MnCl_2$ - 1 mM-mercapto-ethanol - 50 mM-Tris-HCl buffer, pH 7.5, for 12 h. Then the preparation was applied to a Sephadex G-150 column (40 × 2 cm) equilibrated with 100 mM-KCl - 50 mM-Tris-HCl buffer, pH 7.5, and eluted with the same buffer (Fig. 2). The pooled active fractions were concentrated. The arginase preparation obtained in this way was purified about 100-fold.

RESULTS

Properties of the enzyme

The purification of arginase from human lung is summarized in Table 1. All the enzyme activity present in the homogenate was found in the $MnCl_2$-KCl-Tris buffer (pH 7.5) extract, provided the homogenate was kept frozen at $-10°C$ for 12 h. The arginase activity in human lung was found to be very low (0.4 - 0.9 unit/g wet wt.).

Table 1

Purification of arginase from human lung

The values for total enzyme units are normalized to a preparation from 1 kg of lung.

Step	Total activity (units)	Spec. act. (units/mg protein)	Yield (%)
Crude extract	700	0.01	
Ppt. at 0.9 $(NH_4)_2SO_4$ sat.	580	0.01	83
DEAE-cellulose chromatography	320	0.2	45
CM-cellulose chromatography	300	0.33	43
Sephadex G-150 gel filtration	210	1.0	30

In the course of arginase isolation, the presence of 5 mM-Mn^{2+} during extraction, concentration and dialysis appeared to stabilize the enzyme. On dialysis against water the activity decreased by 15% and this decrease was not reversed by Mn^{2+} ion. In the presence of Mn^{2+} ion the enzyme was stable for 4 - 6 days at 2°C, and even for several months at $-10°C$.

The pooled arginase fractions from Sephadex G-150 showed on gel electrophoresis at pH 8.9 a single protein band with a mobility cor-

responding to that of rat liver arginase. The protein band was coincident with the activity.

The molecular weight of lung arginase determined by Sephadex G-150 gel filtration, was found to be 120 000 daltons (Fig. 3).
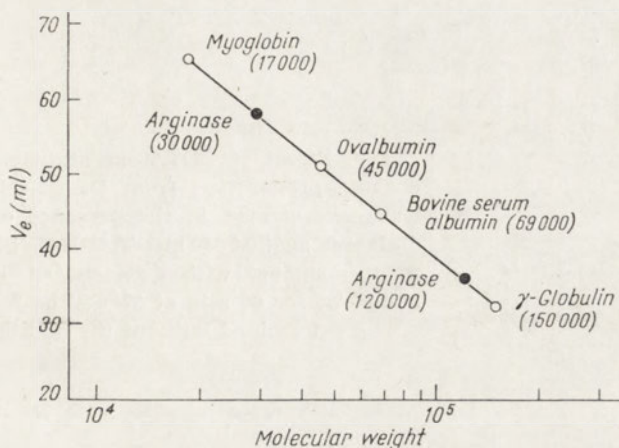

Fig. 3. Molecular weight determination by Sephadex G-150 gel filtration. Sephadex G-150 column (40×2 cm) was equilibrated with 100 mM-KCl in 50 mM-Tris-HCl buffer, pH 7.5. Native and EDTA-treated arginase preparations (4 - 6 mg protein) were applied to the column and eluted with the same buffer at a flow rate of 25 ml/h. Fractions of 2 ml were collected. Marker proteins: bovine γ-globulin, bovine serum albumin, chicken ovalbumin and horse myoglobin.

The enzyme was highly specific toward L-arginine; it did not hydro-lyse D-arginine, L-homoarginine and the tested guanidine derivatives (β-guanidinopropionate, α-amino-β-guanidinopropionate, γ-guanidino-butyrate, α-amino-γ-guanidinobutyrate); with L-canavanine only trace of the activity was observed.

The effect of EDTA on the activity and molecular weight of arginase

The experiments on the effect of EDTA were carried out with the arginase preparation obtained after DEAE-cellulose chromatography, concentrated and dialysed against 50 mM Tris-HCl buffer, pH 7.5.

Incubation at pH 7.5 for 60 min at 37° with 0.5 μmole of EDTA per 100 μg enzyme protein caused 50% inhibition, and with 4 μmoles EDTA the inhibition was complete (Fig. 4). The enzyme inactivated by an excess of EDTA (5 μmoles/100 μg protein) was fully reactivated by the addition of an equimolar amount of Mn^{2+} ion directly to the assay mixture. However, a fourfold excess of Mn^{2+} ion lowered somewhat the reactivation of arginase (Table 2). When EDTA was allowed to act at pH 7.5 for a longer time or at a higher temperature, only partial re-

activation by Mn^{2+} was observed. At 0 - 2°C EDTA had but little effect on arginase activity. At lower pH, the inactivation was not reversible by Mn^{2+} ion (Table 2).

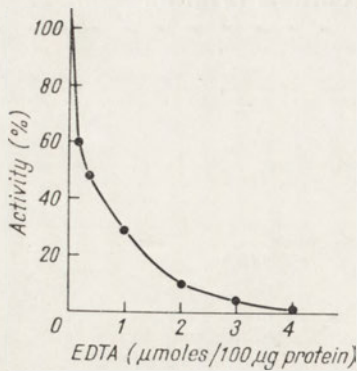
Fig. 4. Effect of EDTA on arginase activity. The enzyme preparation from DEAE-cellulose column was concentrated in the presence of 5 mM-$MnCl_2$, dialysed against 50 mM-Tris-HCl buffer, pH 7.5, then incubated with different amounts of EDTA at pH 7.5 for 60 min at 37°C. The residual activity was determined without the addition of $MnCl_2$.

Table 2

Effect of EDTA and Mn^{2+} on arginase activity

The enzyme preparation from DEAE-cellulose column concentrated in the presence of 5 mM-$MnCl_2$ and dialysed against 50 mM-Tris-HCl buffer, pH 7.5, was incubated for 60 min at 37°C, at pH 7.5 or 4, with or without EDTA. The amount of EDTA was 50 μmoles per 1 mg of protein. The arginase activity was determined in a sample containing 100 μg of protein, with or without $MnCl_2$.

	Mn^{2+} added (μmoles/sample)	Activity	
		units/sample	%
Control	0	0.2	(100)
Control	5	0.2	(100)
EDTA treatment at pH 7.5	0	0.0	0
	3	0.0	0
	5	0.2	100
	10	0.2	100
	20	0.16	80
EDTA treatment at pH 4	0	0.0	0
	5	0.0	0

The arginase inactivated by EDTA treatment separated into two fractions of mol. wt. 120 000 and 30 000 daltons when submitted to gel filtration on Sephadex G-150 equilibrated with KCl-Tris buffer (Fig. 5B). The first fraction showed some enzymic activity in the absence of Mn^{2+} but the addition of Mn^{2+} distinctly enhanced this activity. The second fraction was inactive in the absence of Mn^{2+}.

When the EDTA-inactivated preparation was chromatographed on Sephadex G-150 equilibrated with KCl-Tris buffer containing 0.05 mM-$MnCl_2$, only one active fraction with mol. wt. of 120 000 was obtained
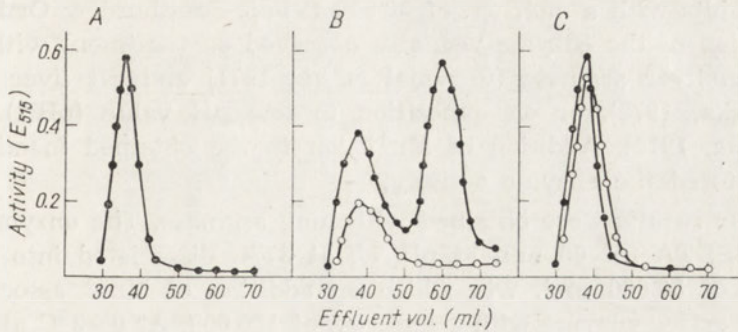
Fig. 5. Sephadex G-150 gel filtration of *A*, native and *B*, *C*, EDTA-treated arginase. The enzyme preparation from DEAE-cellulose column concentrated in the presence of 5 mM-MnCl₂ and dialysed against 50 mM-Tris-HCl buffer, pH 7.5, was inactivated by treatment with EDTA (50 μmoles per 1 mg protein) at pH 7.5 for 60 min at 37°C. The samples, 2 ml, containing 4 mg of protein were loaded on Sephadex G-150 column (40×2 cm) and eluted with 100 mM-KCl - 50 mM-Tris--HCl buffer, pH 7.5. Fractions of 2 ml were collected at a flow rate of 25 ml/h. Arginase activity measured: (●), in the presence of 5 mM-MnCl₂ and (○), in the absence of MnCl₂. *A*, *B*, the column was equilibrated with 100 mM-KCl in 50 mM-Tris-HCl buffer, pH 7.5; *C*, the column was equilibrated and protein eluted with 0.05 mM-MnCl₂ - 100 mM-KCl - 50 mM-Tris-HCl buffer, pH 7.5.

(Fig. 5C). This indicates that under these conditions Mn²⁺ present in the Sephadex G-150 column was sufficient for reassociation of the inactive subunits into the native form of the enzyme (Fig. 5A), with a molecular weight of 120 000 daltons.

DISCUSSION

The partially purified arginase from human lung is very stable in the presence of Mn²⁺ ion, and, like arginases from the tissues of other mammals, it is highly specific towards L-arginine. On polyacrylamide--gel electrophoresis at pH 8.9 the enzyme migrates slowly towards anode, resembling in this respect human (Carvajal *et al*, 1971) and rat (Porembska *et al*., 1971) liver arginase, and differing from the enzyme from horse liver (Greenberg *et al*., 1956), calf liver (Grassman *et al*., 1958), rabbit liver (Vielle-Breitburd & Orth, 1972) and rat kidney (Porembska *et al*., 1971) which have a lower isoelectric point.

The molecular weight of human lung arginase is 120 000 daltons which corresponds to the mol. wt. (110 000 - 136 000) reported for arginases from mammalian liver (for ref. see Porembska, 1973).

The tetrameric structure of liver arginase was first demonstrated by Hirsch-Kolb & Greenberg (1968). The enzyme from rat liver dissociated in 8 M-urea into inactive subunits with a mol. wt. of 30 800. Under the influence of dodecyl sulphate the enzyme from rabbit liver dissociated

into subunits with a mol. wt. of 36 500 (Vielle-Breitburd & Orth, 1972). Dissociation of the enzyme was also observed on treatment with EDTA of human liver arginase (Carvajal *et al.*, 1971) and rat liver enzyme (Porembska, 1973), or on exposition to low pH value (pH 2) at 0°C (Hosoyama, 1972). Addition of Mn^{2+} ion to the obtained inactive subunits restored the enzyme activity.

Similar results were obtained with lung arginase. The enzyme treated with EDTA for 60 min at pH 7.5 at 37°C dissociated into inactive subunits of 30 000 mol. wt., which on addition of Mn^{2+} associated to form the active enzyme with a mol. wt. of 120 000. At 0 - 2°C at pH 7.5, treatment with EDTA did not inactivate the enzyme, and in an acidic medium, at pH 4, the inactivation by EDTA was not reversed by the addition of Mn^{2+} ion even after incubation for 5 - 10 min. Thus it seems that EDTA and low pH value, which separately cause only the dissociation of the enzyme, when acting together affect the conformation of the polypeptide chains in such a way that the subunits are unable to reassociate even in the presence of Mn^{2+} ion.

The molecular weight of arginase from rat kidney, brain and submaxillary gland was found to be 120 000 daltons (Gąsiorowska *et al.*, 1970). This together with the results for lung arginase, suggests that the extrahepatic arginases do not differ in molecular weight from the liver enzyme. However, Glass & Knox (1973) demonstrated the occurrence in rat lactating mammary gland of two active forms of arginase, with mol. wt. of 42 000 and 94 000 daltons.

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WŁASNOŚCI ARGINAZY Z PŁUC CZŁOWIEKA

Streszczenie

Częściowo oczyszczona arginaza z płuc człowieka zdrowego w obecności jonów Mn^{2+} jest białkiem stabilnym. Enzym wykazuje wysoką specyficzność wobec L-argininy. Ciężar cząsteczkowy enzymu oznaczony sączeniem na Sephadex G-150 wynosi 120 000. Enzym inkubowany przez 60 min z EDTA w pH 7.5 w temp. 37°C dysocjuje na nieaktywne podjednostki o m. cz. 30 000. Dodanie jonów Mn^{2+} przywraca aktywność i powoduje reasocjację do natywnej formy enzymu o m. cz. 120 000.

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PREPARATIVE ENZYMIC SYNTHESIS OF NUCLEOSIDE-5'-PHOSPHATES *

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1. Wheat shoot phosphotransferase has been employed, with p-nitrophenylphosphate as a phosphate donor, to specifically phosphorylate the 5'-position of a variety of nucleosides and nucleoside analogues. The specificity of the enzyme towards the 5'-position of pentose nucleosides is testified to by the complete resistance to phosphorylation of 5'-O-methylcytidine. 2. With the use of ion-exchange chromatography, the foregoing procedure has been applied to the large-scale preparation of nucleoside-5'-phosphates with overall yields of the order of 80 - 90%. Quantitative recovery of unreacted nucleoside makes it possible to use this method without risk of losses either on a small or large scale with rare nucleosides. It is also applicable to acid- and alkali-labile nucleosides which cannot readily be phosphorylated by chemical procedures. 3. The wheat shoot phosphotransferase also phosphorylated a galactopyranosyl nucleoside, as well as such derivatives as 1-(β-hydroxyethyl)cytosine and 5-(β-hydroxyethyl)uracil, showing that the enzyme does not have an absolute requirement for a 5-membered sugar ring, but rather for the presence of a primary hydroxyl group. 4. The phosphorylated derivatives of galactopyranosyluracil, and of both hydroxyethyl pyrimidines, were resistant to 5'-nucleotidase. *E. coli* alkaline phosphatase converted all three nucleotides quantitatively to the starting compounds. 5. A synthesis of 1-(β-hydroxyethyl)cytosine is described.

Chemical phosphorylation of nucleosides to the 5'-monophosphates frequently involves a several-step synthesis, resulting from the need for protecting groups, with consequent reduction in yields. In some instances

the widely used cyanoethylphosphate method is applicable to unprotected nucleosides (Tener, 1961; Kulikowski et al., 1969). Undoubtedly the simplest procedure for specific phosphorylation of unprotected nucleosides at the 5'-position (Tsuchiya et al., 1965; Yoshikawa et al., 1967, 1969; Irie, 1969; Haga et al., 1971), makes use of the selective reactivity towards phosphoryl oxychloride of the primary hydroxyl group of nucleosides (Seita et al., 1972, 1973). But this procedure cannot be applied to acid-labile analogues; while the cyanoethylphosphate method cannot be used with compounds susceptible to acid and alkaline hydrolysis. An additional inconvenience of the $POCl_3$ method is the occasional formation of 2',5'- and 3',5'-diphosphates (Perini & Hampton, 1970), and phosphorylation of the aglycone (Hagenberg et al., 1973).

Although a number of observers have reported the use of phosphotransferases with a suitable phosphate donor for preparation of nucleoside-5'-phosphates (Barner & Cohen, 1959; Strider et al., 1968; Harvey et al., 1970; Janion et al., 1970; Hagenberg et al., 1973), the procedure has usually involved the use of paper chromatography for isolation of the product, with its attendant inconveniences, particularly for quantities in excess of several milligrams. Nonetheless, this procedure has been successfully applied to phosphorylation of quantities of the order of 50 - 100 mg nucleoside (Janion et al., 1970; Janion & Shugar, 1973). A more practical procedure for preparative purposes would be the use of column chromatography and one "large-scale" phosphorylation by this method has been reported, viz. 1-β-D-arabinosylcytosine-5'-phosphate, using a Dowex 1 column for isolation of the product (Cardeilhac & Cohen, 1964).

We describe here the use of a crude extract of wheat shoot phosphotransferase, originally proposed by Barner & Cohen (1959), for the specific 5'-phosphorylation of a variety of nucleosides, followed by chromatography of the reaction product on Dowex 1. This enabled quantitative recovery not only of the product of phosphorylation, but also of the unreacted starting material. Some of the attendant problems, such as specificity of the reaction, are also considered.

MATERIALS AND METHODS

Nucleosides and analogues: Cytidine and 6-chloropurine riboside were products of Pharma-Waldhof (Düsseldorf, G.F.R.). The latter compound was converted to 6-methoxypurine riboside by the method of Johnson et al. (1958), for which we are indebted to Z. Kazimierczuk. Pseudouridine was a gift from Dr. W. E. Cohn. Formycin samples were kindly donated by Dr. R. K. Robins and by Meijii Seika Kaiska (Tokyo). We are also indebted to Dr. R. K. Robins for a gift of 6-methyluridine. The 2'-O-methyl, 3'-O-methyl, 5'-O-methyl, and 2',3'-di-O-methyl deri-

vatives of cytidine were prepared as elsewhere described (Kuśmierek *et al.*, 1973), as were also the *α*- and *β*-anomers of 5-ethyl-2'-deoxycytidine (Kulikowski & Shugar, 1974). The sample of 1-*β*-D-galactopyranosyluracil was that previously employed for spectral investigations (Fox & Shugar, 1952). We are indebted to Krystyna Bolewska for a sample of 5-(*β*-hydroxyethyl)uracil, prepared according to the procedure of Chkhikvadze & Magidson (1964).

1-(*β*-Hydroxyethyl)cytosine was obtained as follows: N^4-acetylcytosine (765 mg, 5 mmoles), prepared according to Wheeler & Johnson (1903), was suspended in 30 ml dimethyl sulphoxide, followed by addition of 5 mmoles (690 mg) anhydrous K_2CO_3 and 5.5 mmoles (400 μl) 2-bromoethanol. The mixture was heated, with constant stirring, on an oil bath at 120 - 130°C for 3 h, cooled to room temperature, 20 ml water added, and the pH brought to about 7 by addition of dilute HCl. The reaction mixture was then brought to dryness under reduced pressure, and the residue dissolved in chloroform and loaded on 20×2 cm column of Al_2O_3 in chloroform. The column was washed with chloroform until removal of dimethyl sulphoxide was complete. Elution was then carried out with chloroform-methanol (85:15, v/v). The fractions containing the product were pooled, brought to dryness, and the residue crystallized from aqueous methanol to give 450 mg (50%) of 1-(*β*-hydroxyethyl)-N^4-acetylcytosine in the form of colourless needles, m.p. 232 - 233°C. The product was dissolved in 45 ml ammoniacal methanol and stored at room temperature for 4 h. The solution was then brought to dryness under reduced pressure and the residue crystallized from anhydrous ethanol to yield 330 mg (94%) of 1-(*β*-hydroxyethyl)cytosine as colourless platelets, m.p. 209 - 212°C, chromatographically homogeneous (Table 1). The spectral properties of the product were similar to those of 1-methylcytosine (Fox & Shugar, 1952) with the exception that, at pH 14 (1M-NaOH), there was a slight shift in the spectrum due to ionization of the primary hydroxyl of the hydroxyethyl group, an effect which is observed also with nucleosides due to dissociation of the sugar hydroxyls (Fox & Shugar, 1952).

All compounds were in crystalline form, chromatographically homogeneous in several solvent systems, and exhibited correct spectral properties.

The sodium salt of p-nitrophenylphosphate, used as a phosphate donor, was obtained from Serva (Heidelberg, G.F.R.) and Merck (Darmstadt, G.F.R.).

Enzymes: Purified snake venom 5'-nucleotidase was obtained from Sigma (St. Louis, Mo., U.S.A.), and *E. coli* alkaline phosphatase from Worthington (Freehold, N.J., U.S.A.). Wheat shoot phosphotransferase was prepared as follows: Wheat shoots were cultivated for 5 - 7 days in the dark at room temperature on a cellulose medium saturated with

distilled water. The shoots were homogenized with a high-speed homo-
genizer with a tenfold volume of 0.02 M-acetate buffer, pH 4. The
homogenate was passed through a gauze filter and the clear filtrate was
stored at -20°C. Under these conditions, there was no loss of enzymic
activity over a period of several months. Protein content of the filtrate
was determined by the method of Kalckar (1947).

Chromatography: Paper chromatography, ascending, was with
Whatman no. 1 paper; and thin-layer chromatography with Merck
(Darmstadt, G.F.R.) PF_{254} plates. Solvent systems were (*A*) isopropanol -
- conc. NH_4OH - water (7:1:2, v/v), and (*B*) ethanol - 1 M-ammonium
acetate, pH 5.0 (5:2, v/v).

Column chromatography made use of Sigma (St. Louis, Mo., U.S.A.)
Dowex 1X2 (Cl^-) (200 × 400 mesh) and Bio-Rad (Richmond, Ca., U.S.A.)
Dowex 1X4 (Cl^-) (200 × 400 mesh). Analytical columns were 8 × 1.2 cm,
and preparative columns 20×1.5 cm; both were prepared by equilibration
with 1 M-$HCOONH_4$ or NH_4HCO_3 and then washed with water. An LKB
(Stockholm, Sweden) Ultrograd-Uvigrad assembly was used for pre-
paration of elution gradients and u.v. control of eluates at 283 nm.

U.v. absorption spectra were run on a Zeiss (Jena, G.D.R.) VSU-2P
instrument or a Unicam (Cambridge, England) SP-8000 recording
spectrophotometer. A Radiometer PHM-4d compensating instrument,
with glass electrode, was employed for pH measurements.

Products of phosphorylation were tested by treatment with 5'-
-nucleotidase as described by Kuśmierek & Shugar (1973), and with
E. coli alkaline phosphatase according to Garen & Levinthal (1960),
followed by chromatography to identify the product(s) of hydrolysis.

RESULTS

For purposes of simplicity the results are grouped into several
subsections dealing with conditions of phosphorylation, isolation of the
products of phosphorylation, and the yields obtained with various
nucleosides and related compounds.

Phosphorylation conditions: The nucleoside and *p*-nitrophenyl-
phosphate were both dissolved in a volume of water equal to half that
employed for incubation. This solution was brought to pH about 4 by
careful addition of 50% acetic acid. To this solution was added an equal
volume of enzyme solution containing 6.6 mg protein/ml. The final
concentrations of the components of the reaction mixture were: nucleoside
substrate, 0.02 M; *p*-nitrophenylphosphate, 0.3 M; enzyme protein,
3.3 mg/ml; acetate buffer, pH 4, 0.6 M.

Incubation was at 37°C for periods of up to 24 h. The progress of
the reaction was followed by paper chromatography with solvents *A*
and *B*, with which the R_F values of the nucleosides were 0.4 or higher,

and the nucleotides about 0.1 to 0.3 (Table 1). Elution of the appropriate spots, followed by spectrophotometry, gave the phosphorylation yield as a function of time of incubation. In some instances recovery and estimation of the amount of unreacted nucleoside was hampered by the fact that its R_F was close to that of liberated p-nitrophenol, e.g. both anomers of 5-ethyl-2'-deoxycytidine (Table 1). In these instances a small column was used for separation and estimation of the constituents.

Table 1

Ascending paper chromatography of starting compounds and their phosphorylated derivatives

Whatman paper no. 1 and solvents A and B were used. The Roman numeral I refers to the non-phosphorylated derivative, and II to the phosphorylated analogue.

| Compound | R_F with solvent system | | | |
| | A | | B | |
	I	II	I	II
Cytidine	0.50	0.09	0.64	0.20
2'-O-Methylcytidine	0.64	0.12	0.76	0.22
3'-O-Methylcytidine	0.64	0.12	0.76	0.22
2',3'-Di-O-methylcytidine	0.74	0.16	0.85	0.26
5'-O-Methylcytidine	0.64	—	0.76	—
β-Pseudouridine	0.44	0.07	0.71	0.28
Formycin	0.41	0.08	0.63	0.22
6-Methyluridine	0.50	0.10	0.73	0.32
α-5-Ethyl-2'-deoxycytidine	0.71	0.15	0.77	0.25
β-5-Ethyl-2'-deoxycytidine	0.71	0.15	0.77	0.25
6-Methoxypurine riboside	0.77	0.17	0.83	0.30
1-β-D-Galactopyranosyluracil	0.37	0.08	0.68	0.27
1-(β-Hydroxyethyl)cytosine	0.65	0.11	0.73	0.32
5-(β-Hydroxyethyl)uracil	0.57	0.09	0.73	0.27
p-Nitrophenol	0.73	—	0.95	—
p-Nitrophenylphosphate	—	0.33	—	0.48

Time-course of reaction: The course of phosphorylation as a function of incubation time, as followed by paper chromatography, is illustrated in Fig. 1 for several nucleosides. Two points of interest emerge here. First, that maximum yields of nucleotide are obtained after about 10 h; further prolongation of incubation time is not only not advantageous, but may even lead to a small reduction in overall yield. Second, that incubation time for maximal yield of nucleotide is apparently little dependent on the nature of the nucleoside acceptor; it is rather the rate of phosphate transfer which varies with the nucleoside acceptor.

Isolation of nucleoside-5'-phosphates: Following incubation, the reaction was terminated by heating to 100°C for several seconds. The

reaction mixture was then cooled and extracted several times with ether to remove liberated p-nitrophenol. The aqueous phase was diluted fivefold with water and loaded on a column of Dowex 1X2 (HCO_3^- or $HCOO^-$) (200 × 400 mesh). The column was then washed with water to quantitatively remove unreacted nucleoside.
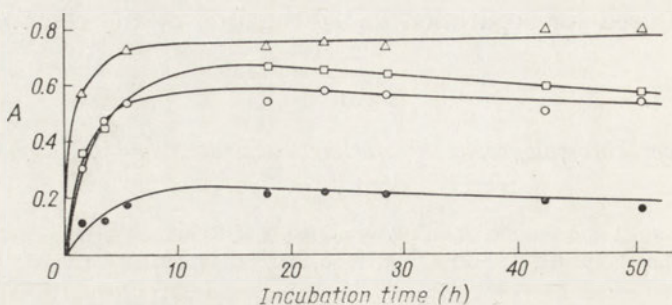

Fig. 1. Time-course of enzymic phosphorylation of several nucleosides with wheat shoot phosphotransferase, using p-nitrophenylphosphate as phosphate donor. The ordinate scale gives the optical density of an aliquot of the nucleoside-5′-phosphate at its λ_{max}; for values of absolute yields see Table 2. Incubation conditions as in text: (□), β-pseudouridine; (○), 1-β-D-galactopyranosyluracil; (●), α-5-ethyl-2′--deoxycytidine; △, β-5-ethyl-2′-deoxycytidine.

The recovered nucleoside may be concentrated to smaller volume, and subjected once again to phosphorylation, without removal of salt, since the phosphotransferase reaction is little affected by the ionic strength of the solution. Or the nucleoside solution may be simply desalted on Sephadex, on activated charcoal, or on an ion exchanger by elution with carbonate or formate, which may be subsequently readily removed under reduced pressure.

The nucleoside-5′-phosphate is then eluted from the Dowex column with a gradient of acid ammonium carbonate or formic acid. For a column in the carbonate form, acid ammonium carbonate was employed for purposes of simplicity to avoid the technical complications involved in the preparation of acid triethylammonium carbonate. Following elution of the nucleotide, its ammonium carbonate solution was concentrated to small volume, a slight excess of triethylamine was added, and the buffer (as acid triethylammonium carbonate) was quantitatively removed by 2 to 3 evaporations with methanol (Symons, 1973). The resulting triethylammonium salt of the nucleotide was converted, with the aid of a Dowex 50W column, to the sodium salt or free acid and, following concentration once again to small volume, precipitated by addition of ethanol-acetone. In those instances where it was possible to do so, a column in the formate form was employed (Fig. 2). The eluted nucleotide in formic acid was concentrated to small volume under reduced pressure,

and then evaporated several times from small volumes of water to remove formic acid. The free acid nucleotide was then precipitated from a concentrated aqueous solution by addition of ethanol or ethanol--acetone.
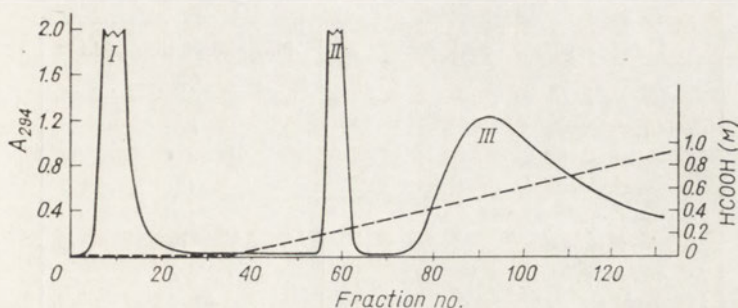

Fig. 2. Fractionation of the phosphorylation products of formycin with a formic acid gradient, on an 8.5×1.5 cm column of Dowex 1X2 (HCOO⁻). Following termination of the reaction, liberated p-nitrophenol was removed by ether extraction. *I*, Unreacted formycin; *II*, formycin-5'-phosphate; *III*, unreacted p-nitrophenyl-phosphate.

Unreacted p-nitrophenylphosphate, under the foregoing conditions, is strongly retained on the column (Fig. 2), and is eluted only at higher concentrations of formic acid.

It should be noted that, if liberated p-nitrophenol is not removed from the reaction mixture by ether extraction prior to loading on the column, then the p-nitrophenol is eluted stepwise throughout the entire gradient in Fig. 2.

Phosphorylation yields: Phosphorylation yields, following incubation for approximately 10 - 12 h, are given in Table 2 for a variety of nucleosides which include α- and β-anomers; one nucleoside in the *syn* conformation, 6-methyluridine (Schweizer *et al.*, 1971); two nucleosides with C—C glycosidic linkages, of which one is presumed to be in the *syn* conformation, formycin (Ward *et al.*, 1969); one with a 6-membered pyranosyl sugar ring; another, 6-methoxypurine riboside, which is acid--labile; and several in which the sugar hydroxyls are etherified.

Some interesting conclusions emerge from the data in Table 2. The complete absence of phosphorylation of 5'-*O*-methylcytidine, even on prolongation of the incubation time, testifies to the high specificity of the enzyme for the 5'-hydroxyl of ribonucleosides. The fact that 1-β-D--galactopyranosyluracil undergoes phosphorylation in high yield demonstrates that the specificity is directed not so much *versus* a 5'-hydroxyl, but rather a primary hydroxyl (see below).

Table 2

Phosphorylation yields of various nucleosides and analogues with wheat shoot phosphotransferase system

Yields are given in % of nucleoside added to incubation medium.

Nucleoside or analogue	Phosphorylation yield (%)
Cytidine	60
2′-O-Methylcytidine	41[a]
3′-O-Methylcytidine	12
2′,3′-Di-O-methylcytidine	10
5′-O-Methylcytidine	0
β-Pseudouridine	36
Formycin	40 - 55
6-Methyluridine	45
α-5-Ethyl-2′-deoxycytidine	13
β-5-Ethyl-2′-deoxycytidine	43
6-Methoxypurine riboside	47
1-β-D-Galactopyranosyluracil	38
1-(β-Hydroxyethyl)cytosine	4.5
5-(β-Hydroxyethyl)uracil	3 - 4
2-Aminopurine riboside	60[b]

[a] Yield under similar conditions previously reported (Janion *et al.*, 1970) was 45 - 50%.
[b] Reported by Janion & Shugar (1973) under similar conditions.

The lower yield (13%) for α-5-ethyl-2′-deoxycytidine, as compared to 43% for β-5-ethyl-2′-deoxycytidine, should not be generalized, since there are other reports on the phosphorylation of α-anomers in reasonably good yields (e.g. Tunis & Chargaff, 1960). From the relatively high yields for formycin and 6-methyluridine, it is clear that the enzyme does not discriminate against a nucleoside in the *syn* conformation, nor against one with a glycosyl C—C bond.

The appreciably lower yield of phosphorylation of 3′-O-methylcytidine and 2′,3′-di-O-methylcytidine ($\sim 10\%$) as compared to 2′-O-methylcytidine ($\sim 40\%$), is undoubtedly due to steric hindrance by the 3′-O-methyl group. This is in accord with the observation that 3′-O-methylcytidine is poorly phosphorylated chemically with $POCl_3$ (J. T. Kuśmierek, personal communication). Also in accord with this is the finding of Tunis & Chargaff (1960) that nucleoside-3′-phosphates are completely resistant to carrot phosphotransferase, an effect clearly due to the larger bulk of the phosphate substituent as compared to a methyl.

Particularly interesting were the results obtained with two non-nucleoside model compounds, each containing a primary hydroxyl group, viz. 1-(β-hydroxyethyl)cytosine and 5-(β-hydroxyethyl)uracil

(Scheme 1). Under standard phosphorylation conditions, as applied to the nucleosides, both of these underwent phosphorylation with yields varying from 3 to 4.5% (Table 2).
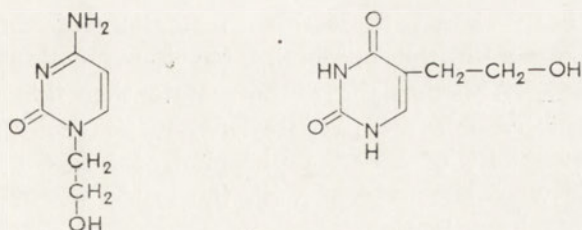
Scheme 1

Both of the foregoing phosphorylated derivatives were fully resistant to snake venom as well as to purified 5'-nucleotidase. To check whether they were indeed phosphorylated derivatives, each was subjected to the action of *E. coli* alkaline phosphatase, which resulted in quantitative conversion to the starting compound, as shown by chromatography in several solvent systems in addition to those indicated in Table 1.

Large-scale phosphorylations: Two nucleosides were selected for trial "large-scale" phosphorylations, viz. formycin (1 mmole, 265 mg) and 6-methyluridine (0.25 mmole, 60 mg), with regeneration of unreacted nucleoside by column chromatography as described above, followed by repeated phosphorylation. In this way three cycles of phosphorylation gave overall yields for both phosphorylated nucleosides of more than 80%.

DISCUSSION

Some instances have been reported of very high phosphorylation yields with phosphotransferase enzymes, e.g. Harvey *et al.* (1970) reported more than 90% phosphorylation of thymidine with the carrot enzyme, but this required the presence of a 40-fold excess of the p-nitrophenylphosphate donor. The same authors state that their average yields with some nucleosides ranged between 40% and 50% following 24 h incubation, hence not substantially different from our results with the wheat shoot enzyme.

Furthermore, the application of column chromatography for isolation of the purified nucleotide products, and recovery of unreacted nucleosides, is shown in the present study to be a fully practical and simple procedure for the preparation of a variety of nucleoside-5'--phosphates, either on a small or large scale. It is also applicable to acid- or alkali-labile nucleosides (e.g. 6-methoxypurine riboside), and may be employed without risk of unforeseen losses with either small or large samples of rare nucleosides.

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Some further comment is called for with regard to the enzyme specificity. The complete absence of phosphorylation with 5'-O-methylcytidine as substrate testifies to the specificity for the 5'-position (cf. Harvey *et al.*, 1970). However, long incubation periods with the carrot enzyme have been shown to lead to formation of the 2'- and 3'--nucleotides along with the predominating 5'-nucleotide (Brunngraber & Chargaff, 1967). Although the authors state that this occurred only with highly purified enzyme preparations, it points to the need for checking the specificity of a carrot phosphotransferase preparation. No such difficulties were encountered with the crude wheat shoot enzyme employed in this investigation.

The results obtained with 1-(β-hydroxyethyl)cytosine and 5-(β--hydroxyethyl)uracil demonstrate that the specificity of the wheat shoot enzyme is not limited to a 5-membered sugar ring system with a hydroxymethylene group, as suggested by Harvey *et al.* (1970) for the carrot enzyme, but is considerably broader. This is further underlined by the high phosphorylation yield with 1-β-D-galactopyranosyluracil as substrate (Table 2), although this broader specificity may possibly be limited to the wheat shoot enzyme. Wu & Chargaff (1969) reported phosphorylation of 9-β-D-glucopyranosyladenine with the carrot enzyme, but Harvey *et al.* (1970) were unable to confirm this with their preparation of the same enzyme.

It appears reasonable to conclude from our findings that the enzyme requires the presence of a primary hydroxyl group. It is consequently of interest to note that glucose was found to be converted to glucose--6-phosphate by the phosphotransferase activity associated with *E. coli* phosphatase, using pyrophosphate as a donor (Anderson & Nordlie, 1967). The same enzymic system has been shown to also phosphorylate Tris buffer and various aminoalcohols (Dayan & Wilson, 1964; Wilson *et al.*, 1964).

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PREPARATYWNA ENZYMATYCZNA SYNTEZA NUKLEOZYDO-5'-FOSFORANÓW

Streszczenie

1. Fosfotransferazę z kiełków pszenicy zastosowano do specyficznej fosforylacji 5'-pozycji różnych nukleozydów i ich analogów, używając *p*-nitrofenylofosforanu jako donora grupy fosforanowej. Specyficzność enzymu wykazano poprzez całkowitą oporność 5'-*O*-metylocytydyny na fosforylację.

2. Użycie chromatografii jonowymiennej pozwoliło na zastosowanie powyższej metody do preparatywnej syntezy nukleozydo-5'-fosforanów z ogólną wydajnością 80 - 90%. Ilościowe odzyskanie nieprzereagowanego nukleozydu umożliwia zastosowanie powyższej metody, na małą lub dużą skalę, do fosforylacji rzadkich nukleozydów bez ryzyka strat. Metodę tę można również stosować używając jako substratów kwaso- i alkali-labilnych nukleozydów, które nie mogą być fosforylowane metodami chemicznymi.

3. Fosfotransferaza z kiełków pszenicy fosforyluje również galaktopiranozylo nukleozyd, jak też takie pochodne jak 1-(β-hydroksyetylo)cytozynę i 5-(β-hydroksyetylo)uracyl, co wskazuje na to, że enzym nie jest absolutnie specyficzny do

pięcioczłonowego pierścienia cukrowego a raczej do I-rzędowej grupy hydroksylowej.

4. Fosforylowane pochodne galaktopiranozylouracylu i obu hydroksyetylowych pirymidyn były oporne na działanie 5'-nukleotydazy. Fosfataza alkaliczna z *E. coli* hydrolizuje ilościowo wszystkie trzy pochodne do związków wyjściowych.

5. Opisano syntezę 1-(β-hydroksyetylo)cytozyny.

RECENZJE KSIĄŻEK

THE MOLECULAR REPLACEMENT METHOD. A collection of Papers on the
Use of Non-Crystallographic Symmetry. M. G. Rossmann (Editor). International
Science Review Series. Gordon and Breach, Science Publishers, New York, London,
Paris 1972; stron VIII+267; cena £ 6,25.

Redaktor tej książki, Michael G. Rossmann, jest znanym badaczem w dzie-
dzinie rentgenografii strukturalnej białek, pracującym na Uniwersytecie Purdue,
w U.S.A. Jest on m.in. twórcą rozwijającej się od kilku lat metody t.zw. „zastą-
pienia molekularnego", która pozwala na określanie lub porównywanie struktur
białkowych na podstawie dyfrakcji promieni rentgenowskich i która jest uzupeł-
nieniem lub alternatywą stosowanej powszechnie metody podstawień izomorficz-
nych.

Informacja, jaką zawiera w sobie dyfraktogram pojedynczego kryształu biał-
kowego, nie pozwala na bezpośrednie wyliczenie położeń atomów w komórce
elementarnej (a stąd w cząsteczce białka), brak jest bowiem informacji o fazach
poszczególnych rozproszonych promieni rentgenowskich. Tej informacji nie może
dać obraz fotograficzny ani licznik promieniowania; obie te metody mierzą jedynie
natężenia (a więc pośrednio amplitudy), a nie fazy promieni. Ten niedostatek
danych ominął Perutz, wprowadzając w latach pięćdziesiątych metodę podstawień
izomorficznych. Jeżeli jesteśmy w stanie otrzymać pochodne badanego białka, za-
wierające metale ciężkie w ściśle określonych miejscach cząsteczki, i białka takie
krystalizują w tej samej sieci przestrzennej (są izomorficzne), to sumaryczna in-
formacja — z dyfraktogramów białka wyjściowego i kilku pochodnych izomorficz-
nych — pozwala już na wyliczenie faz, a w dalszej konsekwencji na ustalenie po-
łożeń atomów (z wyjątkiem atomów wodoru) w cząsteczce.

Sukces tej metody w poszczególnych przypadkach zależy od „alchemii" — jak
wyraża się Rossmann we wstępie książki — uzyskania dobrych pochodnych izo-
morficznych. Około roku 1960 zauważył on, że skoro liczne białka posiadają pełne
cząsteczki złożone z kilku identycznych, lub prawie identycznych podjednostek,
to dyfraktogram takiego kryształu zawiera w pewnym sensie „nadmiar" infor-
macji, ponad minimum niezbędne dla określenia struktury pojedynczej podjed-
nostki. Zasadniczą ideą Rossmanna było, iż nadmiar ten można wykorzystać dla
określenia faz, omijając w ten sposób konieczność preparowania pochodnych izo-
morficznych, lub wykorzystując znacznie mniejszą ich liczbę.

Aby idea ta dała praktyczne rezultaty należało opracować programy pozwala-
jące na drodze operacji matematycznych (translacji i rotacji podjednostek) prze-
kształcać diagramy gęstości elektronowych (lub diagramy Pattersona) jednych pod-
jednostek w inne, związane z nimi elementami nie-krystalograficznej symetrii.
Opracowanie właściwych programów komputerowych i odpowiedniej metodyki
obliczeniowej stanowiło główny problem w rozwoju tej metody i zagadnienia te
są nadal opracowywane.

W analogiczny sposób metodę zastąpienia molekularnego można stosować, gdy
zamiast jednego rodzaju kryształów białka, składającego się z identycznych pod-

jednostek, mamy białko (niekoniecznie o strukturze podjednostkowej) krystalizujące w kilku formach różniących się typem sieci przestrzennej. Rozwinięcie tej metody w innym kierunku pozwoliło na określanie identyczności lub stopnia podobieństwa podjednostek drobin białkowych, lub fragmentów drobin niżej cząsteczkowych.

Omawiana książka daje opis podstaw metody zastąpienia molekularnego i teksty oryginalnych prac, obrazujące jej rozwój i zastosowania. W kilkustronicowym wstępie przedstawił Rossmann zasadnicze idee tej metody i historię (niekiedy anegdotyczną) ich rozwoju. W części A omówiony jest bardziej szczegółowo problem funkcji rotacji, ilustrowany pięcioma pracami oryginalnymi. W części B omówiony jest problem translacji, ilustrują go zaś cztery prace. Część C zawiera teksty sześciu prac dotyczących aparatu matematycznego metody. W części D podano teksty 17 prac (w tym dwa manuskrypty prac nie opublikowanych do czasu wydania książki) dotyczących zastosowań tej metody do badania struktur konkretnych białek oraz kilku substancji niskocząsteczkowych — alkaloidów. Sam Rossmann jest współautorem ponad 1/3 zebranych tu publikacji.

Książka ta ze względu na formę jest raczej nietypowym dziełem, ale pomysł takiego wydawnictwa należy uznać za szczęśliwy. Uzyskaliśmy żywą ilustrację narodzin i rozwoju nowej idei w dziedzinie krystalografii białek. Książka daje czytelnikom interesującym się tym zagadnieniem gotową, pełną dokumentację piśmiennictwa do r. 1972. Metoda ta rozwija się nadal i przynosi szereg praktycznych korzyści, czego dowodem są dalsze publikacje, nie ujęte już w tej książce, jakie ukazały się w piśmiennictwie naukowym od czasu jej wydania.

Książka przeznaczona jest dla krystalografów, głównie dla tych, którzy zajmują się strukturami białek. Skorzystają z niej niewątpliwie także krystalografowie zajmujący się innymi typami substancji, a także badacze struktur białek nie będący krystalografami, lecz posiadający znajomość podstaw techniki rentgenografii strukturalnej.

Andrzej Morawiecki

H. Sajonski, A. Smollich: ZELLE UND GEWEBE. Eine Einführung für Mediziner und Naturwissenschaftler, 2 Auflage. S. Hirzel Verlag, Leipzig 1973; str. 274 + viii; cena 33.90 DM.

Zelle und Gewebe jest drugim wydaniem podręcznika ocenianego uprzednio w *Acta Biochimica Polonica*. W porównaniu z pierwszym wydaniem podręcznik został rozszerzony o rozdział poświęcony budowie chemicznej żywej materii, a w rozdziale zajmującym się strukturą komórki wprowadzono kilka nowych podrozdziałów.

W części pierwszej poświęconej metodom badawczym autorzy przedstawiają współczesne metody, dostępne nowoczesnemu badaczowi struktury, poprzez różne rodzaje mikroskopów do skaningowego włącznie z jednej strony, a histochemię, autoradiografię i immunohistochemię z drugiej. W tym rozdziale, zapoznającym studenta za wspomnianymi wyżej współczesnymi metodami, brak jest wprowadzających wiadomości do techniki hodowli tkanek i frakcjonowania komórek do badań biochemicznych, metod bez których uzyskanie całego szeregu wyników podawanych w dalszych rozdziałach omawianego podręcznika byłoby niemożliwe. Rozdział zakończony jest sięgającym do 1972 r. spisem piśmiennictwa będącego podstawą opracowania.

Bardzo cenną nowością w podręczniku jest rozdział wprowadzający w budowę chemiczną tkanek żywych.

Część podręcznika o budowie komórki przedstawia strukturę organelli komórkowych, podkreślając strukturalne odpowiedniki stanów czynnościowych oraz sprzężone z frakcjami komórkowymi aktywności biochemiczne. W rozdziale tym omówiony jest bardzo szeroko i wyczerpująco podział komórkowy.

W ostatniej, najszerszej części podręcznika przedstawiona jest budowa tkanek z podkreśleniem mechanizmu ich czynności.

Podręcznik zakończony jest spisem literatury do dwóch ostatnich rozdziałów oraz indeksem rzeczowym.

Praca jest bogato ilustrowana schematami, licznymi mikrofotografiami i elektronogramami, co bardzo ułatwia zrozumienie i przyswojenie podawanych wiadomości. Jednak część elektronogramów budzi zastrzeżenia swą jakością techniczną. Poza tym podręcznik zyskałby na zamieszczeniu większej liczby zdjęć wykonanych przy pomocy mikroskopu elektronowego.

Podręcznik został pomyślany jako wprowadzenie dla studentów w strukturę i funkcję organizmu na poziomie tkanki, komórki i organelli komórkowych w połączeniu z podstawami biologii molekularnej. W przekonaniu recenzenta cel postawiony przez autorów został osiągnięty; podręcznik może służyć jako dobre źródło podstawowych wiadomości o komórce.

Teresa Żawrocka-Wrzołkowa

W. W. C h r i s t i e, LIPID ANALYSIS. Pergamon Press, Oxford, New York, Toronto, Sydney, Braunschweig 1973; str. 338; cena £ 6.00.

Omawiana książka jest nowością na rynku wydawniczym. Przeznaczona jest głównie dla biochemików zajmujących się dziedziną lipidów. Może stanowić również dobre źródło informacji z tej dziedziny dla biochemików innych specjalności ze względu na jasny i wyczerpujący sposób informacji.

Cenną zaletą książki jest zwięzłość w opisach izolowania i identyfikacji lipidów i ich składowych, uwzględnienie dużej ilości nowych metod, głównie fizykochemicznych metod spektroskopowych, oraz zwrócenie uwagi na korzyści z ich stosowania. Szereg tradycyjnych metod analizy i preparatyki lipidów zostało omówionych przez autora w sposób krytyczny. Całość materiału książki stanowi pełny, wszechstronny i systematyczny wykład o współczesnych, wartościowych i użytecznych metodach stosowanych w biochemicznych laboratoriach lipidowych. Szereg metod preparatyki i analizy lipidów i ich składowych opracowano w formie użytecznych, praktycznych opisów ich wykonania, przy czym, jak zaznaczono, podana wersja została sprawdzona w laboratorium autora. Książka zawiera dużo materiału ilustracyjnego oraz obszerny spis literatury źródłowej obejmujący rok 1972.

Spis tytułów rozdziałów książki obejmuje wszystkie zagadnienia, z którymi można się zetknąć w biochemicznym laboratorium lipidowym (Rozdz. 1 - 10): budowa chemiczna i występowanie lipidów; izolowanie lipidów z tkanek; ogólne zasady chromatograficznej i spektroskopowej analizy lipidów; preparatyka lotnych pochodnych lipidów; analizy kwasów tłuszczowych; analizy prostych lipidów; analizy lipidów złożonych; analizy molekularne rodzin lipidów; enzymatyczne hydrolizy lipidów; analizy lipidów znakowanych izotopami promieniotwórczymi.

Książka stanowi wartościową pozycję wydawniczą i będzie bardzo użyteczną dla biochemików zajmujących się dziedziną lipidów, jak również dla pracowników innych specjalności.

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ACTA BIOCHIMICA POLONICA

PRENUMERATA KRAJOWA

PRENUMERATA ZAGRANICZNA