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ACTA BIOCHIMICA POLONICA

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EFFECT OF DIFFERENT FACTORS ON LIVER THYMIDINE MONOPHOSPHATE KINASE *IN VIVO* AND THE SYNTHESIS OF THYMIDINE 5′-TRIPHOSPHATE *IN VITRO*

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1. Infiltration of liver with lymphoblasts in leukaemic mice results in four- to sevenfold enhancement of liver thymidine monophosphate kinase. The enzyme is inactivated rapidly during incubation of the cell-free extract. The optimum conditions for stabilization of the enzyme with thymidine *in vitro* have been established. 2. Increased synthesis of thymidine 5′-phosphates is observed in the cell-free extract from the 48-h regenerating liver of rats which had received L-tryptophan 24 h after partial hepatectomy. The enhancement of thymidine and thymidine monophosphate kinase activities is associated with a simultaneous decrease in the activity of thymidine monophosphate 5′-nucleotidase. The changes in enzymic activities are prevented by 5-azacytidine.

In the synthesis ofdeoxyribonucleic acids, the enzymes responsible for the synthesis of thymidine 5′-phosphates are of special importance (Ives, Morse & Potter, 1963; Bresnick, Thompson, Morris & Lievelt, 1964). The increased activity of these enzymes is noted in the rapidly proliferating tissues e.g. in regenerating liver (Bollum & Potter, 1959; Fausto & Lancker, 1965), in some tumours (Bianchi, Buther, Crathorn & Shooter, 1961; Sneider & Potter, 1969) and in embryonal tissue (Hiatt & Bojarski, 1960).

TMP-kinase[1] (ATP:thymidine monophosphate phosphotransferase, EC 2.7.4.9) is considered a key regulatory enzyme (Davidson, 1969) in the synthesis of thymine nucleotides. This enzyme participates in the conversion of TMP to TTP by the intermediary formation of TDP (Ives, 1965), which is rapidly equilibrated with TTP by the highly active diphosphonucleoside kinase.

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[1] Abbreviation used: TMP-kinase, thymidine monophosphate kinase.

In our previous work (Čihák, Veselá & Šorm, 1968) we have demonstrated that the enhancement of the activity of TMP-kinase in regenerating rat liver can be completely prevented by administration of 5-azacytidine. Following oral administration of L-tryptophan an opposite effect was observed: both thymidine phosphorylation and synthesis of TTP were increased (Čihák, Seifertová & Veselý, 1971a).

In this report we are dealing with phosphorylation of thymidine and TMP-kinase activity under various conditions *in vitro* and *in vivo*. Since in adult liver tissue the activity of this enzyme is low, we have chosen as a convenient source the liver of leukaemic mouse and the regenerating rat liver.

MATERIALS AND METHODS

Chemicals. Thymidine 5′-phosphate, thymidine 5′-triphosphate and adenosine 5′-triphosphate were purchased from Calbiochem (Los Angeles, Calif., U.S.A.). 1-(2′-Deoxy-*a*-D-ribofuranosyl)thymidine and 1-(2′-deoxy-*β*-L-ribofuranosyl)thymidine were synthesized in this Institute. [2-^{14}C]Thymidine (25 μCi/μmol) was supplied by the Institute for Research, Production and Uses of Radioisotopes (Prague, Czechoslovakia). [2-^{14}C]Thymidine 5′-phosphate was prepared enzymically as described by Šorm, Veselý & Čihák (1966). L-Tryptophan was a product of Loba-Chemie (Vienna, Austria).

Animals. AKR inbred female mice (weighing 22 - 24 g) kept under standard conditions were inoculated intraperitoneally with 2×10^7 leukaemic cells. The degree of infiltration was evaluated using liver imprint preparations stained with May-Grünwald-Giemsa. Partial hepatectomy in rats was performed under ether narcosis (Higgins & Anderson, 1931) on groups of 6 - 8 female animals (170 - 175 g). L-Tryptophan was dissolved in 1 N-NaOH, neutralized with 1 N-HCl to pH 8.5 - 9.0 and administered by intubation in a maximum volume of 5 ml under ether narcosis. Before being killed, the animals received standard chow and drinking water *ad libitum*.

Liver cell-free extracts. The animals were killed by decapitation. The excised livers were homogenized with simultaneous cooling in a glass homogenizer with a tight-fitting Teflon pestle in 3 vol. (w/v) of ice-cold 0.025 M-tris-HCl buffer, pH 7.5, containing 0.005 M-Mg^{2+} and 0.025 M-KCl. The homogenate was centrifuged (10 000 *g*, 20 min, 3°C), the fat layer was removed and the supernatant fraction was directly used for the enzyme assay.

Enzyme assay. Thymidine kinase and TMP-kinase were assayed at 40°C by incubation of 0.1 ml of the enzyme extract for 10 or 15 min in the reaction mixture (0.5 ml) containing 40 mM-tris-HCl buffer, pH 7.4, 2.5 mM-ATP, 1.25 mM-Mg^{2+} and alternatively 0.05 mM-[2-^{14}C]thymidine or 0.05 mM-[2-^{14}C]TMP. In the assay of TMP-5′-nucleotidase the reaction mixture contained in a total volume of 0.5 ml: 10 mM-tris-maleate buffer, pH 6.0, 0.05 mM-[2-^{14}C]TMP and 0.1 ml of the liver extract. The reaction mixture was incubated as in other enzymic assays.

Samples of the incubation mixture were separated on Whatman no. 1 paper using the isobutyric acid - NH_4OH - H_2O (66:1.5:33, by vol.) solvent system. The

amount of newly formed radioactive thymidine 5′-phosphates was determined in a liquid scintillation counter (Packard Nuclear Corp., Chicago, U.S.A.). The amount of the nucleoside and nucleotides formed was proportional to the concentration of the enzyme protein during the 15 min incubation period, and the enzyme activities were expressed in nmol/min/0.1 ml of extract equivalent to 25 mg of liver tissue.

RESULTS

Effect of thymidine derivatives on TMP-kinase in leukaemic mouse liver. Infiltration of mouse liver with leukaemic lymphoblasts resulted in increased activity of TMP--kinase (Fig. 1) which was the highest at the terminal stage of the disease. Administration of 5-azacytidine, which is known to exert a considerable cancerostatic effect

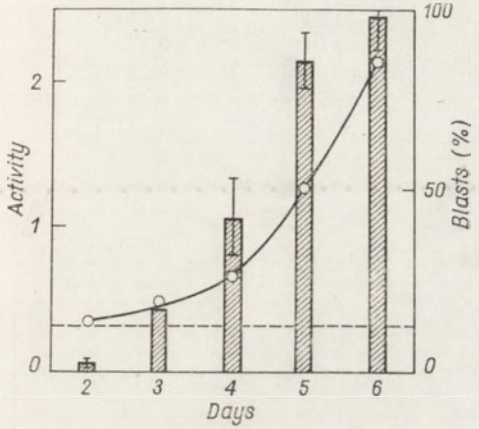

Fig. 1

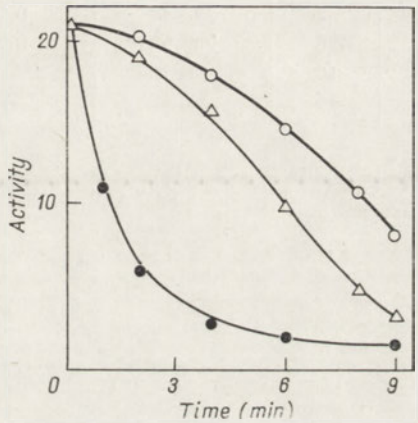

Fig. 2

Fig. 1. Activity of TMP-kinase in mouse liver infiltrated with leukaemic lymphoblasts. Groups of 4 - 7 mice were killed at different time intervals and the enzymic activity (○) was assayed at 37°C during a 15 min incubation period and expressed in nmol of TDP and TTP formed/min/0.1 ml of extract. Hatched bars, percentage of lymphoblasts determined using liver imprints; S.D. values are also indicated. Dashed line, enzyme activity in the control liver corresponding to 25 mg of liver tissue.

Fig. 2. Effect of preincubation on the activity of TMP-kinase. The cell-free extract from leukaemic mouse liver was preincubated at: ●, 37°C; △, 27°C; and ○, 20°C. The enzyme activity was assayed as in Fig. 1.

(Veselý, Čihák & Šorm, 1968) resulted in slowing down of infiltration of the liver and consequently decreased the activity of TMP-kinase (Table 1).

During preincubation of the cell-free extract at 20 - 27°C without substrate, the activity of TMP-kinase was decreased, and at 37°C it diminished very abruptly (Fig. 2). This phenomenon (Bojarski & Hiatt, 1960) was one of the reasons why TMP-kinase has been only recently isolated in a highly purified state (Kielley, 1970). Stabilization of the enzyme *in vitro* by thymidine and TMP (Bianchi *et al.*,

Table 1

Effect of 5-azacytidine on the development of leukaemia in AKR mice

Groups of 6 - 10 mice were inoculated i.p. with 2×10^7 leukaemic lymphoblasts. 5-Azacytidine (4 mg/kg body wt.) was administered i.p. 24 h after inoculation on 4 consecutive days. The activity of TMP-kinase is expressed in nmol/min/0.1 ml extract. The reaction mixture contained: 5×10^{-5}M-[2-^{14}C]thymidine 5′-phosphate, 2.5×10^{-3}M-adenosine 5′-triphosphate, 2.5×10^{-3}M--Mg^{2+}, and 0.1 ml of the cell-free liver extract corresponding to 25 mg of liver tissue.

Days after inoculation	Control		5-Azacytidine-treated	
	Blasts (%)	TMP-kinase	Blasts (%)	TMP-kinase
0	0	0.15	0	0.15
4	30 - 54	0.31	0 - 4	0.13
6	90 - 98*	0.97	0 - 15	0.17
10	death	—	10 - 37	0.25
15	death	—	78 - 92**	0.84

* Survival 7.0 ± 0 days.
** Survival 15.2 ± 0.7 days (Veselý *et al.*, 1968).

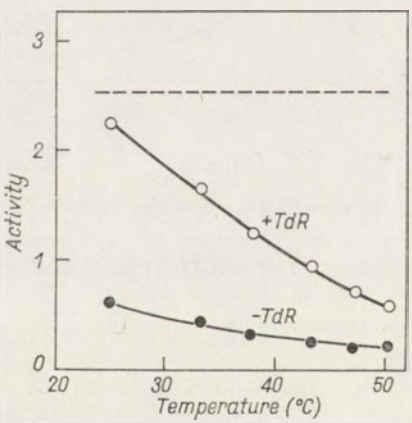

Fig. 3

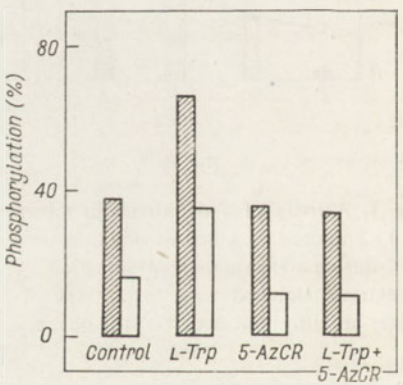

Fig. 4

Fig. 3. Stabilization of TMP-kinase by thymidine during 15 min preincubation at the indicated temperature: ●, without thymidine, and ○, with 50 mM-thymidine. Dashed line, enzyme activity without preincubation.

Fig. 4. Effect of L-tryptophan and 5-azacytidine administered *in vivo* on phosphorylation of thymidine, as measured by the concentration of TMP, TDP and TTP. Twenty four hours after partial hepatectomy, groups of 4 - 8 female rats (175 g) received L-tryptophan (100 mg/100 g body wt., 5 ml, per os) and/or 5-azacytidine (1 mg/100 g, i.p.). After another 24 h the animals were killed and thymidine phosphorylation was assayed in the liver extracts. Time of incubation 10 min. Hatched bars, TMP; outlined bars, TDP + TTP. Control, phosphorylation in the extract from 24-h regenerating liver.

1961; Bojarski & Hiatt, 1960), very pronounced at elevated temperature (Fig. 3), was found to be very specific since none of the structural analogues of thymine tested (Table 2) was effective.

Table 2

Effect of thymine derivatives on TMP-kinase activity

The cell-free liver extract was preincubated at 37°C for 10 min in 0.05 M-tris-HCl buffer, pH 7.4, then 5×10^{-5} M-[2-^{14}C]thymidine 5′-phosphate, 2.5×10^{-3} M-ATP and 2.5×10^{-3}M-Mg^{2+} were added and the mixture was incubated at 40°C for another 15 min. The amount of TMP phosphorylated on direct incubation with the enzyme (34.6%) was taken as 100%.

Addition (0.3 μmol/ml)	Phosphorylation of TMP (%)	Decrease (%)
None	3.5	89.9
1-(2-Deoxy-α-D-ribofuranosyl)thymine	4.6	86.7
1-(2-Deoxy-β-L-ribofuranosyl)thymine	3.6	89.6
Thymidine	23.4	32.3
Thymine riboside	3.5	89.9
Thymine	3.4	90.2

In contrast to the stabilizing effect of thymidine *in vitro*, we were able to observe an only insignificant enhancement of the enzyme activity in the cell-free liver extract of leukaemic mice following oral administration of thymidine. A much more pronounced effect of thymidine administration on TMP-kinase *in vivo* has been observed in the liver, and especially in kidneys, of adult rats (Hiatt & Bojarski, 1961).

Synthesis of thymidine 5′-phosphates in regenerating liver after oral administration of L-*tryptophan to hepatectomized rats.* The enhanced synthesis of thymidine 5′--phosphates from thymidine in the cell-free liver extract following administration of tryptophan to hepatectomized rats (Čihák *et al.*, 1971a), is, similarly as in leukaemic mice, abolished by 5-azacytidine (Fig. 4, Table 3). This inhibitor has been used in the studies on the synthesis of different liver enzymes (Čihák *et al.*, in preparation).

At 24 h after partial hepatectomy, rats were injected alternatively with L-tryptophan, 5-azacytidine, or both these compounds together; the animals were killed after another 24 h. The amount of thymidine 5′-phospates formed *in vitro* by the extract from the 48-h regenerating liver was lower than in the extract from the 24-h regenerating one. Oral administration of tryptophan 24 h after hepatectomy prevented the decrease in the phosphorylation rate. 5-Azacytidine abolished completely the effect of tryptophan (Fig. 4) whereas it was ineffective when administered alone.

5-Azacytidine had a similar effect on stimulation of TMP-kinase by tryptophan (Table 3). Concomitantly with the higher activity of this enzyme, degradation of TMP was decreased. The combined administration of 5-azacytidine and tryptophan prevented both the enhancement of the activity of TMP-kinase and impairment of TMP degradation.

Table 3

Effect of tryptophan on the metabolism of thymidine 5-monophosphate in the liver extract of partially hepatectomized rats

Nineteen groups of 4 -5 female rats (170 - 180 g) 24 h after partial hepatectomy were given L-tryptophan (100 mg/100 g body wt.; 5 ml, per os) and/or 5-azacytidine (2 mg/100 g; i.p.); after another 24 h the animals were killed. Enzymic activities were assayed at 40°C during 15 min incubation, and are expressed in nmol of reacted thymidine 5′-monophosphate per minute per 0.1 ml of liver extract.

Regeneration (h)	Administration	TMP-kinase		TMP-nucleotidase	
			(%)		(%)
0	None	0.51 - 0.58	100	2.06 - 2.14	100
24	None	1.98 - 2.11	371	1.45 - 1.59	73
48	None	1.27- 1.60	252	2.73 - 2.89	139
48	L-Tryptophan	1.60 - 2.08	346	1.67 - 1.95	82
48	5-Azacytidine	1.28 - 1.29	234	2.57 - 2.61	123
48	L-Tryptophan + 5-azacytidine	1.14 - 1.18	214	2.56 - 2.58	122

The time-course of thymidine phosphorylation and the amount of TMP and TDP+TTP formed by the cell-free extract of 48-h regenerating liver 24 h following administration of tryptophan, is given in Fig. 5. The amount of TMP increased

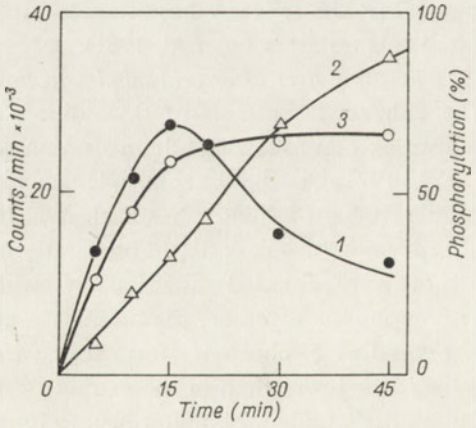

Fig. 5

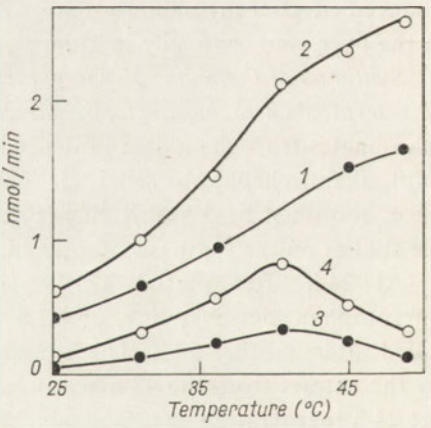

Fig. 6

Fig. 5. Time-course of thymidine phosphorylation in the cell-free extract from 48-h regenerating liver following administration of L-tryptophan (100 mg/100 g body wt., per os) 24 h after partial hepatectomy. The amount of *1*, TMP and *2*, TDP + TTP formed during incubation is expressed as counts/min × 10^{-3}. *3*, Total phosphorylation of thymidine (%).

Fig. 6. Effect of temperature on phosphorylation of thymidine. The cell-free extracts from rat liver 48 h following partial hepatectomy (●) and 24 h following administration of tryptophan (○) were used. *1* and *2*, TMP; *3* and *4*, TDP + TTP.

during 15 min of incubation and then decreased abruptly; the amount of TDP and TTP increased throughout the whole incubation period. From Fig. 6 it is evident that the profile of thymidine 5′-phosphates changed depending on the temperature of incubation; their formation following tryptophan treatment was considerably higher. The optimum temperature for synthesis of TDP and TTP was between 37 and 42°C, and the amount of TMP increased up to 48°C during the 15-min incubation period.

DISCUSSION

The presented results point to a high specificity of the stabilizing effect of thymidine on TMP-kinase (Table 2). It has been postulated (Littlefield, 1965) that this effect is of considerable biological importance *in vivo* also in the case of thymidine kinase. The enhanced activity of the enzyme in the presence of thymidine was regarded as due to stabilization by the substrate and not as a result of the enzyme induction. However, the increased activity of thymidine kinase in regenerating rat liver resulted from the synthesis of the enzyme *de novo* (Maley, Lorenson & Maley, 1965). Hiatt & Bojarski (1961) suggested that administration of thymidine *in vivo* influenced in adult liver the steady-state conditions of the synthesis and degradation of this enzyme by inhibiting its breakdown. The enhanced activity of TMP-kinase in regenerating liver (Fausto & Lancker, 1965) or during leukaemia (Table 1) is due to its synthesis *de novo*, since the enhancement of the enzyme activity is prevented by 5-azacytidine (Table 1), actinomycin D (Hiatt & Bojarski, 1961) or irradiation (Beltz, 1962). However, the inhibitory effect of actinomycin D or X-rays has not been observed in the case of increased enzyme activity on administration of thymidine *in vivo* (Adelstein & Kohn, 1967). This can be explained by the proposed substrate protection of the enzyme by thymidine (Fig. 3).

The increased synthesis of thymidine 5′-phosphates in cell-free liver extracts following tryptophan administration occurs only during some phases of liver regeneration (Čihák *et al.*, 1971a). The maximum increase was obtained at 24 h following tryptophan administration after partial hepatectomy, i.e. during active synthesis of the enzyme. It should be stressed that simultaneous administration of 5-azacytidine with tryptophan completely abolished the enhancement of TMP-kinase as well as the decrease of the activity of TMP-5′-nucleotidase (Table 3 and Fig. 4). The effect of tryptophan was observed neither in the resting liver nor at the later stages of liver regeneration.

The effect of tryptophan on the activity of the investigated liver enzymes may be related to its effect on general hepatic protein synthesis (Fleck, Shepherd & Munro, 1966; Sidransky, Sarma, Bongiorno & Verney, 1968) and on liver polyribosomes (Wunner, Bell & Munro, 1966). There is ample evidence that administration of 5-azacytidine *in vivo* results in the degradation of large polyribosomal aggregates and inhibition of protein synthesis in liver (Čihák *et al.*, 1968; Čihák & Veselý, 1969; Levitan & Webb, 1969). These and other findings (Staehelin, Verney & Sidransky, 1967) suggest that the effect of tryptophan on various liver enzymes is achieved by a cytoplasmic mechanism at the translational level.

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WPŁYW RÓŻNYCH CZYNNIKÓW NA TMP-KINAZĘ *IN VIVO* ORAZ NA SYNTEZĘ TYMIDYNO 5′-TRÓJFOSFORANU *IN VITRO*

Streszczenie

1. Zaobserwowano 4-7-krotny wzrost aktywności TMP-kinazy podczas białaczki limfatycznej u myszy, indukowanej przez wprowadzenie limfoblastów. Stwierdzono labilność TMP-kinazy i opracowano warunki stabilizacji enzymu *in vitro* w obecności tymidyny.

2. L-Tryptofan podany doustnie szczurom w 24 godziny po częściowej hepatoktomii powoduje wzmożenie syntezy TTP w tym narządzie. Wzrost stężenia TTP jest prawdopodobnie następstwem zarówno podwyższonej aktywności kinazy tymidyny oraz TMP-kinazy, jak i obniżenia aktywności TMP-nukleotydazy. 5-Azacytydyna zapobiega zmianom aktywności wspomnianych enzymów.

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BINDING OF MERCURY IN RAT LIVER BY METALLOTHIONEIN*

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The low-molecular-weight protein of rat liver, binding selectively cadmium and mercury administered *in vivo* was proved to be identical with metallothionein. Its properties were close to those of the protein isolated from the kidney: metallothionein from rat liver had a molecular weight of about 10 000, was rich in SH groups, contained cadmium which dissociated at pH 2 and showed characteristic absorption band at 250 nm.

It seems at present to be well documented that metallothionein, a low-molecular-weight protein (~10 000), plays a role in the protection of the organism from harmful effects of heavy metals, especially cadmium. The high affinity of heavy metals towards metallothionein results from the extremely high content (up to 30%) of cysteine residues in the molecule of this protein (Kägi & Vallee, 1960, 1961).

Cadmium is a component of the native metallothionein molecule. It is selectively bound to metallothionein present in the kidney of horse (Kägi & Vallee, 1960, 1961) and man (Pulido, Kägi & Vallee, 1966). Cadmium, when administered in high doses, is also bound by metallothionein in the kidney of rat (Wiśniewska, Trojanowska, Piotrowski & Jakubowski, 1970) and man (Wiśniewska-Knypl, Jabłońska & Myślak, 1971) as well as in the liver of rabbit (Piscator, 1964), rat (Wiśniewska-Knypl & Jabłońska, 1970) and man (Wiśniewska Knypl *et al.*, 1971).

Pulido *et al.* (1966) have demonstrated that metallothionein isolated from human kidney contained also mercury. The same was found to be true for rat kidney (Wiśniewska *et al.*, 1970).

Jakubowski, Piotrowski & Trojanowska (1970) and also Piotrowski & Bolanowska (1970) have found that in the liver of rats there also exists a low-molecular-weight (~10 000) protein fraction, binding mercury with high efficiency.

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* This paper was presented at the 8th Annual Meeting of the Polish Biochemical Society, Szczecin, May 28 - 30, 1970; *Abstr. Commun.* p. 74 - 75.

The results of the present work demonstrated the identity of the Hg-bound low-molecular-weight protein in rat liver with the metallothionein found in renal cortex

MATERIALS AND METHODS

Animals. Two groups of male albino rats of Wistar strain (200 - 300 g) were used: control and $CdCl_2$-treated ones (a single subcutaneous dose of Cd^{2+}, 0.5 mg per kg of body wt.). The rats of both groups (the Cd^{2+}-treated ones after 24 h) were given a single low dose of $^{203}HgCl_2$ (0.02 mg of Hg^{2+} per kg of body wt., activity of about 1 μCi per rat), and one day later the rats were killed and the livers excised.

Isolation of the metallothionein fraction. The ^{203}Hg-labelled metallothionein fraction was isolated after Kägi & Vallee (1960) from pooled homogenates (20%, w/v) of livers of 15 rats. The procedure included extraction of the protein with phosphate buffer (0.05 M, pH 7.4), precipitation of high-molecular-weight protein with ethanol - chloroform (1.2 : 0.094, v/v), and dialysis. The fine precipitate formed on dialysis was removed by centrifugation, and the clear supernatant after concentration *in vacuo* at 30°C was subjected to Sephadex G-75 gel filtration. The protein fractions showing high radioactivity were pooled and submitted either to refiltration, or chromatography on DEAE-cellulose in a step-wise concentration gradient of tris-HCl according to Kägi & Vallee (1961). Identification tests were performed on the low-molecular-weight (~10 000) protein fraction with a high content of $^{203}Hg^{2+}$, Cd^{2+} and -SH groups.

Identification tests for metallothionein. The following determinations were carried out using the procedures of Kägi & Vallee (1960, 1961) as described by Wiśniewska *et al.* (1970): (1), molecular weight by gel filtration on Sephadex G-75, using as reference proteins: cytochrome *c*, RNase and ovalbumin; (2), stability of mercury- and cadmium-metallothionein in neutral and acidic media; (3), u.v. absorption spectra at pH 7 and 2; (4), content of thiol groups.

Analytical methods. At each step of purification the following analyses were carried out: (1) ^{203}Hg γ counting in a well-type scintillation counter with NaI (Tl) crystal (counter USB-2, Biuro Urządzeń Techniki Jądrowej, Warszawa); (2) Colorimetric determination of cadmium in the mineralized samples, using the method of Elkins (1958); (3) Protein determination according to Lowry, Rosebrough, Farr & Randall (1951); since tris interferes with this method, in the tris eluates protein was determined by the tannin method of Mejbaum-Katzenellenbogen (1955). The correspondence of both methods was proved on the metallothionein fraction obtained from the formate buffer eluates; (4) Colorimetric estimation of thiol groups with β-hydroxyethyl-1,2-dinitrophenyl disulphide according to Bitny-Szlachto, Kosiński & Niedzielska (1963); (5) u.v. absorption measurements using Spectromom 201 spectrophotometer and 1-cm light-path quartz cells; where necessary, the samples were diluted with the appropriate buffer.

Reagents. $^{203}HgCl_2$, spec. act. 170 mCi/g of Hg (Nuclear Research Institute, Świerk, Poland); Sephadex G-75 and G-25 gels, blue Dextran (Pharmacia, Uppsala,

Sweden); DEAE-cellulose, medium (Sigma Chem. Co., St. Louis, Mo., U.S.A.); RNase from bovine pancreas (Zawod Medicinskich Preparatov, Leningrad, U.S.S.R.); cytochrome *c* and ovalbumin (Polfa, Kraków, Poland); β-hydroxyethyl-1,2-dinitrophenyl disulphide (HEDD), was synthesized by Professor S. Bitny-Szlachto.

RESULTS AND DISCUSSION

Hg-*metallothionein from rat liver after* ^{203}Hg *administration*. The dialysed fraction of low-molecular-weight proteins from liver was passed through Sephadex G-75 column. As it can be seen in Fig. 1, the peaks of ^{203}Hg, Cd^{2+} and -SH groups coincide with the elution volume of the protein fraction of about 10 000 mol. wt. This

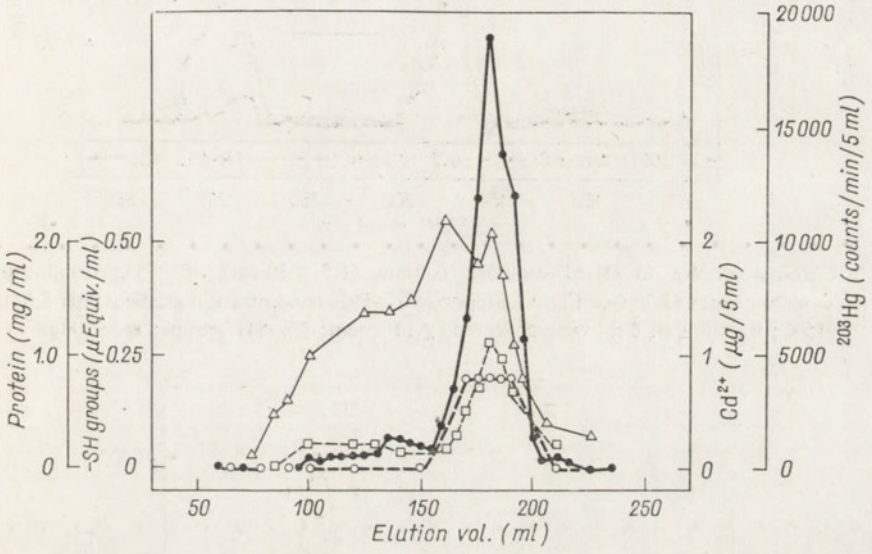

Fig. 1. Sephadex G-25 gel filtration of the low-molecular protein from liver extract of rats given a single dose of $^{203}HgCl_2$. Column 2.6 × 56 cm, 0.1 M-ammonium-formate buffer, pH 8; temp. 15°C; flow-rate 10 ml/hour; fractions of 5 ml were collected. △, Protein; □, -SH groups; ○, Cd^{2+}; ●, ^{203}Hg.

fraction (25 ml) was pooled, concentrated *in vacuo* at 30°C to the volume of about 8 ml and subjected to chromatography on DEAE-cellulose. Two main protein fractions were obtained (Fig. 2). The first one, eluted with 0.25 M-tris-HCl (pH 8.6), contained all cadmium and almost all mercury tracer and showed high content of -SH groups. The second peak eluted with 1 M-tris-HCl contained no cadmium and showed only traces of ^{203}Hg radioactivity. The first protein peak, unlike the second one, had an absorption maximum at 280 nm; it showed a characteristic broad shoulder of absorption between 250 and 280 nm. Since a similar elution pattern has been reported for metallothionein from equine renal cortex (Kägi & Vallee, 1961) it could be inferred that the material eluted in the first peak was metallothionein.

Because of the very low quantity of cadmium present in the metallothionein

fraction obtained from DEAE-cellulose column, it was impossible to perform the additional identification test based on the stability of Cd- and ^{203}Hg-protein complex

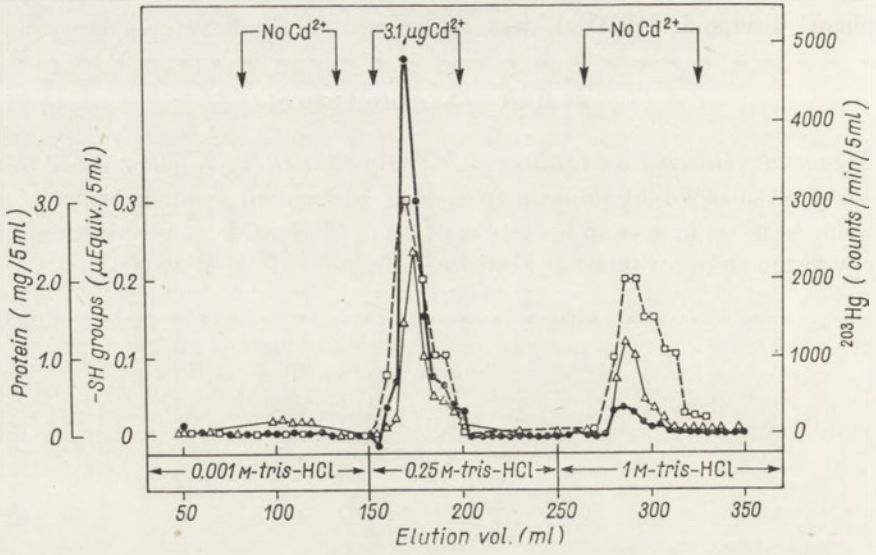

Fig. 2. Chromatography on DEAE-cellulose column (1.5 × 30 cm) of ^{203}Hg-metallothionein fraction from Sephadex G-75 (see Fig. 1). Step-wise tris-HCl concentration gradient; pH 8.6; temp. 15°C; fractions of 5 ml were collected. △, Protein; □, -SH groups; ●, ^{203}Hg.

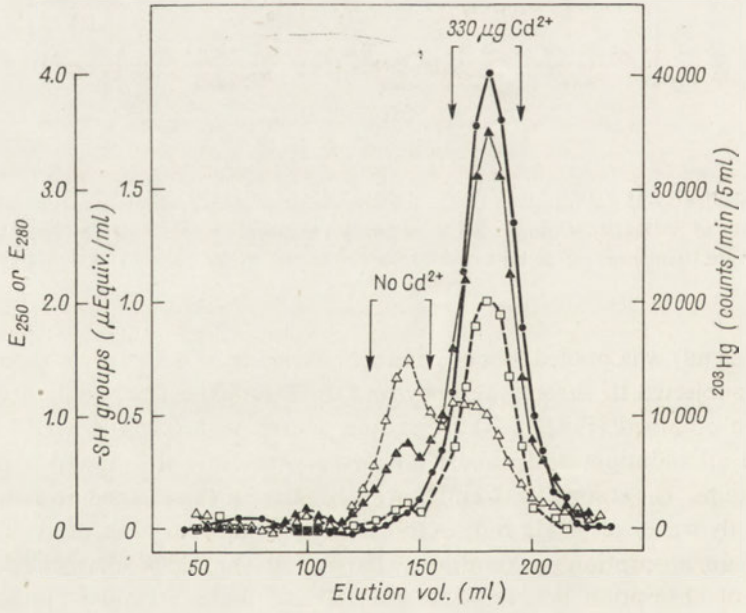

Fig. 3. Sephadex G-75 gel filtration of the low-molecular protein from liver extract of rats given a single dose of $CdCl_2$ followed by $^{203}HgCl_2$. Details as in Fig. 1. ▲, E_{250}; △, E_{280}; □, -SH groups; ●, ^{203}Hg.

in neutral and acidic media. For this reason it was necessary to enrich the liver metallothionein in cadmium, by a single administration of $CdCl_2$ *in vivo*.

^{203}Hg-*metallothionein from the liver of rats after administration of a single dose of cadmium.* It has been demonstrated that the administration of cadmium ions induces the biosynthesis of metallothionein (Piscator, 1964; Shaikh & Lucis, 1970; Wiśniewska-Knypl & Jabłońska, 1970). In the present experiments an increase in the content of this protein was also observed (Fig. 3; for comparison see Fig. 1 and Table 1). The content of -SH groups was doubled, and a marked u.v. absorption peak at 250 nm, due to high cadmium content, appeared. The intended enrichment of the protein in cadmium was achieved, a 17-fold increase in its content being

Table 1

Purification of ^{203}Hg-*labelled metallothionein from rat liver.*

Two groups of rats were used: A, non-treated; B, treated with Cd^{2+} as described in Methods. For comparison, standard equine renal cortex metallothionein (C) is included.

Expt. no.	Fraction	Percentage content			Metal content per mg protein		—SH groups	Degree of concentration	
		^{203}Hg	Cd^{2+}	Protein	^{203}Hg (c.p.m.)	Cd^{2+} (ng)	μEquiv/mg protein	^{203}Hg	Cd^{2+}
A	Liver homogenate	100	100	100	43	1.5	—	1	1
	Dialysed supernatant after ethanol-chloroform treatment	18.2	—	1.6	470	—	—	11	—
	Sephadex G-75 eluate	6.0	18.1	0.2	1 547	140	0.13	36	93
	DEAE-cellulose eluate (0.25 M-tris-HCl)	5.0	16.4	0.09	2 500	500	0.20	60	330
B	Liver homogenate	100	100	100	49	29	0.11	1	1
	Dialysed supernatant after ethanol-chloroform treatment	26.0	38.2	2.2	573	510	0.12	12	18
	Sephadex G-75 eluate	19.0	34.7	0.4	2 305	2 407	0.25	47	83
	Second Sephadex G-75 eluate	10.3	31.2	0.13	4 350	8 600	0.54	89	297
C	Standard equine renal cortex metallothionein	—	—	—	—	9 200	0.78	—	—

obtained. The data on the efficiency of purification at each step of the procedure were given in Table 1 for both groups of rats: treated with cadmium (B) and untreated (A). For comparison, the content of cadmium and -SH groups in standard equine renal cortex metallothionein are included in the Table (part C); the preparation

was obtained in this laboratory according to Pulido *et al.* (1966). For technical reasons the purification procedure for group B was changed, and the second step of purification consisted of refiltration on Sephadex G-75 gel, instead of chromatography on DEAE-cellulose. The comparable data for the groups A and B in Table 1 show that the two procedures resulted in a similar purification efficiency. The final enrichment of metallothionein was almost the same in both groups and the degree of concentration was 60 and 90 for ^{203}Hg and about 300 for Cd.

It is worthwhile to mention that about 16 and 30% of cadmium and only a few percent of the mercury present in the homogenate, were recovered in the final metallothionein preparation. This is due to the fact that *in vivo* cadmium is bound by metallothionein more selectively than mercury (Wiśniewska-Knypl & Jabłońska, 1970).

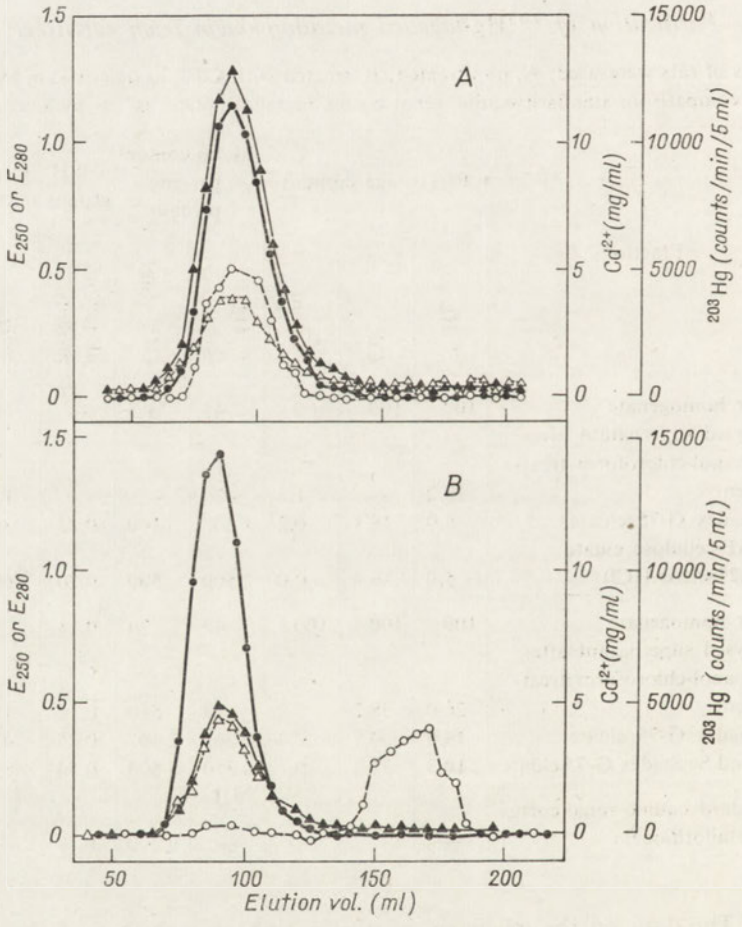

Fig. 4. Stability of ^{203}Hg and Cd-metallothionein in *A*, neutral (1 mM-tris-HCl, pH 7), and *B*, acidic media. Sample *B* was adjusted to pH 2 with 0.1 N-HCl before application and eluted with 0.01 N-HCl. Sephadex G-25, 2.6 × 56 cm bed; flow rate 10 ml/hour; fractions of 5 ml were collected. ▲, E_{250}; △, E_{280}; ○, Cd^{2+}; ●, ^{203}Hg.

Identification of the metallothionein. The content of cadmium in the final preparation of metallothionein from liver of cadmium-treated rats was sufficient for performing the necessary identification tests.

The molecular weight of the ^{203}Hg-labelled metallothionein determined by gel filtration was found to be about 10 000, close enough to that found for the metalloprotein from kidney (Wiśniewska *et al.*, 1970).

Cadmium was firmly bound by the protein at neutral pH. When, however, the metallothionein solution was acidified to pH 2, cadmium dissociated and could be separated by gel filtration on Sephadex G-25; mercury, in contrast to cadmium, did not dissociate under these conditions (Fig. 4A, B). Uncoupling of Cd-cysteine chromophore at pH 2 resulted in disappearance of the characteristic u.v. absorption shoulder at 250 nm (Fig. 5).

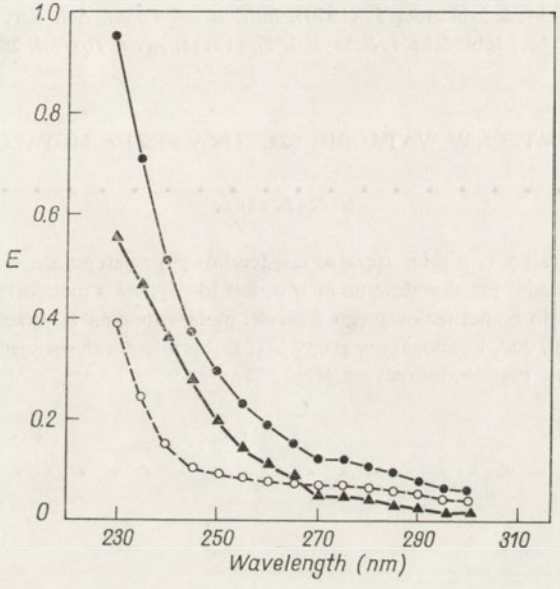

Fig. 5. U.v. absorption spectra of the metalloprotein fraction at ●, pH 7; and ○, pH 2; ▲, difference spectrum of the protein at pH 7 and pH 2.

The presented data agree well with the characteristics of the metallothionein from other sources (Kägi & Vallee, 1960, 1961; Piscator, 1964; Pulido *et al.*, 1966; Wiśniewska *et al.*, 1970; Wiśniewska-Knypl *et al.*, 1971).

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WIĄZANIE RTĘCI W WĄTROBIE SZCZURA PRZEZ METALOTIONEINĘ

Streszczenie

Wykazano, że białko o niskim ciężarze cząsteczkowym występujące w wątrobie szczurów, wiążące selektywnie kadm i rtęć podawane *in vivo*, jest identyczne z metalotioneiną. Białko to ma właściwości zbliżone do białka izolowanego z nerek: metalotioneina z wątroby szczura ma ciężar cząsteczkowy około 10 000, jest bogata w grupy SH, zawiera kadm dysocjujący przy pH 2 i wykazuje charakterystyczne pasma absorpcyjne przy 250 nm.

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CHANGES IN VISCOSITY AND T_m OF CHROMATIN PARTIALLY DEPLETED OF HISTONES

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1. Calf thymus chromatin was partially depleted of histones in sulphuric acid solutions at 0°C in the concentration range of 0.05 to 0.30 N, and the viscosity and thermal denaturation properties of the depleted samples were measured. 2. It has been established that the removal of only histone *f1* had a small effect on the viscosity and melting properties of chromatin. The pronounced changes in melting profiles and viscosity of chromatin molecules have been shown to be associated with the removal of histones *f2a1*, *f2a2*, *f2b* and *f3*. 3. The results are discussed in terms of the role of particular histones in maintaining the structure of chromatin.

The structure of the isolated interphase chromatin has been recently studied by different methods. The results of these investigations point to the vital structural role of histones. Histones have been shown to stabilize DNA towards thermal denaturation (Lee, Peacocke & Walker, 1963; Huang, Bonner & Murray, 1964). The data of analyses of hydrodynamic properties of chromatin (Zubay & Dotty, 1959; Henson & Walker, 1970), and of X-ray diffraction studies (Zubay, 1964; Pardon, Wilkins & Richards, 1967; Richards & Pardon, 1970) indicated that histones maintain the DNA of chromatin in a compact, supercoiled configuration. According to Henson & Walker (1970) the histones *f2a2*, *f2b* and *f3* (or at least one of them) are implicated in supercoiling, while Toczko & Jaźwiński (1971) suggested such a function for histones *f2a1*, *f2a2* and *f3*.

The purpose of this study was to investigate the changes in viscosity and thermal denaturation properties of chromatin accompanying the successive removal of histones in order to obtain further evidence concerning the participation of particular histones in maintaining supercoiled configuration of DNA in chromatin.

MATERIALS AND METHODS

Preparation of chromatin. Chromatin was prepared from calf thymus according to the method of Marushige & Bonner (1966), except that sedimentation through 1.7 M-sucrose was omitted. For each preparation about 25 g of tissue was used

The pellet after last centrifugation was resuspended in 25 ml of deionized water by homogenizing in a glass-teflon homogenizer. The resulting suspension usually had a concentration of about 200 extinction units at 260 nm in 1 cm light path.

Partial extraction of histones from chromatin. Ten grams of cold (0°C) chromatin suspension, equivalent to about 2000 extinction units at 260 nm, was mixed for 60 min with 240 ml of sulphuric acid of appropriate concentration (final concentrations of acid selected for extraction were: 0.05, 0.10, 0.14, 0.22 and 0.30 N) and centrifuged at 10 000 *g* for 20 min. The sediment was resuspended in 100 ml of 0.01 M-tris-HCl buffer, pH 8.0, and centrifuged as above. The temperature during the whole procedure was kept at 0°C.

Solubilization of chromatin. The chromatin sample (native or partially depleted of histones), equivalent to 1200 - 1500 extinction units at 260 nm, was dispersed in 50 ml of 0.7 mM-sodium phosphate buffer, pH 8.0, and dialysed at 0°C for 48 h against 5 litres of the same buffer. The obtained chromatin preparation was then sheared in the Unipan type 309 homogenizer for 90 sec at 120 V and centrifuged at 12 000 *g* for 25 min. By this procedure more than 90% of chromatin became soluble and remained in supernatant. The resulting chromatin solution was used for viscosity and denaturation studies.

Viscosity measurements. Relative viscosity was measured at 25° (±0.1°)C in a Ubbelohde viscometer in 0.7 mM-sodium phosphate buffer, pH 8.0. For each chromatin sample, viscosity was estimated at five different concentrations.

Melting profiles. Melting profile was determined at 260 nm in 0.7 mM-sodium phosphate buffer, pH 8.0, with Zeiss VSU-1 (Jena, German Democratic Republic) spectrophotometer. The rate of temperature increase was 1° per 3 min.

Polyacrylamide-gel electrophoresis. Gel electrophoresis of histones was performed according to Johns (1967), except that the gel containing 15% (w/v) of acrylamide instead of 20%, was used. To the solution of native or partially depleted chromatin, 6 N-HCl was added at 0°C to the final concentration of 0.3 N. After 1 h the precipitate was removed by centrifugation (10 000 *g*, 20 min) and the clear supernatant was used for electrophoresis. The gel was stained overnight with 1% Amido Black in 7% acetic acid and destained by exhaustive washing with 7% acetic acid. The protein bands were scanned using an ERJ 65 Densitograph (Zeiss, Jena, German Democratic Republic) adapted for gel scanning.

Determination of DNA and RNA. DNA and RNA of native or depleted chromatin were fractionated and determined according to Munro & Fleck (1966).

Determination of histones and non-histone protein. Histones were extracted with 0.3 N-HCl for 30 min at 0°C and assayed by the microbiuret method of Goa (1953). After removal of nucleic acids by heating in 10% trichloroacetic acid at 95°C for 10 min, the non-histone protein was determined on the 0.3 N-HCl-precipitable material by the same method. In both cases bovine serum albumin (Michrome, Gurr, London, England) was used as standard.

Determination of cysteine. The content of cysteine in histones was determined by the colorimetric method of Ellman (1959).

RESULTS AND DISCUSSION

The data of chemical analysis of whole and acid-extracted chromatin preparations (Table 1) indicate that treatment with increasing concentrations of sulphuric acid in the range of 0.05 to 0.30 N, at 0°C, resulted in progressive dissociation of histones from chromatin. The quantity of histones extractable by 0.05, 0.10, 0.14, 0.22 and 0.30 N-sulphuric acid solutions was found to be, respectively, 15, 34, 45, 63 and 90% of total histone content. Non-histone protein and RNA were not extracted by acid.

Table 1

Chemical composition of whole and partially depleted chromatin of calf thymus

For details see under Materials and Methods.

Chromatin preparation	Preparation no.	Mass ratios			
		DNA	Histone	Non-histone protein	RNA
Whole	1	1.00	0.98	0.41	0.06
	2	1.00	1.00	0.46	0.06
	3	1.00	0.99	0.42	0.06
	4	1.00	0.98	0.38	0.06
Depleted by 0.05 N-H_2SO_4	1	1.00	0.82	0.39	0.06
	4	1.00	0.84	0.38	0.06
Depleted by 0.10 N-H_2SO_4	1	1.00	0.67	0.38	0.06
	4	1.00	0.64	0.38	0.06
	5	1.00	0.67	0.40	0.07
Depleted by 0.14 N-H_2SO_4	4	1.00	0.58	0.40	0.07
	5	1.00	0.55	0.39	0.07
Depleted by 0.22 N-H_2SO_4	4	1.00	0.38	0.40	0.07
	5	1.00	0.36	0.40	0.07
Depleted by 0.30 N-H_2SO_4	1	1.00	0.10	0.40	0.08
	4	1.00	0.09	0.40	0.08
	5	1.00	0.11	0.41	0.09

The histones remaining in chromatin after acid treatment were identified and quantitatively determined by means of polyacrylamide-gel electrophoresis. The densitometer scans of electrophoretic patterns are shown in Fig. 1. It is evident that the protein removed from whole chromatin by 0.05 N-H_2SO_4 was only histone *f1*. The acid solutions of concentrations 0.10 - 0.14 N removed also large amounts of histone *f2a2* (peak *1*) and one of histones of peak *2* (*f2b* or *f3*). Analysis of cysteine content in histones isolated from equivalent amounts of whole and 0.14 N-H_2SO_4--extracted chromatin preparations allowed to conclude that it was histone *f2b*. The remaining two histones (*f2a1* and *f3*) dissociated in acid solutions of concentrations above 0.14 N. The relationship between the sulphuric acid concentration and the percentage of individual histones remaining in chromatin, calculated from

the data of electrophoretic analysis, is shown in Fig. 2. The order of dissociation of particular histones as a function of acid concentration was: very lysine-rich (*f1*), lysine rich (*f2a2* and *f2b*) and arginine-rich (*f2a1* and *f3*) histones.

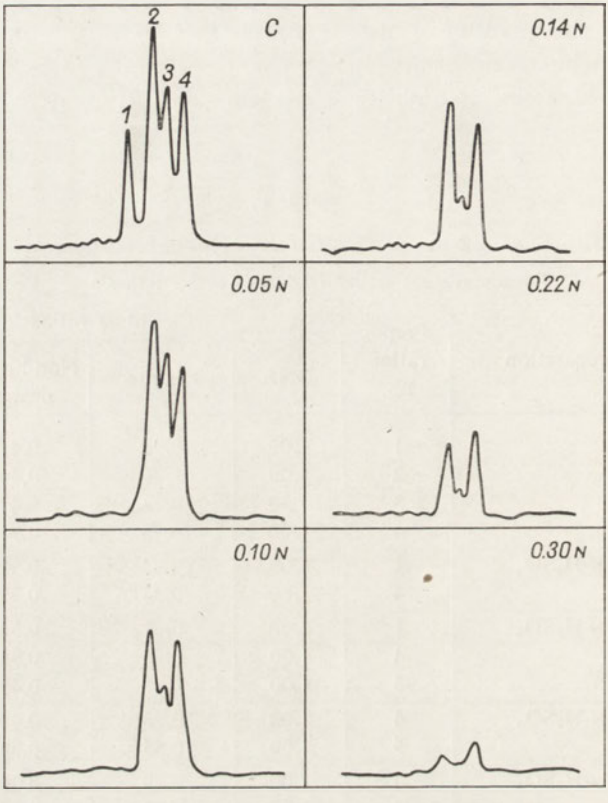

Fig. 1. Densitometer scans of electrophoretic patterns of whole histones of calf thymus chromatin (*C*), and histones of chromatin preparations after extraction with the indicated concentration of sulphuric acid. The sample in all gels was equivalent to 20 μg of DNA of chromatin. *1*, Histone *f1*; *2*, histones *f2b*+*f3*; *3*, histone *f2a2*; *4*, histone *f2a1*.

Progressive removal of histones produced a displacement of the melting profiles of depleted chromatin to lower temperature. Figure 3 presents the melting profiles, determined in 0.7 mM-sodium phosphate buffer, pH 8.0, for whole chromatin and for depleted chromatin preparations. The removal of only histone *f1* by 0.05 N-H_2SO_4 had little effect on the melting properties of chromatin. The main effect of removal of *f1* was to increase the portion of material (about 20% of the total hyperchromicity) melting at a lower temperature. Much more pronounced changes in melting profiles were observed after removal of further histones, particularly after removal of histones *f2a2* and *f2b* by 0.14 N-H_2SO_4.

The variation of the reduced viscosity with concentration for preparations of whole chromatin and of depleted chromatin preparations is shown in Fig. 4. As

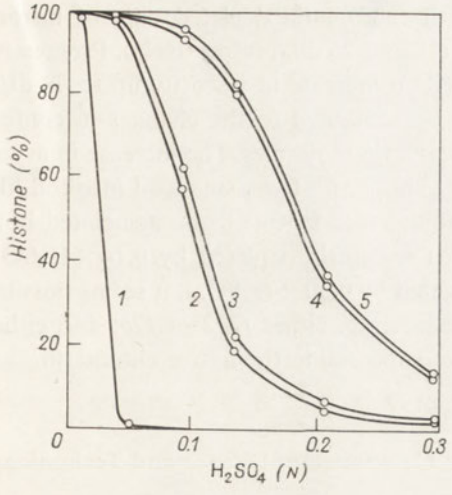

Fig. 2

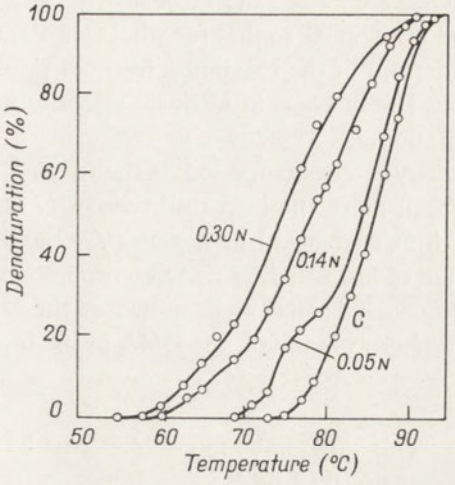

Fig. 3

Fig. 2. Effect of sulphuric acid concentration on the extractability of individual histones from calf thymus chromatin. The data are calculated from densitometer scans of electrophoretic patterns. *1*, Histone *f1* (peak *1*); *2*, histone *f2a2* (peak *3*); *3*, histone *f2b* (peak *2*); *4*, histone *f3* (peak *2*); *5*, histone *f2a1* (peak *4*).

Fig. 3. Melting profiles of whole chromatin preparations (*C*) and chromatin preparations extracted with the indicated concentrations of sulphuric acid. The determinations were made in 0.7 mM-Na--phosphate buffer, pH 8.0.

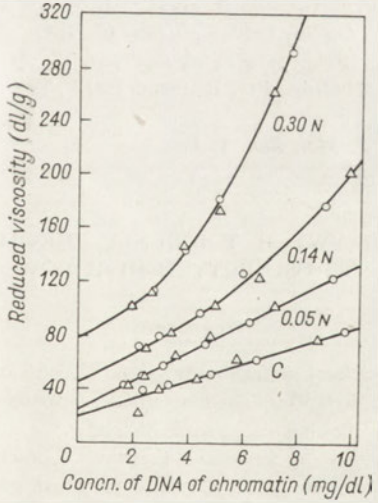

Fig. 4. Reduced viscosity as a function of concentration of DNA of chromatin. The viscosity was determined in Ubbelohde viscometer at 25°C, in 0.7 mM-Na-phosphate buffer, pH 8.0, for whole chromatin preparations (*C*) and chromatin preparations extracted with the indicated concentrations of sulphuric acid. The results of two independent experiments are given.

it may be seen, the intrinsic viscosity of the chromatin depleted only of histone *f1* was similar to that for whole chromatin (18 and 26 dl/g, respectively). Progressive removal of the remaining histones produced an increase in viscosity up to 78 dl/g.

The increase in intrinsic viscosity may be attributed to the changes in conformation, i.e. to increase in asymmetry of chromatin molecules. The increase in asymmetry can be interpreted as the transition of chromatin from supercoil into rod-like form. Since the increase in viscosity was found (see Fig. 4) to be associated both with the removal of histones *f2a2* and *f2b* (chromatin depleted by 0.14 N-H_2SO_4) and of histones *f2a1* and *f3* (chromatin depleted by 0.30 N-H_2SO_4), it seems possible to conclude that at least two of these histones, i.e. either *f2a2* or *f2b*, and either *f2a1* or *f3* maintain the DNA in the form of supercoil in the native chromatin.

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ZMIANY LEPKOŚCI I KRZYWYCH TOPNIENIA CHROMATYNY W ZALEŻNOŚCI OD USUNIĘTYCH HISTONÓW

Streszczenie

1. Z chromatyny grasicy cielęcej ekstrahowano stopniowo histony roztworami kwasu siarkowego w zakresie stężeń od 0,05 do 0,30 N i zbadano zmiany lepkości i krzywych topnienia chromatyny towarzyszące usuwaniu z niej poszczególnych histonów.

2. Stwierdzono, że istotne zmiany lepkości i krzywych topnienia związane są z usunięciem z chromatyny zarówno pary histonów *f2a2* i *f2b*, jak i pary histonów *f2a1* i *f3*, natomiast zmian takich nie obserwowano po usunięciu z chromatyny wyłącznie histonu *f1*.

3. Uzyskane dane pozwalają wnioskować, że conajmniej dwa histony, a mianowicie jeden z histonów *f2a2* lub *f2b* oraz jeden z histonów *f2a1* lub *f3*, utrzymują DNA w chromatynie w postaci superspirali.

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EFFECTS OF IONIZING RADIATIONS ON POLYRIBOADENYLIC ACID AND ITS CONSTITUENTS, AND DEPENDENCE OF RADIOSENSITIVITY ON SECONDARY STRUCTURE

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1. Radiochemical yields have been determined for the destruction of adenine (G_a) and ribose (G_r) in free adenine and ribose, adenosine, adenosine monophosphates, as well as in two structural forms of polyriboadenylic acid (poly A), viz. the neutral single-stranded, and the acid twin-stranded, forms.

2. The results were profited from to determine the dependence of radiation sensitivities of the various components in poly A on its secondary structure. The effect of irradiation on polynucleotide structure was followed by means of the temperature-induced helix-coil transition. Irradiations were performed in 0.005 M-acetate buffer, pH 5, which is equivalent to a monoradical OH· system.

3. The values of G_a were found to be quite uniform in all the above compounds; but G_r decreased even in the nucleoside, to attain a value more than one order of magnitude lower in acid twin-stranded poly A.

4. In acid poly A, G_r increases with radiation dose, testifying to the influence of secondary structure on the course of radiolysis of the polymer.

5. Temperature-induced helix-coil transition profiles of poly A made it feasible to examine the influence of relatively low doses (as low as 2 kR) on the secondary structure. Radiation-induced modifications of the structure of poly A are dependent on the conformation and the presence or absence of oxygen.

6. Measurements of G values demonstrated that radiolysis of adenine most affected secondary structure up to doses of 10 kR. Radiolysis of ribose intervened at higher doses.

7. The value of G_a for adenosine mononucleotides is markedly dependent on the position of phosphorylation. Phosphorylation on the $C_{(2')}$ or $C_{(3')}$ positions markedly protects adenine.

8. Citrate buffer, commonly used as a standard solvent for following helix-coil transitions in nucleic acids, was found to exhibit a pronounced radioprotective effect on poly A. This is related to radiation-induced effects on citrate, which are reflected in changes in UV absorption of the buffer.

The present communication deals with the effect of gamma irradiation on a homopolyribonucleotide, poly A[1], and on its constituent monomers. This readily accessible synthetic polymer provides a convenient model system for an examination of the dependence of radiation susceptibility of the component monomeric constituents on polynucleotide secondary structure.

Poly A exists in two distinct structural forms: (a) in neutral or alkaline medium it has a random-coiled structure with short fragments of single-stranded helix in which the nitrogenous bases interact by means of the base stacking and with no hydrogen bonding between the bases (Griffin, Haslam & Reese, 1964; Luzzati, Mathis, Masson & Witz, 1964; Witz & Luzzati, 1965; Holcomb & Tinoco, 1965; Leng & Felsenfeld, 1966); (b) in slightly acid medium it forms a twin-stranded helix (Rich, Davies, Crick & Watson, 1961) resulting from formation of two hydrogen bonds between adenine bases (both involving $N_{(7)}$ and $N_{(10)}$) and a supplementary hydrogen bond between a phosphate oxygen in one strand and an amino hydrogen in the complementary strand. The presence of a positive charge on the ring $N_{(1)}$ of the adenine residues and a negative charge on the phosphate hydroxyl of the neighbouring strand leads to formation of a strong internal bond of a salt-like nature. This twin-stranded acid form of poly A is considerably more compact than a DNA helix but, at low ionic strengths, it is sensitive to small variations in pH and at elevated temperatures undergoes thermal degradation, *via* a mechanism elsewhere described (Barszcz & Shugar, 1964). It was consequently necessary to eliminate these effects, as described below, so that the temperature-induced helix-coil transition could be used as a criterion for radiation-induced modifications of secondary structure. Irradiations were conducted in 0.005 M-acetate buffer, pH 5. With reference to the compounds studied, this may be treated as a monoradical system containing uniquely OH· at a concentration about 20% that of the initially formed radicals. The remaining OH· radicals, as well as H· and e^-_{aq}, are removed by scavenging by other components of the system. This follows from calculations of radical yields in water at pH 5 ($G_{OH} = 2.2$; $G_H = 1.9$; $e^-_{aq} = 0.9$, as reported by Spinks & Woods, 1964), the concentrations of the individual components, and the values of the rate constants for the reaction of these components with water radicals (Anbar & Neta, 1967).

Hence, on the one hand, poly A and its monomers provide a useful system for examining the radiosensitivity of both adenine and ribose as a function of secondary structure. On the other hand the relative radioresistance of adenine, as compared

[1] The following abbreviations are used in this text: 3′-CMP, cytidine-3′-phosphate; 5′-AMP, adenosine-5′-phosphate; 2′(3′)-AMP, yeast adenylic acid, consisting of a mixture of the adenosine-2′--phosphate and adenosine-3′-phosphate; poly A, polyriboadenylic acid; oligo A, a low-molecular--weight polymer of adenylic acid (see Materials and Methods); poly U, polyribouridylic acid; poly (A+U), 1:1 complex of poly A with poly U; poly (I+C), 1:1 complex of polyriboinosinic acid with polyribocytidylic acid; G, number of molecules damaged per 100 eV absorbed energy; G_a, G_u and G_r, G value for adenine, uracil and ribose, respectively; kR, kiloroentgen; P_i, inorganic phosphate; T_m, temperature corresponding to mid-point of melting profile; ΔT_m, and $T_{50\%}$, see Table 3.

to the other nitrogenous bases encountered in nucleic acids, suggested the possibility of observing modifications in secondary structure resulting from doses provoking little damage to the linear structure of the polynucleotide chains.

MATERIALS AND METHODS

Adenine, ribose, adenosine, 2′(3′)-AMP and 5′-AMP were commercial preparations (Fluka, A.G., Buchs SG, Switzerland or Sigma Chemical Co., St. Louis, Mo., U.S.A.), checked for purity by chromatographic and spectral methods. Oligo A was prepared by chemical methods (Michelson, 1959), and consisted of chains containing up to 15 residues, with a mixture of 2′,5′ and 3′,5′ internucleotide linkages. Poly U was a commercial preparation (Miles Chemical Co., Elkhart, Indiana, U.S.A.) and poly A was prepared with the aid of polynucleotide phosphorylase from *Micrococcus lysodeikticus* (Beers, 1958). Snake venom phosphodiesterase (*Crotalus adamanteus*) was purified by Mrs F. Rzendowska by elution from a cellulose column with an NaCl gradient.

The irradiation source was a 30 curie cylinder of ^{60}Co (Tramer & Shugar, 1961). Dosimetry was according to standard procedures (Weiss, Allen & Schwarz, 1956) using for the ferric ion G = 15.6 and a value for A = 2201 (molar absorption coefficient of the ferric ion, see Allen, 1961). Solutions were irradiated either in thin-walled test tubes with a diameter of 5 mm or, when irradiations were to be performed under nitrogen, in 15 mm ampoules which were sealed off. In the latter case the solutions were freed of oxygen by stirring and passing over them for 30 min commercial nitrogen purified by passage through alkaline pyrogallol solution and then water. With this procedure, no loss by evaporation occurred. Polynucleotides were hydrolysed in 1 N-HCl in sealed ampoules at 100°C for 2 hours[2]. Ribose was determined according to the method of Dische (1957), some samples being initially heated in 0.1 N-NaOH for 5 min at 100°C to decompose both free and phosphorylated ribose, so that only glycosidically bound ribose reacted. Inorganic phosphate was determined on paper chromatograms by a modification of the procedure of Fletcher & Malpress (1953; cf. Barszcz & Shugar, 1964).

Melting profiles were run on a Beckman DU spectrophotometer fitted with a heating block, temperatures being determined with the aid of a thermistor fitted to a dummy cuvette with an accuracy of a 0.1°C. A Radiometer PHM 22 instrument with glass microelectrode was employed for pH measurements. Paper chromatography was ascending, with Whatman no. 1 paper, using two solvent systems: (*A*), *n*-butanol - CH_3COOH - H_2O (5:2:3, by vol.); (*B*), *n*-butanol - HCOOH - H_2O (77:10:3, by vol.).

[2] Attempts to apply milder hydrolysis methods, e.g. with snake venom phosphodiesterase, were unsuccessful because of a significant decrease in rate of enzymic hydrolysis with increasing radiation doses, due to radiation-induced modifications in the poly A.

RESULTS

Radiochemical yields for destruction of adenine (G_a) *and ribose* (G_r)

These are presented in Tables 1 and 2 and merit the following comments:

a) The value of G_r is appreciably reduced in the presence of additional radical acceptors.

b) The value of G_r in acid poly A, while increasing with dose, is initially more than an order of magnitude lower than for free ribose or adenine ribosides. The role of polynucleotide helical structure is best illustrated by the fact that this decrease is not observed in oligo A, which exhibits little, if any, secondary structure under these conditions. For the neutral form of poly A, the value of G_r is dose-independent.

c) The value of G_a is relatively constant except for acid poly A in the absence of oxygen.

Table 1

Radiochemical yields for destruction of adenine (G_a) *and ribose* (G_r) *in monomers and polymers irradiated in air in* 0.005 M-*acetate buffer*, pH 5.0

Concentration of solutes was 5×10^{-5}M, for polymers it was expressed as mononucleotide concentration. The G values were calculated from the plot of the percentage of non-modified substance *versus* dose in kR/ml. Where such a plot was not linear, i.e. when G was not constant, the G values were calculated for several doses shown by the figures in brackets. The numbers in this and the next table are averages of at least three sets of experiments; deviations of less than 5% of average are not shown.

Compound	G_a	G_r
Ribose	—	0.59 (10 kR) 0.46±0.05 (30 kR) 0.41 (60 kR)
Adenine	0.16	
Adenine+ribose (1:1)	0.16±0.02	0.33±0.02
Adenosine	0.13±0.01	0.31
5′-AMP	0.12	0.25
2′(3′)-AMP	0.06±0.005 (10 kR) 0.07 (30 kR) 0.11±0.009 (60 kR)	0.25
Oligo A	0.13	0.32 (10 kR) 0.21 (30 kR) 0.18 (60 kR)
Poly A	0.12	0.01 (10 kR) 0.05 (30 kR)
Poly A in nitrogen	0.09*	0.03 (30 kR) 0.07 (60 kR)

* This value is not corrected for the presence of UV-absorbing products other than adenine resulting from irradiation.

d) The G_a value for 2′(3′)-AMP is dose-dependent, whereas it is relatively constant for 5′-AMP, while the value for G_r is identical in both isomers (see also below).

Table 2

Radiochemical yields for destruction of adenine (G_a) *and ribose* (G_r) *in irradiated neutral poly A in the presence of sodium acetate*

Concentration of poly A, expressed as mononucleotide concentration, was 5×10^{-5} M.

Irradiation conditions	G_a	G_r
0.01 M-Acetate, pH 7.6, in air	0.07	0.05
0.005 M-Acetate, pH 7.0, in air	0.08	0.05
0.005 M-Acetate, pH 7.0, in nitrogen	0.05*	0.05

* Uncorrected for UV-absorbing products other than adenine resulting from irradiation.

Radiation induced modifications of secondary structure of acid poly A

Poly A in 0.005 M-acetate buffer, pH 5, was irradiated in the presence of air or nitrogen with doses as indicated in Figs. 2 and 3, below.

Absorption spectra. These were determined as soon as possible after irradiation directly on the irradiated solutions (Fig. 1), and clearly demonstrate the influence of the saturating gas. In the presence of oxygen (i.e. air), it will be seen from Fig. 1A that irradiation leads to the same modifications of the absorption spectrum as neutralization of the solution (Fresco & Klemperer, 1959) or heating under conditions which lead to fragmentation of the chains and/or their dissociation (Barszcz & Shugar, 1964). In the absence of oxygen the effect is somewhat different (Fig. 1B).

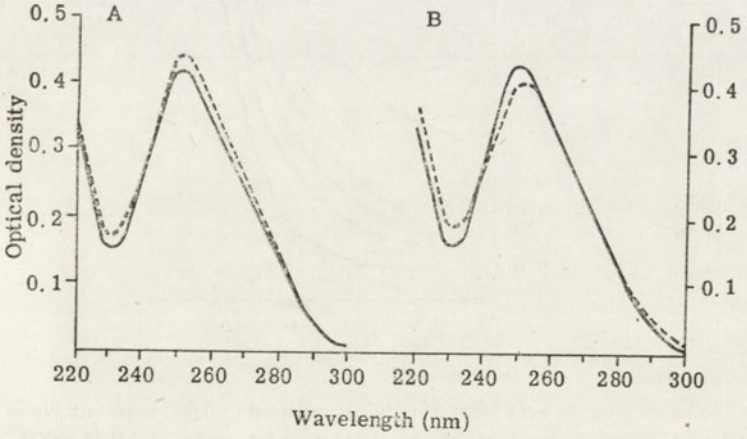

Fig. 1. Absorption spectra of the acid form of poly A irradiated: *A*, in 0.005 M-acetate buffer at pH 5.02 saturated with air, dose 21.8 kR; *B*, in 0.005 M-acetate buffer at pH 4.97 saturated with nitrogen, dose 22.0 kR. ——, Control, - - -, irradiated.

Following completion of the above operation, to each of the irradiated solutions was added the appropriate volume of a solution of 5 M-NaCl and 0.4 M-acetate buffer so that the final concentrations of these were 0.35 M-NaCl in 0.033 M-acetate buffer, pH 4.97. The resultant increase in ionic strength led to a rise in optical density of the irradiated solution, this being independent of the nature of the gas present during irradiation. Control solutions were unaffected by this treatment. The changes observed in absorption of the irradiated solution, relative to that of a control, are exhibited by the heights of the initial portions of the melting profiles shown in Figs. 2 and 3. It should be noted that the above increase in ionic strength of the medium following irradiation reveals modifications of the polynucleotide structure resulting from doses as low as 2.2 kR.

Melting profiles. Profiles of poly A irradiated in the presence of air are exhibited in Fig. 2. Following doses of 2.2 kR and 4.3 kR they are shifted to slightly lower temperatures, but are still closely parallel to the control profile. Melting of the secondary structure also begins at a lower temperature. Such modifications in profiles

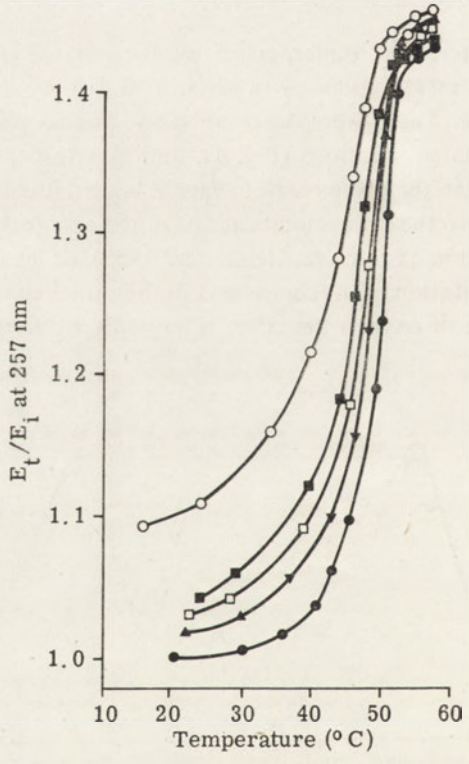

Fig. 2. Melting profiles of the acid form of poly A irradiated in 0.005 M-acetate buffer at pH 5.02 saturated with air. The profiles were measured in 0.033 M-acetate buffer with 0.35 M-NaCl at pH 4.97. In E_t/E_i (in this and subsequent figures), E_i is optical density of a control measured at room temperature before melting and E_t is optical density of a given solution measured at temperature t. ●, Control; ▲, 2.2 kR; □, 4.35 kR; ■, 8.7 kR; ○, 21.8 kR.

are consistent with the changes normally observed for complexes of high-molecular-weight polynucleotides at reduced ionic strengths (e.g. for poly (I+C) in 0.2 M to 0.1 M-Na^+; Szer & Shugar, 1966), i.e. when the decrease in stability of the complex is related to the uniform weakening of secondary structure resulting from changes in intermolecular forces and solvent interaction, but not with a decrease in average molecular weight. It appears reasonable to assume that for doses up to about 4 kR, the resulting damage is randomly distributed in the individual molecules, without appreciable fragmentation of the chains.

When the radiation dose is increased to 9 kR, it will be seen that the initial portion of the melting profile undergoes marked modifications. Following a dose of 22 kR, dissociation of the twin chains is initiated at temperatures below 20°C, the profiles lose their co-operative character up to about 40°C, and complete dissociation of the twin strands occurs at a lower temperature than for the control. Hence, although after a dose of 9 kR the melting profile points to the presence of some fragmented chains which are still capable of forming a secondary structure, a dose of 22 kR leads to the appearance of an appreciable number of fragments with a low degree of secondary structure or devoid of ability to form a twin-stranded complex. Finally, it should be noted that, following the running of a temperature profile on irradiated solutions, and cooling to room temperature, a second heating profile coincided with the initial ones in all instances.

It should be noted, from Fig. 2, that the upper plateaux of all the melting profiles for irradiated poly A are above those for the control, notwithstanding the destruction in the irradiated samples of some of the nitrogenous bases. This is due to the fact that the melting of acid poly A proceeds as follows: (acid twin-stranded poly A) → (single-stranded structured poly A) → (single-stranded less structured poly A), (Massoulié, 1965; Holcomb & Tinoco, 1965). The profiles embrace the entire melting region for acid poly A; but, at 55°C, the single-stranded form still possesses a certain degree of structure, characteristic for the neutral form at this temperature. It is this structure which leads to masking of the expected decrease in absorption due to destruction of some of the adenine residues. The overall increase in absorption of the irradiated solutions is the consequence of damage to the linear structure of the polymer.

Figure 3 exhibits the melting profiles of poly A irradiated in the absence of oxygen. Under these conditions the doses required to produce the effects resulting from irradiation in air, were higher. A characteristic feature of the profiles, following irradiation under nitrogen, is that they all cross the control profile at optical densities which decrease with increasing dose; furthermore, after crossing the control profile, the absorption of the irradiated solutions do not attain a plateau, but continue to increase in a non-cooperative manner.

There is one additional characteristic feature of these profiles. Figure 4 shows a melting profile, followed by cooling and a second heating cycle, of a sample of poly A irradiated under nitrogen at a dose of 8.8 kR. The difference between the first and second heating profiles was dependent on the irradiation dose. A third heating profile of these samples coincided with the second. The most likely interpre-

tation of this is that irradiation in the absence of oxygen resulted in some modifications which were further accentuated by heating, in agreement with the results described in the previous paragraph (see also absorption spectra). This phenomenon was not further studied.

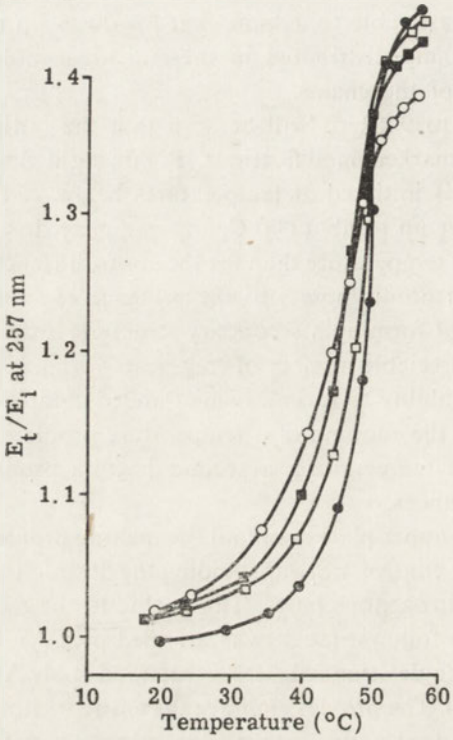

Fig. 3

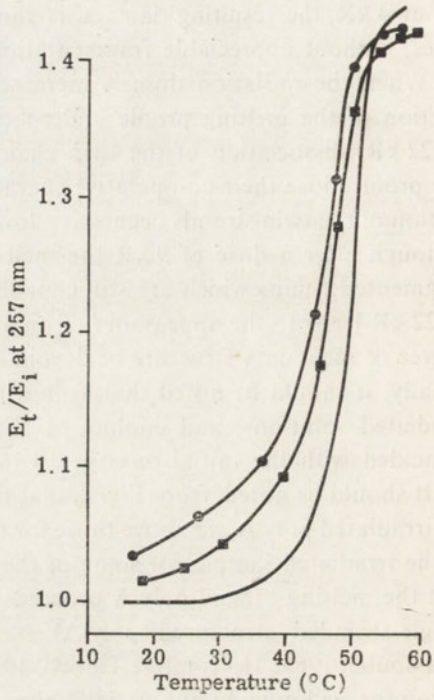

Fig. 4

Fig. 3. Melting profiles of the acid form of poly A irradiated in 0.005 M-acetate buffer at pH 4.97 saturated with nitrogen. Conditions of melting as in Fig. 2. ●, Control; □, 4.4 kR; ■, 8.8 kR; ○, 22.0 kR.

Fig. 4. Two consecutive melting profiles of the acid form of poly A irradiated in nitrogen-saturated solution (for details see Fig. 3) showing irreversibility of the melting.——, Control; ■, first melting; ●, second melting.

Changes in structure of acid poly A following prior irradiation in neutral medium. It has been shown previously that both the absorption spectrum and temperature profile of neutral poly A are relatively insensitive to moderate degrees of chain degradation (Barszcz & Shugar, 1964), but modifications resulting from the effects of degradative agents are readily placed in evidence by subsequent acidification of the solution and examining the properties of the resultant acid poly A.

Solutions of poly A, in 0.005 M-sodium acetate brought to pH 7.5 with diluted HCl, were irradiated in the presence and absence of oxygen. To the irradiated solutions was added 5 M-NaCl in 0.4 M-acetate buffer, pH 5.0, to give a medium identical

with that described in the previous section for determination of the melting profiles of acid poly A. Absorption spectra were recorded both before and after addition of buffered salt. The spectral changes noted in the irradiation medium were negligible and those observed after acidification were similar to the changes shown in Fig. 1A. There was no influence of gas present during irradiation.

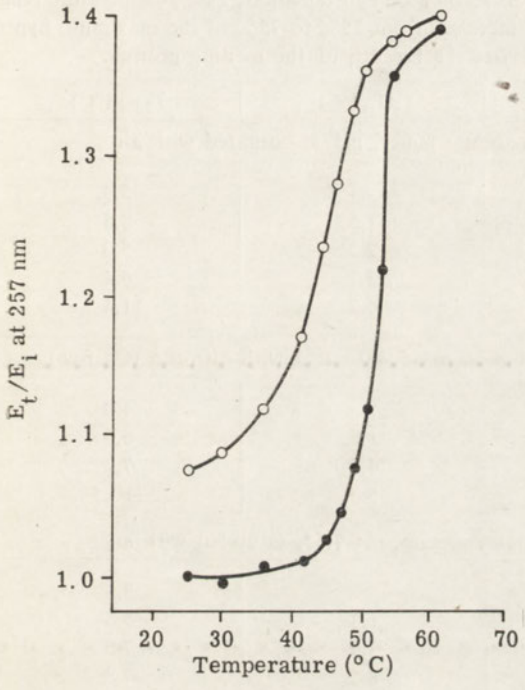

Fig. 5

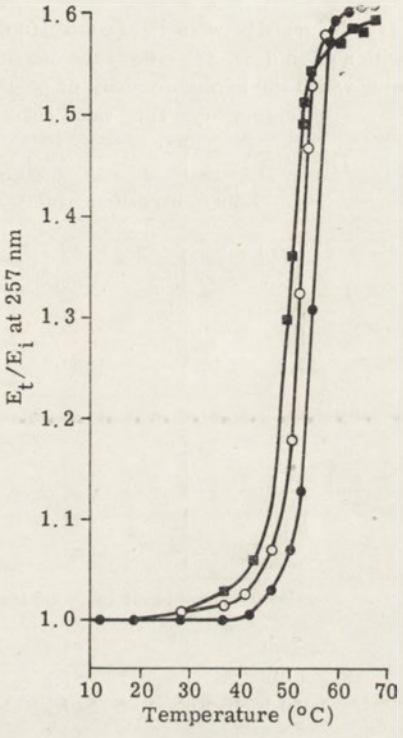

Fig. 6

Fig. 5. Melting profiles of the acid form of poly A after irradiation at pH 7.5 in 0.005 M-sodium acetate solution saturated with nitrogen. Conditions for melting as in Fig. 2. ●, Control; ○, 22.2 kR.

Fig. 6. Melting profiles in 0.08 M-NaCl at pH 7 of 1:1 helical complexes of poly U with poly A previously irradiated in the acid form (0.005 M-acetate buffer, pH 5.05, in air-saturated solution). In E_t/E_i, E_i is optical density of a given solution measured at room temperature before melting and E_t is optical density measured at elevated temperature. ●, Control, non-irradiated poly A; ○, poly A previously irradiated to 11.8 kR; ■, poly A previously irradiated to 22.3 kR.

The temperature profiles of poly A irradiated in air differed from the control in much the same way as irradiated acid poly A, but the changes in T_m were larger with equivalent doses. For poly A irradiated in the absence of oxygen, the melting profiles did not intersect the control profile as in the case of irradiated acid poly A (cf. Figs. 5 and 3). By comparison with a control, the course of the profiles themselves closely resembled those of poly A irradiated in air.

The changes in T_m and the breadth of the melting profiles, as a function of dose under various conditions, are presented in Table 3.

Table 3

Influence of γ-irradiation on characteristic features of melting profiles of acid form of poly A

ΔT_m, difference between T_m of control (non-irradiated) poly A and T_m of profile of poly A irradiated with a given dose. ΔT_m reflects the decrease in T_m induced by irradiation; $T_{50\%}$, temperature range over which the hyperchromicity of poly A increases from 25% to 75% of the maximum hyperchromicity. This value characterizes the breadth of the melting profiles.

Dose (kR)	T_m (°C)	ΔT_m (°C)	$T_{50\%}$ (°C)
Poly A irradiated in 0.005 M-acetate buffer, pH 5, saturated with air			
Control	49.7	—	5.3
2.2	48.2	1.5	5.5
4.3	47.5	2.2	5.0
8.7	46.0	3.7	6.4
21.8	43.5	6.2	11.4
Poly A irradiated in 0.005 M-acetate buffer, pH 5, saturated with nitrogen (O_2 free)			
Control	50.0	—	4.0
4.4	49.0	1.0	6.0
8.8	46.0	4.0	7.7
22.0	44.7	5.3	10.5
Poly A irradiated in 0.005 M-sodium acetate, pH 7.5*, saturated with air			
Control	53.0	—	3.3
4.4	51.7	1.3	5.5
8.9	49.2	3.8	7.5
22.2	44.5	8.5	7.8
Poly A irradiated in 0.005 M-sodium acetate, pH 7.5*, saturated with nitrogen (O_2 free)			
Control	53.5	—	3.5
4.4	51.7	1.8	6.0
8.9	49.0	4.5	6.8
22.2	45.7	7.8	9.0

* Melting profiles were measured in pH 5, i.e. for the acid, twin-stran ded form of poly A (see text for details).

Complexing of irradiated poly A with poly U. Acid poly A (0.005 M-acetate buffer, pH 5.05) was irradiated in air at doses of 11.8 and 22.3 kR. To each sample was added NaCl to final concentration of 0.08 M (resultant pH 4.9), and an equimolar amount of poly U. For the poly A sample subjected to 22.3 kR, mixing with poly U was accompanied by the immediate appearance of several percent hypochromicity (measured at 258 nm). However, after overnight storage following mixing, the absorption of both mixtures of irradiated poly A plus poly U were identical, about

14% below that of the control. Neutralization of the mixtures to pH 7.5 showed that poly A irradiated with 11.8 kR still was able to complex with poly U to the same extent as the control. The absorption of the solution containing poly A after a dose of 22.3 kR was higher than that of the control probably due to incomplete binding of some chains. The resultant melting profiles were as exhibited in Fig. 6. This demonstrates that the stabilities of the complexes poly (A+U) decreased with increasing dose given to poly A.

Chromatographic analysis of irradiated oligo A

Measurements of ultraviolet absorption on an irradiated solution of oligo A are the resultant of two processes, viz. increase in absorption due to chain cleavage, and a simultaneous decrease due to destruction of adenine residues. More precise information, apart from quantitative measurements following hydrolysis (see Table 1) are furnished by chromatographic analysis.

An aqueous solution of oligo A was subjected to doses of 5.3 and 21.5 kR and chromatographed in solvent *A* along with a control. Elution of the spots with 0.1 N-HCl showed that the optical densities of the oligo A samples had decreased by 11% and 20%, respectively. Changes in absorption before chromatography for both solutions were smaller than 2%.

Oligo A was irradiated in acetate buffer, pH 5.0, or in unbuffered aqueous medium and, following total doses of 5 to 231 kR, chromatographed in solvents *A* and *B*, followed by elution of products other than initial oligo A, which demonstrated the release of adenine and inorganic phosphate (P_i), but not free ribose or ribose phosphate.

Radiolysis of adenosine monophosphates

The marked differences in destruction of adenine in 2′(3′)-AMP and 5′-AMP (see Table 1) as compared to the identical values of G_r for the two isomers, indicated the involvement of different radiochemical pathways. These were examined in more detail by means of chromatography (solvent *A*), following irradiation in acetate buffered and unbuffered media at doses of from 13 to 150 kR, with the following results:

Irradiated 5′-AMP was found to yield only free adenine, whereas 2′(3′)-AMP gave free adenine and (in lesser yield) a compound which appeared to be adenosine on the basis of its R_F values and ultraviolet absorption spectrum; however, it is not possible by means of R_F values and absorption spectra to exclude the possibility of some minor modification in the ribose ring. The yields were dependent on dose and medium. In buffered solution, after 150 kR, the ratio of adenine to the adenosine-like product was 2:1, whereas in unbuffered medium only traces of adenosine-like product were observed after a dose of about 80 kR. P_i was identified in all irradiated solutions.

Hence both 2′(3′)-AMP and 5′-AMP release inorganic phosphate on irradiation. But in the case of 2′(3′)-AMP, the P_i is released directly, without degradation of ribose, to give an adenosine-like product, whereas in 5′-AMP the release of P_i is most likely a consequence of destruction of ribose.

The above findings are too scanty to formulate a mechanism for release of P_i from 2′(3′)-AMP which, however, parallels the thermally induced release of P_i from this isomer (Barszcz & Shugar, 1964), although this may be purely coincidental. Referring back to the differences in G values for destruction of adenine in the two isomers (Table 1), it would appear that the lower value of G_a for 2′(3′)-AMP is due to the protective effect of the phosphate at the 2′(3′)-hydroxyl. But it is difficult to conclude whether the adenine moiety is attacked following cleavage of the phosphate ester bond or in the intact nucleotide.

It is worth noting that in the case of 5′-AMP the protective effect of polyphosphate groups on the radiosensitivity of adenine was observed by Barron, Johnson & Cobure (1954), who found that radiation damage to adenine decreased markedly in the range from AMP to ATP.

Protective effect of citrate

Temperature profiles of both natural and synthetic polynucleotides are frequently followed under so-called standard conditions (Rice & Doty, 1957), i.e. in the presence of 0.15 M-NaCl and 0.015 M-sodium citrate. The following observations, noted in the initial stages of this investigation, are of practical interest.

Irradiation of poly A in 0.01 M-citrate buffer, pH 5.2, was found to lead to a dose-dependent increase in absorption over the wavelength range 225 - 300 nm (cf. Fig. 1A). The nature of this effect at first sight suggested extensive destruction of polynucleotide structure, but the temperature profile of the irradiated (up to 10 kR) poly A was unchanged and the T_m unaltered with respect to the control. No modifications were observed in the ribose for doses up to 74 kR; in fact the orcinol reaction was more positive than for the control in the absence of prior alkaline treatment (see Materials and Methods).

These rather surprising findings led to the following experiment: Samples of poly A in water and in 0.01 M-citrate buffer (both at about pH 5.0) were subjected to a dose of 50 kR and submitted to paper chromatography in solvent *A*, along with a control. The control sample did not move from the starting line. The sample irradiated in water exhibited clear evidence of formation of smaller fragments, which formed a faint smudge up to 2.5 cm from the start; whereas the sample in citrate buffer remained at the start.

It follows that irradiation of poly A in citrate buffer did not result in depolymerization of the polynucleotide; whereas for poly A in water, the same doses led to appreciable fragmentation.

Irradiation of the citrate buffer alone, however, led to modifications in the absorption spectrum in the ultraviolet which were proportional to the dose (see Fig. 7).

The observed modifications in absorption of the solutions of poly A over the range 225 - 300 nm must consequently be ascribed to this effect and not to changes in structure of poly A.

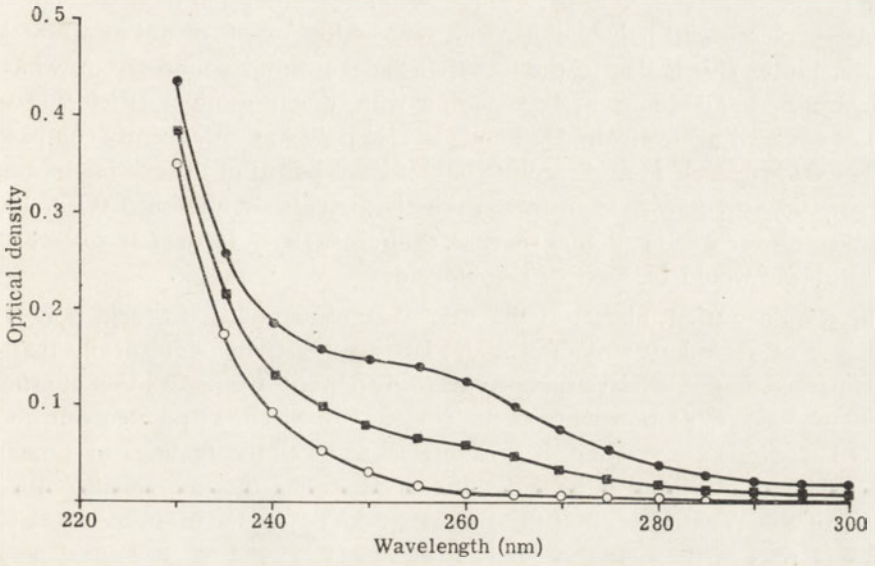

Fig. 7. Influence of γ-irradiation on absorption spectra of 0.01 M-citrate buffer of pH 5.2: ○, before irradiation; ■, after 22.8 kR; ●, after 46.0 kR.

It is clear that both the pronounced protective effect of citrate, as well as the radiation-induced appearance of absorption in ultraviolet, argue against the use of this buffer anion in radiation chemical studies on polynucleotides. A similar effect has been noted under the influence of ultraviolet irradiation (Tramer, Wierzchowski & Shugar, 1969).

DISCUSSION

Notwithstanding that the G values determined in this study are derived from specific model systems, the known structural variations between these systems are such that the results should prove equally applicable to studies on natural nucleic acids.

From the quantitative data presented in Tables 1 and 2 it may be concluded that the secondary structure of both forms of poly A inhibits the radiolysis of the ribose moieties. On the other hand, the variation of G_r for acid poly A calls for comment. The relatively low initial value for G_r increases several-fold at a dose of 30 kR, where it approaches the G_r value for neutral poly A. The resulting conclusion regarding the strict dependence of G_r on the compactness of the polynucleotide structure is strongly supported by the melting profiles (Fig. 2) which show that, following a dose

of 22 kR, the polymer has already been partially converted to the neutral form, even in the medium suitable for the existence of the acid form.

Additional evidence for the formation in irradiated acid poly A of regions containing reduced helical structure is provided by the complexing ability of the irradiated polymer with poly U at pH 4.9. It is well known that well-formed acid helical poly A complexes with poly U either to a very limited extent or not at all (Steiner & Beers, 1961). This is due to the fact that the exogenous amino group, which is the hydrogen bond donor in base-pairing with uracil residues (Rich & Davies, 1956), is engaged in two hydrogen bonds in acid poly A. Consequently complexing with poly U is feasible in acid medium only if involvement of the exogenous amino hydrogens in acid poly A in hydrogen bonding is entirely abolished (Cox, 1963), or abolished over some region(s) (Barszcz & Shugar, 1964). In the latter case, complexing ability would be limited, as observed.

The demonstration, above, of the strict dependence of G_r for acid poly A on the degree of secondary structure of the latter suggests non-equivocally that the closely-packed double helical structure effectively prevents the penetration of primary radicals to susceptible positions on the ribose ring. Since, as pointed out above, only OH· radicals are involved, it is of interest to recall the findings of Loman & Ebert (1968) who utilized pulse radiolysis to determine the rate constant for the reaction of poly A with OH· over the pH range 3.5 - 7.5, i.e. the pH range for existence of acid poly A, the range for the transition of acid poly A to neutral poly A, and that for existence of neutral poly A. They found the rate constant k, lowest for acid poly A, to increase appreciably in the pH range characteristic for the transition to neutral poly A, and to attain its highest (and constant) value for the neutral form of the polymer. The concurrent increase in k with the transition from the acid to the neutral form was, therefore, indicative of the importance of the compactness of the helical structure relative to its reactivity with OH· radicals. It appears reasonable to assume that an analogous increase in reactivity will follow the radiation-induced "loosening" of the secondary structure of acid poly A, in acid medium.

This, in turn, leads to the following: the ribose moiety includes several bonds susceptible to radiochemical attack (Phillips & Criddle, 1962). But, from the known structure of acid poly A (Rich *et al.*, 1961) only a portion of carbohydrate ring, located between phosphodiester linkages, is on the periphery of the helix, while that involving the carbons from $C_{(3')}$ to $C_{(1')}$ is inside. Access of radicals to this portion of the ring is inhibited prior to appreciable collapse of the helical structure. It seems reasonable to expect these bonds to be the ones mainly, or exclusively, attacked in the polymer. On the other hand, Bernhard & Snipes (1968), in a study on radical formation in single crystals of 3'-CMP, were led to conclude that the unpaired electron was located on the carbohydrate moiety. The initial step involved elimination of the hydrogen from $C_{(1')}$, followed by cleavage of $C_{(1')}$-O-$C_{(4')}$ with formation of the double bond $C_{(1')} = O$ to leave the unpaired electron on $C_{(4')}$. Hüttermann & Müller (1969) have likewise postulated that removal of hydrogen from $C_{(1)}$ in irradiated single crystals of deoxyribose is the initial step in radical formation.

A similar process may be regarded as operating during irradiation of poly A in aqueous medium, removal of the hydrogen from $C_{(1')}$ of ribose residues being due in this case to the radiation-induced OH· radicals.

In the case of adenine residues, a certain protective effect of secondary structure is manifested in neutral poly A; but the G_a value for adenine in acid poly A was significantly higher and, in fact, equal to that for the free nucleoside and 5'-AMP (see Tables 1 and 2). Before attempting to draw any conclusions from these observations, it is perhaps worth drawing attention to the findings of Ekert & Tisne (1966), who measured the radiochemical yields for destruction of uracil (G_u) and adenine in double-stranded poly (A+U), in neutral poly A and the random coil form of poly U. G_u in the homopolymer was twice that for poly (A+U); but G_a was identical for both systems, so that the radiochemical susceptibility of adenine was the same in a single strand and a double-stranded helix. One might similarly expect the G_a values for the neutral and acid forms of poly A to be similar. The fact that this is not so, and that G_a is higher for the acid form, may be related to the presence of a positive charge on $N_{(1)}$ of the adenine residues in acid poly A. But this is probably an over-simplification since the G value for adenine itself is unaltered over a wide range of pH (Ranadive, Korgaonkar & Sahasrabudhe, 1956; Scholes, Ward & Weiss, 1960). Furthermore, in the case of the polynucleotide we may be dealing with radical transfer processes which differ for the two structural forms of poly A.

The melting profiles furnish additional information regarding the course of radiolysis of poly A. For all doses, from 2.2 to 22 kR, the melting profiles of the polymer samples irradiated in air were fully reversible, and a second heating cycle reproduced the initial profile. This testifies to the permanent changes resulting from irradiation, which must be linked with the linear structure, i.e. the radiation-induced modifications affect primarily the primary structure of the chains.

The overall results make it possible to calculate, for each irradiation dose, the percentage destruction of adenine and ribose and to correlate these with the modifications in secondary structure of poly A. Such calculations show that submission of acid poly A to a dose of 8.7 kR leads to a loss in ribose of barely 0.15%, corresponding to one molecule per 660 residues. For an analogous dose the number of adenine residues transformed is much higher, about 1.85%, corresponding to one residue in 54. And changes in the profiles, which reflect modifications in the secondary structure of the polynucleotide, are clearly visible after a dose as low as 2.2 kR, and probably could be followed at even lower doses, e.g. by difference spectrophotometry. It may be concluded that the modifications in absorption and melting profiles resulting from the doses of about 9 kR can be ascribed to destruction of adenine residues and the accompanying disruption of a number of hydrogen bonds. Only at doses surpassing 22 kR are the changes in melting profile due to both effects: destruction of adenine (4.6%) and ribose (1.9%). The average length of non-modified chains, estimated from these calculations and the forms of the corresponding profiles, are in good agreement with the data of Brahms, Michel-

son & Van Holde (1966), who determined the melting profiles of oligomers of adenylic acid with chain lengths of 8 residues and higher. The shape of the melting profile for poly A, following a dose of 22 kR, resembles that presented by the foregoing authors for oligo A with a chain length of 30 residues.

While the foregoing results cannot be directly extrapolated to natural nucleic acids, they do point to the fact that, for a polynucleotide possessing a highly ordered structure, the radiolysis of the nitrogenous bases may precede that of the carbohydrate residues and markedly affect the structure. At present considerable attention is being devoted to radiation-induced strand breakage, which is regarded by some authors as the most important consequence of radiation damage in DNA (e.g. Alexander *et al.*, 1970). This is due in part to the ease with which strand breakage (and repair) may be followed by density gradient centrifugation, and by no means excludes the possibility of base radiolysis being an important factor. For example, X-irradiation of double-stranded DNA with a dose of 9.6 kR revealed the presence of single strands containing 250 residues (Das Gupta & Mitra, 1970). It is conceivable that the release of such large fragments may be due to initial radiolytic destruction of bases, followed by breakage of hydrogen bonds in neighbouring regions of the helix. Our knowledge of the effects of low radiation doses on nucleic acids, the sequence of various damaging events, and their effects is still rather meagre.

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WPŁYW PROMIENIOWANIA JONIZUJĄCEGO NA KWAS POLIRYBOADENILOWY I JEGO SKŁADNIKI; ZALEŻNOŚĆ MIĘDZY STRUKTURĄ A WRAŻLIWOŚCIĄ NA PROMIENIOWANIE

Streszczenie

1. Oznaczono radiochemiczne wydajności zniszczenia adeniny (G_a) i rybozy (G_r) w wolnej adeninie i rybozie, adenozynie, kwasach adenilowych oraz w dwóch formach strukturalnych kwasu poliryboadenilowego (poli A), tj. w formie obojętnej, jedno-łańcuchowej i kwaśnej, dwu-łańcuchowej.

2. Otrzymane wyniki posłużyły do określenia zależności między wrażliwością na promieniowanie jonizujące składników poli A, a jego strukturą drugorzędową. Wpływ promieniowania na strukturę polinukleotydu śledzono metodą indukowanego cieplnie przejścia spirala-kłębek. Napromieniano w 0.005 M buforze octanowym o pH 5; jest to praktycznie układ monorodnikowy — zawierający rodniki $OH\cdot$.

3. Stwierdzono, że wydajności G_a są zbliżone we wszystkich badanych związkach, natomiast wydajność G_r jest wyraźnie niższa w nukleozydzie, a niższa o ponad rząd wielkości w kwaśnej, dwu-łańcuchowej formie poli A.

4. W kwaśnej formie poli A wydajność G_r wzrasta z dawką promieniowania, wskazując na istotny wpływ struktury drugorzędowej na przebieg radiolizy polinukleotydu.

5. Metoda oznaczania indukowanych cieplnie profili przejścia strukturalnego pozwala śledzić wpływ stosunkowo niskich dawek promieniowania (nawet 2 kR) na strukturę drugorzędową poli A. Wywołane promieniowaniem zmiany w tej strukturze zależą od konformacji polinukleotydu i środowiska gazowego.

6. Określenie wydajności G pozwala powiązać zmiany w strukturze poli A po dawkach sięgających 10 kR, z radiolizą głównie adeniny; radioliza rybozy odgrywa istotną rolę po wyższych dawkach promieniowania.

7. Wydajność G_a w monofosforanach adenozyny jest w istotny sposób zależna od miejsca fosforylacji. Grupa fosforanowa w pozycji $C_{(2')}$ lub $C_{(3')}$ działa wybitnie ochronnie.

8. Bufor cytrynianowy, powszechnie stosowany jako standardowy roztwór do śledzenia indukowanego cieplnie przejścia strukturalnego spirala-kłębek, w radiolizie poli A wykazuje silne działanie ochronne. Wpływ ten związany jest z radiochemiczną reakcją buforu cytrynianowego, znajdującą swoje odbicie w zmianie widma absorpcji w nadfiolecie.

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BIOSYNTHESIS OF STEROL GLYCOSIDES IN CELL-FREE PREPARATIONS FROM *CALENDULA OFFICINALIS* L.

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1. It was shown that UDPglucose is utilized for the synthesis of sterol 3-*O*-glucosides and acyl derivatives of these glycosides (most probably monoacetyl-3-*O*-glucosides) by the cell-free preparations from *Calendula officinalis*; acetylation involves already formed sterol 3-*O*-glucosides. Optimum pH for glucosylation and acetylation is 7.6 and 6.6, respectively. 2. The occurrence of acyl-3-*O*-glucosides in *C. officinalis* has been proved and it has been found that all the investigated sterol compounds: free sterols, esters with fatty acids, 3-*O*-glucosides and acyl-3-*O*-glucosides contain *β*-sitosterol, stigmasterol and, in smaller quantities, isofucosterol.

It has been shown recently (Hou, Unemura, Nakamura & Funahashi, 1967; Kauss, 1968; Eichenberger & Newman, 1968; Ongun & Mudd, 1970) that sterol glycosides can be synthesized by cell-free preparations from certain plants, UDPglucose or UDPgalactose being utilized as donors of carbohydrate residues in the glycosylation process. It was also found that some of the enzymic preparations, such as mitochondrial fraction from pea roots, chloroplasts of spinach leaves (Ongun & Mudd, 1970), microsomal preparation from soya-bean leaves (Hou *et al.*, 1967) as well as partially purified enzymic preparation from bean shoots (Kauss, 1968) synthesize on incubation with UDP-[U-^{14}C]glucose not only 3-*O*-glucosides of sterols but also unidentified acyl derivatives of these glucosides.

It seems at present that in higher plants sterol glycosides occur commonly together with free sterols and sterol esters, and constitute probably a transport form of sterols. Acyl derivatives of these glycosides have been detected by LePage (1964) but their presence in plants was demonstrated in a few cases only. As it has been shown by Kiribuchi, Mizunaga & Funahashi (1966) and Kiribuchi, Yasumatsu & Funahashi (1967), acylglycosides of *β*-sitosterol occurring in soya-bean contain long-chain fatty acids. There are, however, no data concerning the structure of other acylglucosides of this type. It is known from the previous works from this laboratory (Kasprzyk, Pyrek & Turowska, 1968; Kasprzyk & Turowska, 1969; Kasprzyk & Wojciechowski, 1969; Kasprzyk, Turowska, Grygiel & Kanabus, 1970) that *β*-sitosterol,

stigmasterol and isofucosterol, their 3-*O*-monoglycosides and esters with fatty acids occur in *C. officinalis* during the whole vegetation period. A considerable increase in the content of these compounds in all parts of the plant was observed during formation of flower buds.

The present paper describes the results of studies on biosynthesis of sterol glycosides in cell-free preparations of *C. officinalis*.

MATERIALS AND METHODS

Plants. Calendula officinalis L. var. Radio were cultivated in a lumistate under conditions described by Kasprzyk & Wojciechowski (1969). About 40-day-old flowering plants were used for experiments.

Enzymic preparations. Homogenates were prepared by disintegration of fresh plant material (leaves, roots, flowers) in a Potter homogenizer with 10 volumes of 0.1 M-tris-HCl, pH 8.0. In experiments use was made of a supernatant after centrifugation at 500 *g*. Subcellular fractions were obtained by consecutive centrifugation at 10 000 *g* and 105 000 *g*. The sedimented fractions were resuspended in a buffer to obtain the initial volume. To prepare acetone powders, fresh leaves were frozen in solid CO_2 and ground in a mortar, then five-gram portions were dehydrated by addition of 300 ml of anhydrous acetone chilled to -15°C, followed by ten times repeated extraction with 50 ml portions of acetone at 2°C. The residue remaining after vacuum drying was extracted with 10 ml of 0.2 M-tris-HCl buffer of pH 7.4 at 2°C. The supernatant after centrifugation at 500 *g* was used for the experiments.

Biosynthesis of sterol glucosides. The reaction mixture contained in a total volume of 0.5 ml: 0.2 ml of the enzymic preparation, 0.1 ml of aqueous solution of UDP-[U-^{14}C]glucose (0.1 mCi = 12 000 c.p.m.) and 0.2 ml of water. In some experiments 0.1 ml of ATP (5 mg/ml) was added instead of 0.1 ml of water, and/or 0.1 ml of emulsion of β-sitosterol or stigmasterol in 0.5% aqueous solution of Tween 20 (2 mg/ml). The mixture was incubated at 30°C for 1 - 4 hours and the reaction was terminated by adding 5 ml of methanol and boiling the samples for 3 min. The filtrate obtained therefrom was mixed with 5 ml of water, and methanol was distilled off. The aqueous residue was then extracted three times with 3 ml portions of *n*-butanol. The components of the extract were separated by means of thin-layer chromatography in system *I* or *II*, radioactive compounds being localized by autoradiography. Radioactivity was measured after elution from silica gel with methanol using thin-window G-M counter.

Quantitative determination of sterol derivatives. Three grams of fresh leaves were extracted three times with 20 ml portions of boiling methanol. An equal volume of water was added to the extract, methanol was removed by distillation under reduced pressure and the aqueous residue was extracted with *n*-butanol. One-half of the extract obtained was used for isolation of free sterols and sterol esters by means of thin-layer chromatography in system *III*. The remaining portion of the extract was separated in system *II* to yield sterol glucoside fraction and the fraction of sterol

acylglucosides. Glucosides were hydrolysed by heating with a mixture of hydrochloric acid - acetic acid - water (10:35:55, by vol.) at 100°C for 3 hours. Sterol esters were hydrolysed in 10% methanolic KOH at 100°C for 3 hours. The fraction of free sterols was further purified by means of thin-layer chromatography in system *IV* followed by acetylation with an excess of a mixture of [1,1′-^{14}C]acetic anhydride and pyridine (1:2, v/v) at room temperature for 20 hours. The mixtures of sterol acetates obtained were separated into individual compounds by means of thin-layer chromatography on silica gel impregnated with $AgNO_3$ in system *V*.

Thin-layer chromatography. This was performed on silica gel or on silica gel impregnated with $AgNO_3$ (10%, w/w) in the following solvent systems (all v/v): *I*, *n*-propanol - 14% ammonia (8:2); *II*, chloroform - methanol (15:3), saturated with water; *III*, chloroform - methanol (98 : 2); *IV*, chloroform - methanol (95 : 5); *V*, chloroform.

Paper chromatography of sugars was performed on Whatman no. 1 paper in the system: *n*-butanol - benzene - pyridine - water (5:1:3:3, by vol).

Reagents. [U-^{14}C]Glucose was prepared by the method of Putnam & Hassid (1952). The leaves of bean were supplied with $^{14}CO_2$ and glucose prepared from labelled starch.

UDP-[U-^{14}C]glucose was prepared by the method of Wright & Robbins (1952) using UTP, [U-^{14}C]glucose and enzymic preparation from baker's yeast.

β-Sitosterol, stigmasterol, isofucosterol and sterol glycosides were isolated from the flowers of *Calendula officinalis* and their acetyl derivatives prepared as described by Kasprzyk & Turowska (1969).

Oleanolic acid glycosides were isolated from the flowers of *Calendula officinalis* as described by Kasprzyk & Wojciechowski (1969).

[1,1′-^{14}C]Acetic anhydride, activity 2 mCi/mmol, was from The Radiochemical Centre (Amersham, England); ATP, from Koch-Light Laboratories (Colnbrook, Bucks., England); silica gel, kieselgel G nach Stahl, from Merck AG (Darmstadt, German Federal Republic).

RESULTS AND DISCUSSION

When the supernatant of leaf homogenate of *C. officinalis*, following incubation with UDP-[U-^{14}C] glucose, was submitted to thin-layer chromatography in systems *I* and *II*, two fractions soluble in *n*-butanol were found to be intensively labelled. Radioactivity of these two fractions together corresponded to 15% of the radioactivity of the UDP-[U-^{14}C]glucose applied, and accounted for about 90% of the total radioactivity of the butanolic extract. It was demonstrated by means of paper chromatography of acid hydrolysates of these fractions from which sterols were removed by ether extraction, that glucose was the only recovered radioactive compound. On the basis of its behaviour on cochromatography with appropriate standards, the fraction more polar on thin-layer chromatography was found to be a mixture of sterol 3-*O*-monoglucosides. The less polar fraction migrated on chromatography in system

IV at the same rate as monoacetyl-3-*O*-monoglucosides, and on additional acetylation, as tetraacetyl-3-*O*-monoglucosides. On alkaline hydrolysis this fraction was transformed into the more polar fraction *I* and exhibited the same radioactivity and chromatographic properties as that fraction.

It may be thus concluded that fraction *II* was most probably a mixture of sterol monoacetyl monoglucosides. So far no data were available as to the occurrence of this type of compounds in *C. officinalis*, which are, in addition to free sterols, their esters with fatty acids and 3-*O*-glucosides, the fourth form of sterols occurring in this plant.

The addition to the incubation mixture of the emulsion of β-sitosterol or stigmasterol, which are known to be the main sterols of *C. officinalis*, did not affect the rate of glucoside synthesis. On the other hand, addition of ATP stimulated quite distinctly (by about 15%) the rate of incorporation of glucose from UDP-[U-^{14}C]glucose into sterol glucosides and their acyl derivatives. A similar stimulatory effect of ATP was observed by Kauss (1968) and Hou *et al.* (1967) who studied biosynthesis of sterol glucosides in bean and soya-bean. The incorporation of glucose from UDP-[U-^{14}C]glucose into sterol acylglucosides was distinctly delayed as compared with sterol glucosides (Fig. 1), which implies that in the homogenates sterol 3-*O*-monoglucosides are synthesized first and are then acetylated to form acylglucosides.

This conclusion is supported by an up to 30% transformation of radioactive

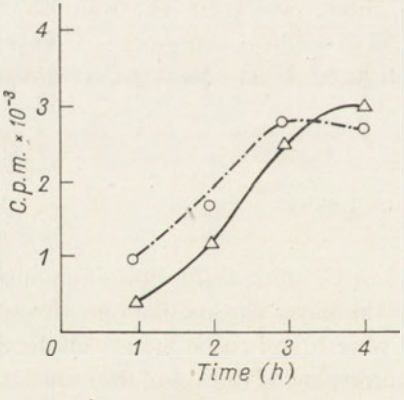

Fig. 1

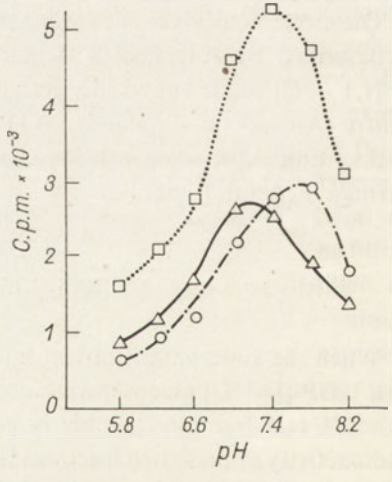

Fig. 2

Fig. 1. Incorporation of UDP-[^{14}C]glucose into sterol glucosides (○) and acylglucosides (△) in homogenates of *C. officinalis* leaves. Incubation at pH 8; for details see Methods.

Fig. 2. Effect of pH on incorporation of UDP-[^{14}C]glucose into sterol glucosides and acylglucosides in homogenates of *C. officinalis* leaves. Incubation time 2.5 h. pH values between 5.0 - 7.0 were adjusted with 0.1 M-phosphate buffer, and those between 7.2 - 8.2, with 0.1 M-tris-HCl buffer. ○, Sterol glucosides; △, sterol acylglucosides; □, sterol glucosides + sterol acylglucosides.

Table 1

Incorporation of UDP-[^{14}C]glucose into sterol glucosides and their acyl derivatives in homogenates of different organs of C. officinalis

Glucosides were separated by thin-layer chromatography as described in Methods. Incubation time 2 h in tris-HCl buffer, pH 8.

Organ	Activity (c.p.m.)	
	Sterol 3-*O*-glucosides	Sterol acyl-3-*O*-glucosides
Root	620	630
Leaf	1980	1660
Stem	2440	3100
Flower	2640	3320

[U-^{14}C]glucosides added to leaf homogenates into their acylderivatives. It was shown that both types of sterol glucoside derivatives are synthesized not only by the leaves but also by roots, stems and flowers of *C. officinalis* (Table 1).

The effect of pH on incorporation of UDP-[U-^{14}C]glucose into both types of compounds is presented in Fig. 2. The maximum incorporation of glucose into the non-separated fraction of sterol glucosides was observed at pH 7.4. The optimum pH for the synthesis of particular sterol glucosides differed slightly. Transformation of sterol [U-^{14}C]glucosides added to the leaf homogenates, into acyl derivatives occurred with the highest rate at a pH value of about 6.6.

To obtain some preliminary information on localization of glycosylation processes, incorporation of UDP-[U-^{14}C]glucose into both types of sterol glucosides was studied using preparations obtained by differential centrifugation of the leaf homogenate (Table 2) as described in Methods. The obtained results indicate that most of the glycosylation activity was found in the 10 000 *g* fraction but a considerable part was also present in the 105 000 *g* fraction, almost no activity being present in supernatant. As in the case of whole homogenate, the addition of sterol emulsion did not stimulate the incorporation of glucose from UDP-[U-^{14}C]glucose into the studied compounds by the enzymic fractions obtained after differential centrifugation. A significant stimulation (20-30%) after addition of β-sitosterol or stigmasterol was observed only with the acetone-dried leaf preparations. This is probably due to the removal of endogenous sterols which are the substrates for these enzymic processes. Acetone-dried powders obtained from leaves showed a considerable ability to synthesize sterol glucosides from UDP-[U-^{14}C]glucose but these preparations did not catalyse transformation of sterol glycosides into their acyl derivatives.

In order to determine the content of individual sterols, all the sterol fractions found in *C. officinalis* leaves separated by thin-layer chromatography were eluted, hydrolysed, acetylated with radioactive acetic anhydride, and rechromatographed (Table 3). It was found that all the examined sterol fractions contained β-sitosterol, stigmasterol and small amounts of isofucosterol. Differences in composition of sterol constituents in glycosides in comparison with free sterol fraction consist first of all

Table 2

Incorporation of UDP-[^{14}C]glucose into sterol glucosides and their acyl derivatives by the subcellular fractions of C. officinalis *leaves*

Glucosides were separated by thin-layer chromatography as described in Methods. Incubation in tris-HCl buffer, pH 8, containing ATP for 2 h.

Fraction	Activity (c.p.m.)	
	Sterol 3-*O*-glucosides	Sterol acyl-3-*O*-glucosides
Homogenate	1100	850
10 000 *g* fraction	840	410
105 000 *g* fraction	130	290
105 000 *g* supernatant	traces	traces

Table 3

The content of free and bound sterols in fresh leaves of C. officinalis

Determinations were made by radioactivity measurements of acetylated sterol compounds with labelled acetic anhydride following chromatographic separation described in Methods. The results are expressed in μg/g of fresh material.

Form	Total sterols	β-Sitosterol	Stigmasterol	Isofucosterol
Free sterols	115	42	65	8
Glucosides	63	42	20	1
Acylglucosides	23	16	7	traces
Esters	6	3	3	traces

in a considerably greater quantity of β-sitosterol in the glycosidically bound sterols. The fact that sterol acylglucosides have not been previously detected (Kasprzyk *et al.*, 1970) can be explained by their greater lability. The data obtained by Wojciechowski, Janiszowska & Kasprzyk (1970) indicate that in *C. officinalis* UDPglucose is a specific donor of glucose residues in biosynthesis of sterol glucosides. It is noteworthy that only insignificant (below 0.2%) incorporation of this precursor to oleanolic acid glycosides was observed, the content of which in *C. officinalis* is severalfold greater than that of sterol glucosides. A considerable ability of the examined preparations from *C. officinalis* to synthesize sterol glucosides, indicates higher turnover of this type of glucosides, connected probably with some important biochemical processes in this plant.

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BIOSYNTEZA GLIKOZYDÓW STEROLI W BEZKOMÓRKOWYCH PREPARATACH Z *CALENDULA OFFICINALIS*

Streszczenie

1. Bezkomórkowe preparaty enzymatyczne z roślin *Calendula officinalis* wykorzystują UDP-glukozę do syntezy 3-*O*-glukozydów steroli i acylopochodnych tych glukozydów (prawdopodobnie mono-acetylo-3-*O*-glukozydów). Z przeprowadzonych badań wynika, że proces acylowania zachodzi na poziomie już gotowych 3-*O*-glukozydów steroli. Optimum pH dla glukozylacji wynosi 7.6 a dla acylowania 6.6.

2. Zbadano skład wszystkich czterech form steroli występujących w liściach *C. officinalis* (t.j. steroli wolnych, estrów z kwasami tłuszczowymi, 3-*O*-glukozydów i acylo-3-*O*-glukozydów). Wszystkie formy zawierają *β*-sitosterol i stigmasterol oraz, w mniejszych ilościach, izofukosterol.

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ENZYMIC FORMATION OF POLYISOPRENOL PHOSPHATE SUGARS

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The formation of lipid-linked sugars from UDP-[6-^{3}H]glucose in microsomal preparations of rat liver and brain was stimulated by dolichol phosphate and ficaprenol phosphate, the latter being less effective. With enzyme preparations from *E. coli* and *Sh. flexneri* the formation of polyisoprenol monophosphate glucose was also observed, and ficaprenol phosphate was a better acceptor of sugar than dolichol phosphate. UDP galactose did not enter the reaction in rat liver and brain microsomes. Enzyme preparations from *E. coli* and *Sh. flexneri* catalysed the labelling of lipids from UDP-[^{14}C] galactose in the presence of polyisoprenol phosphate. A modified procedure for preparation of ficaprenol phosphate is presented.

The formation of bacterial wall polymers was the first biosynthetic pathway in which the role of long-chain polyisoprenols was recognized (Higashi, Strominger & Sweeley, 1967; Wright, Dankert, Fennessey & Robbins, 1967). In these and in several subsequent papers the C_{55} polyisoprenol was identified in several microorganisms as the lipid component of a variety of lipid-linked sugars involved in biosynthetic processes.

A few years earlier the Liverpool group began to study the structure and occurrence of polyisoprenols; Burgos, Hemming, Pennock & Morton (1963) reported on mammalian polyisoprenol, dolichol (C_{100}), and Stone, Wellburn, Hemming & Pennock (1967) isolated large quantities of C_{55} plant polyisoprenol, ficaprenol. Ficaprenol differs from bacterial C_{55} polyisoprenol in having 3 instead of 2 *trans* double bonds. In preparations obtained from leaves of *Ficus elastica* the C_{55} polyisoprenol was accompanied by C_{50} and C_{60} analogues.

The possible involvement of polyisoprenol-containing intermediates in mammalian metabolism has been studied less extensively. Behrens & Leloir (1970) demonstrated the formation of dolichol monophosphate glucose in rat liver microsomes and Alam, Barr, Richards & Hemming (1971) reported on the mannosyl transfer from GDP mannose to various polyisoprenol phosphates.

The function of dolichol monophosphate glucose is still not clear. It seemed that its formation might be the expression of a more general phenomenon. The attractive

possibility that compounds of this type may play a role in the synthesis and transport of intracellularly manufactured glycoproteins to the extracellular space has been raised in the paper of Caccam, Jackson & Eylar (1969).

The aim of the present paper was to study the formation of polyisoprenol phosphate sugars from UDPglucose and UDPgalactose by enzyme preparations from rat liver and brain and from bacteria. Two polyisoprenol phosphates varying in the chain length and in unsaturation (C_{100}, saturated α-terminal isoprene residue; C_{55}, unsaturated α-terminal isoprene residue) were used in view of their possible interchangeability. Some of the preliminary data concerning the specificity of mammalian enzymes synthetizing lipid-linked sugars have been published (Jankowski & Chojnacki, 1972).

A full description of the preparation of ficaprenol phosphate is also included in this paper as the literature data on the preparation of this compound are rather incomplete.

MATERIALS AND METHODS

Reagents. Polyethyleneimine (PEI), 50% solution, acetonitrile (distilled from P_2O_5 and K_2CO_3 before use) and Triton X-100 were from British Drug Houses (Poole, Dorset, England); D-[6-^{3}H]glucose (spec. act. 1000 mCi/mmol), and UDP-D-[U-^{14}C]galactose, ammonium salt (spec. act. 290 mCi/mmol) were from the Radiochemical Centre (Amersham, Bucks., England); phytol was from Calbiochem (Los Angeles, Calif., U.S.A.); ubiquinone (coenzyme Q_{10}), UDP, UDPgalactose, UDPglucose, UMP and UTP (sodium salts) were from Sigma (St. Louis, Mo., U.S.A.); trichloroacetonitrile (Eastman-Kodak, Rochester, N.Y., U.S.A.) was distilled before use. 1,2-D-Diglyceride was obtained from egg yolk lecithin by the action of phospholipase C according to Renkonnen (1966). Phytol phosphate was prepared by the method of Lahav, Chiu & Lennarz (1969). Di-triethylammonium phosphate was prepared from 85% orthophosphoric acid and triethylamine, and crystallized from acetonitrile.

Media for column chromatography. Alumina, acid washed, Brockmann activity V - VI, was prepared from alumina, neutral, Brockmann activity II (POCh, Gliwice, Poland). One kilogram was suspended in 3 litres of cyclohexane and shaken vigorously for 6 hours with 70 ml of 10% aqueous solution of acetic acid (Schneider, Clayton & Bloch, 1957). After filtration the alumina was dried in air. Its Brockmann grade was estimated by running on it a mixture of azobenzene and Sudan red in carbon tetrachloride (Brockmann & Schodder, 1941).

DEAE-cellulose in the acetate form was prepared as described by Dankert, Wright, Kelley & Robbins (1966) with some modifications. DEAE-cellulose (DEAE-SS, Serva, Heidelberg, German Federal Republic) was suspended in water, left for 24 hours, washed with 5 volumes of 1 N-NaOH, then with water until the washings became neutral, the finest particles of the adsorbent being decanted with water. Then the DEAE-cellulose was transferred to a Buchner funnel, washed with 95% ethanol and absolute methanol and dried in a desiccator. The dried material was

ground in a mortar with acetic acid. Before preparing the columns the adsorbent was washed with methanol containing 1% of water, and subsequently with chloroform - methanol (2:1, v/v). Columns were filled with the suspension of DEAE-cellulose in the above chloroform - methanol mixture, the adsorbent being gently pressed with a glass rod.

PEI-cellulose was prepared from cellulose powder for chromatography (POCh, Gliwice, Poland), 200 g, which was mixed with 500 ml of 2.5% aqueous solution of polyethyleneimine that was adjusted to pH 7 with 5 N-HCl. A thick suspension of PEI-cellulose was dried in air. Before preparing the column the adsorbent was washed with 2 volumes of 3 M-NaCl and then with water until the washing was free from chloride ions.

Sephadex G-10, Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and silicic acid, 100 mesh (Mallincrodt, St. Louis, Mo., U.S.A.) were used as supplied.

Enzyme preparations. Wistar rats, both sexes, weighing 150 - 200 g were used for preparing the microsomal fraction from liver and brain. Homogenates (10%) in 0.25 M-sucrose - 1 mM-EDTA were prepared with a motor-driven glass-teflon homogenizer of Potter-Elvehjem type. The homogenates were centrifuged at 8 000 *g* for 10 min in a refrigerated Sorvall centrifuge and from the resulting supernatant the microsomes were spun down by centrifuging at 105 000 *g* for 1 h in a MSE 40 ultracentrifuge at 4°C. The microsomal pellet was resuspended in 0.25 M-sucrose - - 1 mM-EDTA and centrifuged as above. The washed microsomes suspended in sucrose-EDTA solution were stored at -10°C until use.

Shigella flexneri 2a, virulent, and *Escherichia coli* K_{12} HfrC (from the Collection of the State Institute of Hygiene, Warsaw, Poland) were grown on a liquid medium (pepton tryptone 10 g, yeast extract 5 g, NaCl 10 g, glucose 1 g, and water, 1000 ml, adjusted to pH 7.1). Bacteria were collected in the early stationary phase and washed with 0.9% NaCl by centrifuging. Washed cells (wet weight 4 g) were suspended in 30 ml of 0.03 M-NaCl - 0.01 M-tris buffer, pH 7.1, and subjected to sonic oscillation at maximum power for 4 min at 0°C with a MSE probe. The suspension was then centrifuged for 20 min at 4000 *g* to remove unbroken cells and large debris. The supernatant was centrifuged at 105 000 *g* for 1 h and the resulting pellet was resuspended in 0.03 M-NaCl - 0.01 M-tris buffer, pH 7.1. These particulate enzyme preparations were stored at -10°C until use.

Preparation of labelled uridine diphosphate glucoses. UDP-[6-^{3}H]Glucose was prepared from [6-^{3}H]glucose by the method of Wright & Robbins (1965). The isolation of the labelled nucleotide-glucose from the deproteinized reaction mixture was performed on the column of PEI-cellulose (0.9 × 30 cm). The fractions eluted with 0.2 M-LiCl were monitored for u.v. absorbing material and radioactivity. UDPglucose formed the first u.v. absorbing peak. The specific activity of the preparation was 40 mCi/mmol.

[β-^{32}P]UDPglucose was prepared from [^{32}P]glucose-1-phosphate and UTP using a partially purified preparation of UDPglucose pyrophosphorylase from human red blood cells as described by Sawicka & Chojnacki (1969). The product

was isolated on a PEI-cellulose column as above, and its specific activity was 20 mCi/mmol.

The desalting of labelled UDPglucose preparations was performed by chromatography on Sephadex G-10 column (3.5×40 cm).

Enzyme assays. The reaction mixture for studying the formation of lipid-linked sugars was similar to that used by Behrens & Leloir (1970). It contained 0.2 M--glycylglycine buffer, pH 7.5; 0.1 M-mercaptoethanol; 0.01 M-Mg_2EDTA; 0.6% Triton X-100; 16 μM-UDP-[^{3}H]glucose (150 000 c.p.m./sample) or UDP-[^{14}C]galactose (30 000 c.p.m./sample) and the studied sugar-binding lipid (0.8 mM-dolichol phosphate, 1.6 mM-ficaprenol phosphate, 7.0 mM-phytol phosphate) which was dissolved in a small volume of chloroform - methanol (2:1, v/v) and first added to the tube; the solvent was removed *in vacuo*, other components of the reaction mixture were added, and finally the suspension of brain or liver microsomes (1.5 mg of protein) or a suspension of *E. coli* or *Sh. flexneri* particulate enzyme (0.5 mg of protein). The reaction mixture (final volume, 0.2 ml) was shaken vigorously on a Vortex-mixer and incubated at 37°C for 30 min. The reaction was stopped by adding 5 ml of chloroform - methanol (2 : 1, v/v) and immersing the tube in a water bath of 60°C. The protein precipitate was separated by centrifugation and the chloroform - methanol extract was washed with 0.73% NaCl and three times with 3 ml portions of the mixture of chloroform - methanol - water (3:48:47, by vol.). The lower, chloroform phase containing lipids was transferred to Packard vials and the solvent allowed to evaporate at 50°C. The radioactivity of the lipid residue was estimated in a Packard Tri-Carb scintillation spectrometer using dioxane scintillation solvent (Bray, 1960). When the radioactivity of proteins was to be estimated, the protein precipitate after treatment with chloroform - methanol was washed with this mixture, then twice with 5% trichloroacetic acid and twice with water. The washed protein was solubilized by adding 0.3 ml of 90% formic acid and heating at 60°C for 30 min. The radioactivity of the samples was estimate as above.

Analytical. Phosphorus was assayed using the method of Bartlett (1959) and protein by the method of Lowry, Rosebrough, Farr & Randall (1951). Mass spectrometry was conducted with a RMU-6D Hitachi mass spectrometer (ionization energy 70 e.v., source temperature 150°C, probe temperature 220°C). The nuclear magnetic resonance spectrum was recorded in CCl_4 with a JNM-4 H-100 Jeol instrument. Infrared spectra were recorded with a Carl Zeiss UR-10 instrument using solvent-free films of the studied substance.

Thin-layer chromatography. Silica gel G, silica gel H and kieselguhr were from Merck (Darmstadt, German Federal Republic).

For silica gel G plates (0.25 mm thick) the following solvents were used: *A*, isopropyl ether - light petroleum, 1:4, v/v (Stone *et al.*, 1967); *B*, chloroform - - methanol - conc. ammonia - water, 80:30:0.5:3, by vol. (Behrens & Leloir, 1970); *C*, chloroform - methanol - formic acid - water, 70:18.5:8:0.5, by vol. (Behrens & Leloir, 1970); *D*, diisobutyl ketone - acetic acid - water, 40:25:5, by vol. (Higashi, Siewert & Strominger, 1970); *E*, isopropanol - conc. ammonia - water, 6:3:1, by vol. (Pelc, 1970).

Plates of silica gel H (0.5 mm thick), prewashed with chloroform - methanol - - formic acid (2:1:1, by vol.) were developed with solvent *F*: chloroform - methanol - - water, 65:25:4, by vol. (Troy, Frerman & Heath, 1971).

Reversed-phase partition thin-layer chromatography was performed after Stone *et al.* (1967) on plates of kieselguhr (0.25 mm thick) impregnated with 5% (v/v) liquid paraffin in light petroleum using acetone - water (23:2, v/v) saturated with paraffin (solvent *G*).

The spots were detected with anisaldehyde reagent for polyisoprenoids (Dunphy, Kerr, Pennock & Whittle, 1966) and with acid molybdate reagent for phosphorus (Hanes & Isherwood, 1949).

Preparation of dolichol phosphate from pig liver

The method was based on the procedure of Behrens & Leloir (1970). The modifications concerned the preparing of original lipid extract from liver and the use of increasing concentrations of ammonium acetate in chloroform - methanol for eluting the sugar-binding lipid from DEAE-cellulose column.

Two kg of pig liver obtained from the slaughterhouse was passed through a mincing machine and suspended in 6 litres of acetone. The partially defatted and dehydrated tissue was collected on a Buchner funnel and extracted twice with 2 litres of chloroform - methanol mixture (1:1, v/v). The pooled extracts (4 litres) were made alkaline by adding 100 ml of 5 N-NaOH and left at 37°C for 15 min. Then 100 ml of conc. HCl (11 N) was added and the acidified mixture was boiled under reflux for 15 min. The cooled alkali- and acid-treated extract was mixed with 2 litres of chloroform and 1.5 litres of water. The mixture was shaken, the lower, chloroform phase collected by centrifuging, evaporated under a stream of nitrogen, the residue dissolved in 1 litre of chloroform - methanol (2:1, v/v) and shaken with 200 ml of water. After evaporating the chloroform phase, about 50 g of lipid material was obtained.

For isolation of sugar-binding lipid on DEAE-cellulose, 5 g of the above lipid material was dissolved in 50 ml of chloroform - methanol (2 : 1, v/v), applied to the column (1.5 × 15 cm) and eluted with continuously increasing concentrations of ammonium acetate (0 - 0.2 M or 0 - 0.4 M) in chloroform - methanol (Fig. 1). From each fraction, water-soluble substances were removed by adding 1/5 volume of water, shaking and allowing the phases to separate; the chloroform phase was collected and the enzymic test for the presence of sugar-binding lipid as well as the assay for phosphorus were performed. Two fractions of sugar-binding lipids were obtained that stimulated in the presence of rat liver microsomes the incorporation of radioactivity from UDP-[^{3}H]glucose into lipids (Fig.1). The first fraction (peak *I*) was preceded by a phospholipid material; the second, much smaller fraction of sugar- -binding lipid (peak *II*) was eluted at higher concentrations of ammonium acetate and partly coincided with the peak of lipid phosphorus.

To identify the sugar-binding lipid present in fraction *I*, it was submitted to thin-layer chromatography on silica gel G. Several phosphorus-positive spots

were detected in addition to those with R_F 0.0 and 0.8 in solvents *B* and *C*, respectively, which according to Behrens & Leloir (1970) are the R_F values for dolichol phosphate. Both in solvents *B* and *C* the spot corresponding to dolichol phosphate represented only 20% of the total lipid phosphorus present in the fraction. Dolichol phosphate was also identified by estimating the sugar-binding activity after separation

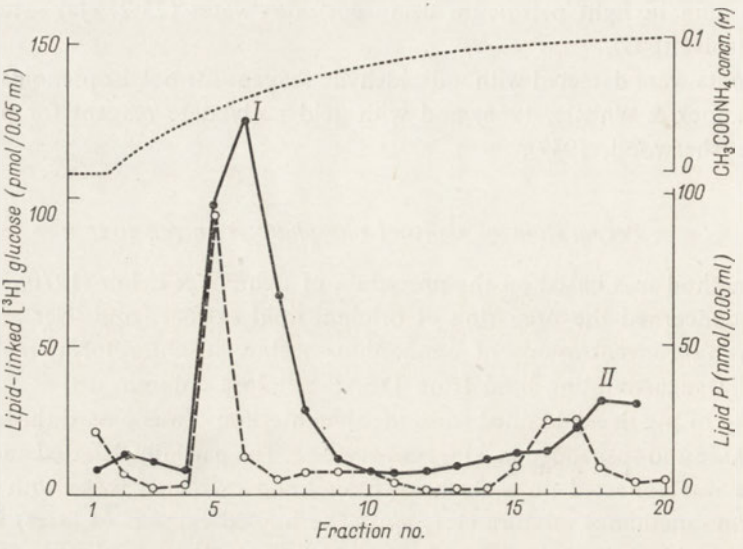

Fig. 1. Column chromatography on DEAE-cellulose of alkali- and acid-stable lipids from pig liver. To the column (1.5×15 cm) of DEAE-cellulose in acetate form lipids dissolved in 5 ml of chloroform - methanol (2:1, v/v) were applied and eluted with continuously increasing concentrations of ammonium acetate (pH 4) in chloroform - methanol, using a constant volume mixer (250 ml of chloroform - methanol, 2:1, v/v). The upper reservoir contained an appropriate solution of ammonium acetate prepared from a stock methanolic solution of 5 M-ammonium acetate - 3% acetic acid, in chloroform - methanol solvent. ———, Sugar-binding activity; - - -, lipid phosphorus; ···, ammonium acetate concentration. The volume of each fraction was 12 ml.

by thin-layer chromatography on silica gel H in solvent *F* and elution after Troy *et al.* (1971). The sugar-binding lipid was found to be present only in the band with R_F 0 - 0.1. On the basis of the above results and of the elution pattern from DEAE-cellulose, the sugar-binding lipid of fraction *I* (Fig. 1) was assumed to be dolichol phosphate.

The preparation of dolichol phosphate (peak *I*) was stored as solution in chloroform - methanol (2:1, v/v) at 4°C for one week without appreciable loss of activity.

Preparation of ficaprenol phosphate

Isolation of ficaprenol. Ficaprenol was obtained from leaves of *Ficus elastica* by modification of the original procedure of Stone *et al.* (1967). The modification consisted in drying the plant material and in a simplification of the procedure of purification of ficaprenol on alumina (Jankowski & Chojnacki, 1971).

Leaves of *Ficus elastica* (90 g) were cut into small pieces and allowed to dry

in open air. Dry leaves (20 g) were extracted three times with fresh 500 ml portions of acetone - diethyl ether (3:1, v/v) in a homogenizer. The pooled extracts were evaporated to dryness on a rotary evaporator. The residue was dissolved in 10 ml of light petroleum and the clear dark-green solution was applied to a column (3.5×25 cm) of acid-washed alumina. The column was washed with 200 ml of light petroleum and then with 150 ml portions of light petroleum containing diethyl ether at concentrations increasing up to 20%. Fifteen 150-ml fractions were collected, the solvent evaporated and the residues examined by thin-layer chromatography in solvent *A* (Fig. 2). The fractions eluted from alumina contained a large variety of compounds stained with the anisaldehyde reagent.

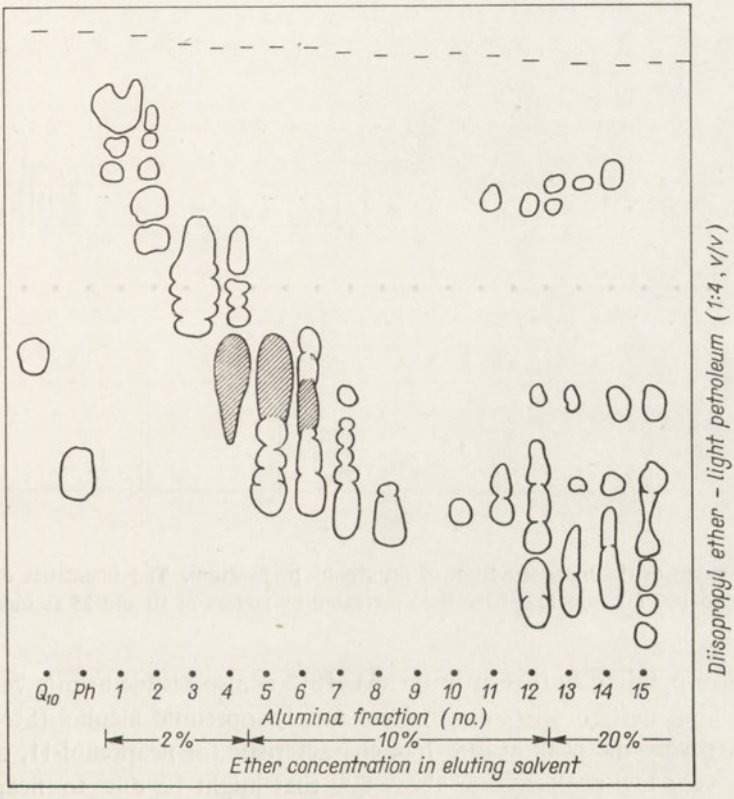

Fig. 2. Thin-layer chromatography on silica gel G of lipids from *Ficus elastica* separated by alumina column chromatography. Solvent system *A*; detection with anisaldehyde reagent. *Q*, ubiquinone; *Ph*, phytol; *1 - 15*, fractions (150 ml each) eluted with increasing concentrations of diethyl ether in light petroleum as indicated. Hatched spots correspond to ficaprenol.

The assay for the presence of ficaprenol was performed using mass spectrometry, nuclear magnetic resonance spectroscopy and infrared spectroscopy. Crude lipid material from alumina fractions nos. *2*, *4*, *8* and *12*, and purified material from pooled alumina fractions *4*, *5* and *6* (cf. Fig. 2) were analysed. The purified material was prepared in the following way. Pooled lipid fractions *4*, *5* and *6* were evaporated,

dissolved in benzene, applied to a column of silicic acid (2×20 cm) and eluted with chloroform. The substance which was first eluted from the column, just after trace amounts of u.v. absorbing material had passed, on thin-layer chromatography on silica gel G with solvent *A* had an R_F value 0.45. The substances which were next eluted from the silicic acid column, with R_F 0.2 - 0.4, were derived mainly from alumina fractions *5* and *6*.

The mass spectrum of the purified material with R_F 0.45 (Fig. 3) was characteristic of long-chain isoprenoid alcohol. Mass spectrometry showed a molecular ion (M^+) peak at m/e 766, consistent with the ficaprenol possessing 11 isoprene residues.

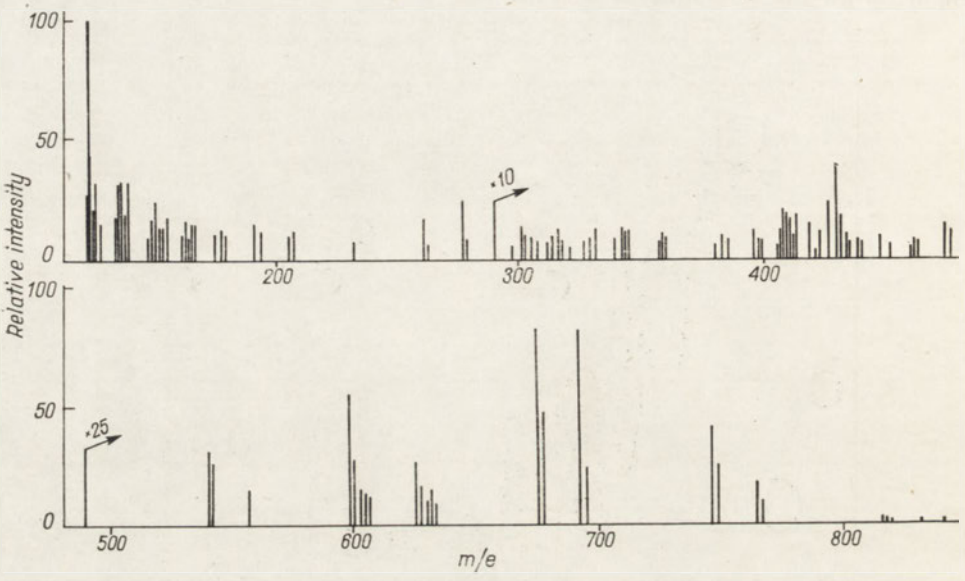

Fig. 3. Histogram of the mass spectrum of ficaprenol preparation. The intensities of the peaks (relative to 100 for m/e 122) have been increased by factors of 10 and 25 as indicated.

The ion corresponding to loss of water $(M-18)^+$ is also visible at m/e 747 as well as the cracking pattern characteristic for a polyisoprenoid alcohol (Stone *et al.*, 1967). Apart from the peak at m/e 766, characteristic for ficaprenol-11, our preparation gave very low peaks at m/e above 800 that might be due to ficaprenol-12.

The results of nuclear magnetic resonance spectroscopy in CCl_4 are shown in Fig. 4. The predominance of isoprene residues in *cis* configuration, the presence of primary allylic alcohol and of *cis* "OH-terminal" isoprene residue is evident from the tracings of the spectrum. The assignment of the peaks to resonating protons in the spectrum of our preparation was taken from the data of Stone *et al.* (1967) for ficaprenol-11. The nuclear magnetic resonance spectrum was identical with that of ficaprenol-11 obtained by the above authors.

Infrared spectroscopy of our preparation demonstrated a weak C=O stretching band at 1730 cm^{-1}, otherwise the spectrum is identical with the spectrum of ficaprenols reported by Stone *et al.* (1967). In our experience the use of i.r. spectroscopy

for identification of polyisoprenols in the course of preparation was of value only at higher stages of purification.

On reversed-phase thin-layer chromatography (solvent *G*) our preparation of ficaprenol exhibited the presence of three substances with R_F values 0.65, 0.50 and 0.40, as expected for the mixture of ficaprenols -10, -11 and -12, respectively, ficaprenol-11 being predominant.

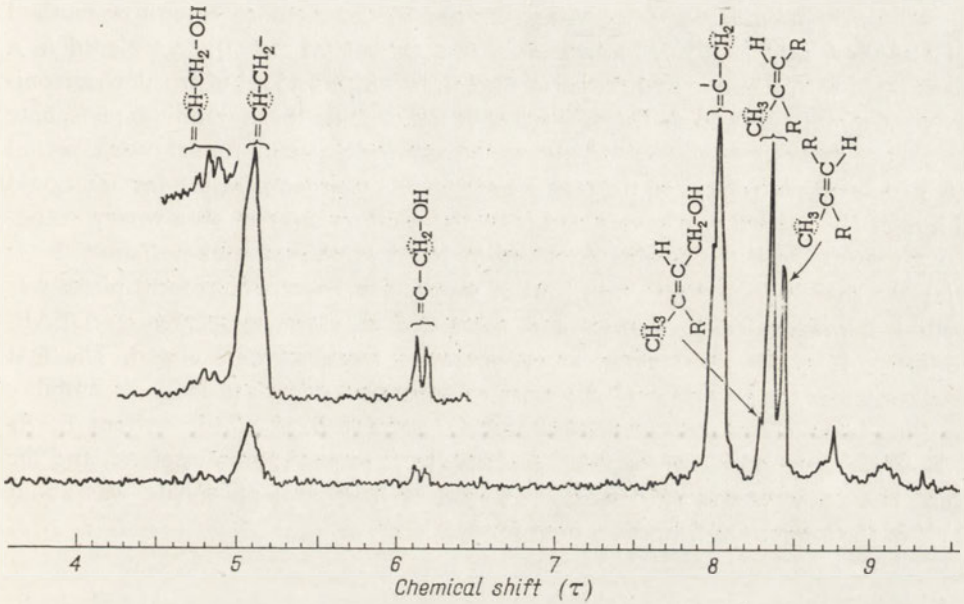

Fig. 4. Nuclear-magnetic-resonance spectrum of ficaprenol preparation in carbon tetrachloride at 100 Mcyc./sec. The assignments of the peaks to resonating protons (encircled with a broken line) are taken from Stone *et al.* (1967).

From 20 g of dried leaves, 100 mg of ficaprenol was obtained. According to Stone *et al.* (1967), *Ficus elastica* is the richest source of polyisoprenols (2.6 g per 1 kg of fresh weight of leaves). We could attain only the yield of 1 g per kg. This was probably due to our having at disposal rather young plants, as the content of polyisoprenols is known to increase with age of a plant (Wellburn & Hemming, 1966), though the effect of drying of leaves on the content of polyisoprenols cannot be excluded.

For preliminary identification of polyisoprenols in the course of isolation and purification, we have applied ubiquinone-10 and phytol as reference substances. On thin-layer chromatography the spot of ficaprenol was found above that of phytol and below that of ubiquinone. It could be distinguished from the former by its characteristic beige colour with anisaldehyde reagent (whereas phytol gives a blue-violet spot), and from ubiquinone by the lack of u.v. absorption. The use of the above two standards was also convenient for isolation of polyisoprenols from *Aesculus hippocastanum* and *Betula verrucosa* which, as reported by Wellburn, Stevenson, Hemming & Morton (1967) and Lindgren (1965), respectively, contain

large amounts of polyisoprenols. It should be added that we were unable to detect any polyisoprenols in the leaves of *Ficus pandurata*.

The described procedure for isolation of ficaprenol simplifies its purification, and the use of dried plant material may be advantageous, especially when it is intended to prepare polyisoprenols from imported exotic rubber-producing plants such as *Hevea brasiliensis* (Dunphy, Kerr, Pennock, Whittle & Fenney, 1967).

Phosphorylation of ficaprenol was performed by the trichloroacetonitrile method of Cramer & Böhm (1959). Ficaprenol, 70 mg (about 0.1 mmol), was placed in a flask fitted with a stirrer and dropping funnel, to which 0.15 ml of trichloroacetonitrile (0.6 mmol) was added. The solution of 70 mg of di-triethylammonium phosphate in 2 ml of acetonitrile was added slowly through the dropping funnel over a period of 30 min with stirring, and the mixture stirred at room temperature for additional 2 hours. The solvent was evaporated from the reaction mixture on a rotary evaporator (<40°C) and the residue dissolved in 10 ml of chloroform - methanol (2 : 1, v/v) and shaken three times with 2 ml of water. The lower, chloroform phase was collected, evaporated to dryness and submitted to chromatography on DEAE--cellulose (Fig. 5). Three phosphorus-containing fractions were eluted. The first one contained the monophosphoric ester of ficaprenol as judged from its mobility on thin-layer chromatograms (silica gel G; solvent *D*, R_F 0.65; solvent *E*, R_F 0.9). The second peak corresponded probably to ficaprenol pyrophosphate, and the third one to inorganic phosphate. The yield of ficaprenol phosphate was about 20% of the amount of ficaprenol used for synthesis.

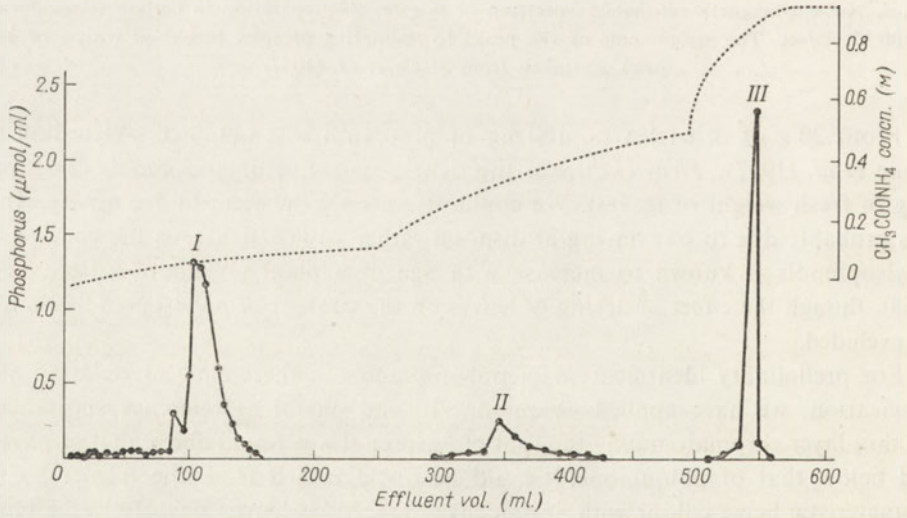

Fig. 5. Purification of ficaprenol phosphate by column chromatography on DEAE-cellulose. The chloroform phase from the phosphorylation reaction mixture was evaporated, the lipids dissolved in 5 ml of chloroform - methanol (2:1, v/v) and chromatographed as described in Fig. 1. Peak *I*, ficaprenol phosphate; peak *II*, unidentified; peak *III*, non-lipid phosphate.

RESULTS

Two fractions of alkali- and acid-stable lipids from pig liver separated by DEAE--cellulose column chromatography, stimulated the incorporation of ^{3}H-labelled glucose from UDPglucose into lipids by rat liver microsomes (Fig. 1). The main fraction (peak *I*) contained dolichol phosphate, and the smaller fraction, another sugar-binding lipid. When UDP-[^{14}C]galactose was used as sugar donor, no radioactive lipids were formed.

The incorporation of labelled sugar into the insoluble residue remaining after extraction with lipid solvents, was also studied (Fig. 6) to find out whether the incorporation of labelled sugar into glycoproteins was dependent on the presence of sugar-binding lipids. When liver microsomes were incubated with UDP-[^{3}H]glucose in the presence of sugar-binding lipids only a small increase of protein labelling was observed, and with UDP-[^{14}C]galactose as the sugar donor no increase of the labelling of proteins occurred.

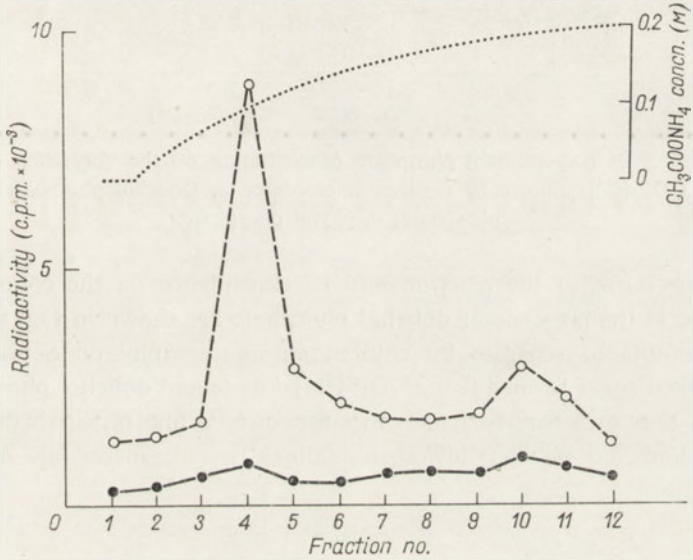

Fig. 6. The effect of alkali- and acid-stable lipid fractions from pig liver separated by DEAE--cellulose chromatography, on incorporation of radioactivity from UDP-[6-^{3}H]glucose into lipids and proteins by rat liver microsomes. For the enzymic assays 1 ml samples of DEAE-cellulose eluate were used. Incorporation: — — —, into lipids; ——, into proteins; ···, concentration of ammonium acetate.

The dependence on the amount of polyisoprenol phosphate of the formation of lipid-linked sugars from UDPglucose was studied with liver microsomes. As the dolichol phosphate preparation contained also other phospholipid substances, to study the effect of dolichol phosphate its actual concentration was calculated on the basis of the phosphorus assay after thin-layer chromatographic separation (see Methods). The dependence of the incorporation of radioactivity on the concentration of dolichol phosphate was linear (Fig. 7, curve *a*); in the case of ficaprenol phosphate,

the labelling of lipids first increased with the rising content of this compound, then decreased (curve *b*); with phytol phosphate, no stimulation was observed (curve *c*). For further experiments, the following concentrations of the polyisoprenol phosphates studied were used: dolichol phosphate, 0.8 mM; ficaprenol phosphate, 1.6 mM, and phytol phosphate, 7.0 mM.

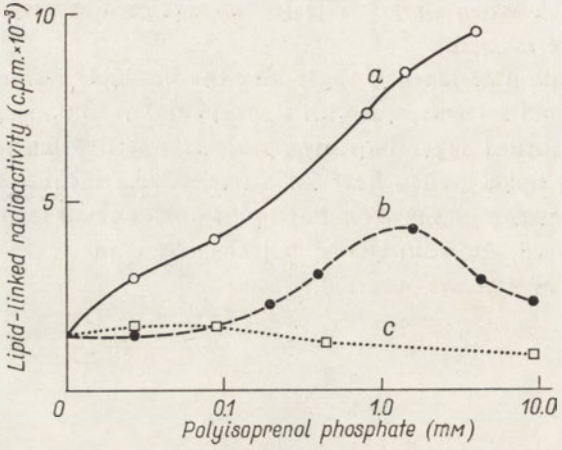

Fig. 7. The effect of polyisoprenol phosphate concentration on the formation of lipid-linked sugars from UDP-[6-^{3}H]glucose by rat liver microsomes. *a*, Dolichol phosphate; *b*, ficaprenol phosphate; *c*, phytol phosphate.

The time-course of the reaction and its dependence on the concentration of UDPglucose in the presence of dolichol phosphate are shown in Fig. 8.

The attempts to separate by column chromatography on Sephadex LH-20 the lipid-linked sugar formed from UDP-[^{3}H]glucose and dolichol phosphate in the presence of liver microsomes, from other microsomal phospholipids (mainly phosphatidylcholine and phosphatidylethanolamine) were unsuccessful. Although the

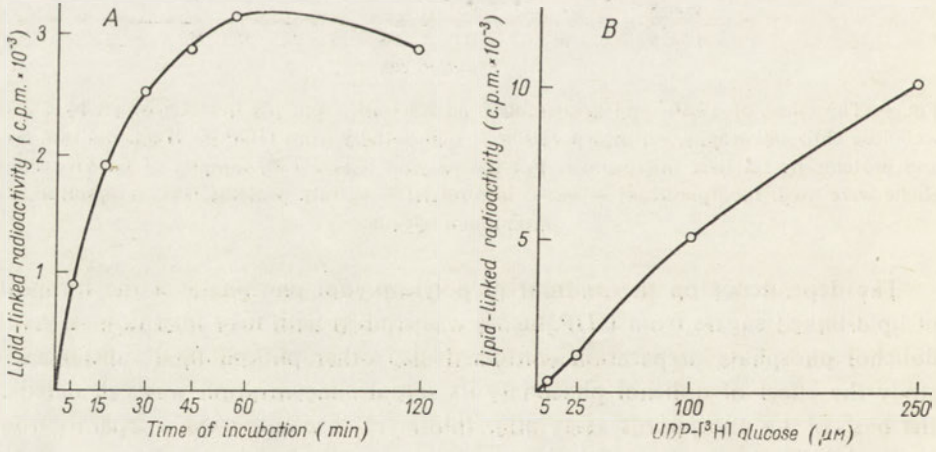

Fig. 8. *A*, Time-course of lipid-linked sugar formation from UDP-[6-^{3}H]glucose (16 μM) and 0.8 mM-dolichol phosphate by rat liver microsomes. *B*, Effect of UDP-[6-^{3}H]glucose concentration.

radioactivity appeared in the eluate prior to phospholipids, as expected for large molecule of dolichol monophosphate glucose, subsequently a considerable tailing of radioactive material was observed (Fig. 9).

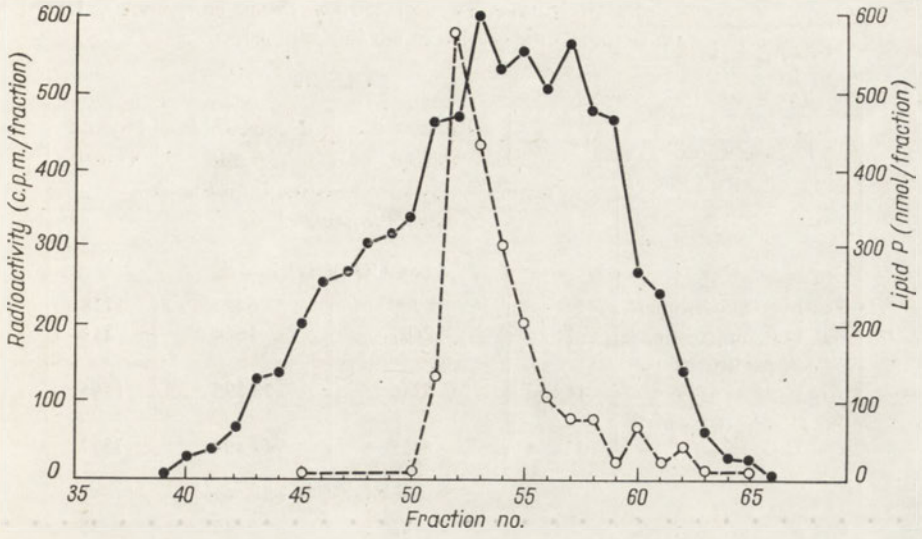

Fig. 9. Column chromatography on Sephadex LH-20 of radioactive lipids formed from UDP-[6-^{3}H]glucose and dolichol phosphate by rat liver microsomes. Lipid extract from a typical incubation mixture was applied on a 0.9×60 cm column and eluted with 0.1 M-ammonium acetate in 99% methanol. Fractions of 0.36 ml were collected and assayed for ○, phosphorus and ●, radioactivity.

The effect of various polyisoprenol phosphates on formation of lipid-linked sugars from UDPglucose by microsomes of rat liver and brain or enzyme preparations from *E. coli* and *Sh. flexneri* is presented in Table 1. Dolichol phosphate and ficaprenol phosphate increased the formation of lipid-linked sugar with every studied enzyme preparation. However, dolichol phosphate was more effective with rat microsomal preparations, and ficaprenol phosphate with bacterial enzymes. Phytol phosphate had no effect.

The transfer of glucose from UDPglucose to lipids, catalysed by liver microsomes, was not accompanied by the transfer of the phosphate group adjacent to glucose. This is evident from the results on the incorporation of radioactivity into lipids from UDP-[^{3}H]glucose and [β-^{32}P]UDPglucose in the presence of polyisoprenol phosphates (Table 2). However, with ficaprenol phosphate, trace incorporation of ^{32}P was observed with bacterial enzymes.

The effect of 1,2-diglyceride on the incorporation of ^{3}H-labelled glucose from UDPglucose into lipids is presented in Table 3. Diglyceride alone did not stimulate the labelling of lipids, it enhanced, however, the increase in labelling of lipids by polyisoprenol phosphate with preparations of rat liver microsomes and particulate enzyme from *E. coli*, but had no effect in the case of *Sh. flexneri*.

The effect of UMP and UDP on the formation of lipid-linked sugar from UDP-[^{3}H]glucose is shown in Table 4. With liver and brain microsomes, with either of

Table 1

The effect of various polyisoprenol phosphates on the formation of lipid-linked sugar from UDP-[^{3}H]glucose and UDP-[^{14}C]galactose

Substrate concentrations: UDP-[^{3}H]glucose 16 μM (150 000 counts/min/sample), UDP-[^{14}C] galactose 16 μM (30 000 counts/min/sample).

Enzyme source	Addition			
	None	Dolichol-P (0.8 mM)	Ficaprenol-P (1.6 mM)	Phytol-P (7 mM)
	Incorporation of radioactivity into lipids (counts/min/sample)			
	from UDP-[^{3}H]glucose			
Rat liver microsomes	1163	18 906	6426	1128
Rat brain microsomes	357	2497	1644	211
E. coli particulate enzyme	1647	2246	33 395	1465
Sh. flexneri particulate enzyme	3745	8325	40 494	3377
	from UDP-[^{14}C]galactose			
Rat liver microsomes	38	42	48	35
Rat brain microsomes	32	34	55	33
E. coli particulate enzyme	201	244	3238	280
Sh. flexneri particulate enzyme	1446	1357	5429	1820

Table 2

Incorporation of ^{32}P and ^{3}H into lipids from labelled UDPglucose

The data were obtained in separate experiments with [β-^{32}P]UDPglucose and with UDP-[6-^{3}H] glucose, with or without 0.8 mM-dolichol phosphate or 1.6 mM-ficaprenol phosphate.

Source of enzyme	Polyisoprenol phosphate added	Incorporation into lipids (pmol/sample) ^{32}P	^{3}H
Rat liver microsomes	None	3.4	25
	Dolichol-P	3.4	403
	Ficaprenol-P	3.4	135
E. coli particulate enzyme	None	3.2	35
	Dolichol-P	3.6	48
	Ficaprenol-P	5.3	710
Sh. flexneri particulate enzyme	None	3.4	80
	Dolichol-P	3.4	177
	Ficaprenol-P	4.0	860

Table 3

The effect of UMP and UDP on the formation of lipid-linked sugars from UDP-[^{3}H] glucose

Dolichol phosphate was added to the final concentration of 0.8 mM, ficaprenol phosphate 1.6 mM, and UMP or UDP, 0.25 mM, as indicated.

Enzyme preparation	Polyisoprenol phosphate	Incorporation of ^{3}H into lipids (counts/min/sample)		
		Control	with UMP	with UDP
Rat liver microsomes	None	1163	892	778
	Dolichol-P	18 906	10 027	6435
	Ficaprenol-P	6426	4869	4031
Rat brain microsomes	None	357	134	124
	Dolichol-P	2497	877	437
	Ficaprenol-P	1644	822	495
E. coli particulate enzyme	None	47	—	—
	Ficaprenol-P	2326	1017	47
Sh. flexneri particulate enzyme	None	1839	—	—
	Ficaprenol-P	24 483	18 922	175

Table 4

The effect of polyisoprenol phosphates and diglyceride on the formation of lipid-linked sugars from UDP-[6-^{3}H]glucose

Dolichol phosphate was added to the final concentration of 0.8 mM; ficaprenol phosphate, 1.6 mM, and diglyceride, 0.25 mM as indicated.

Source of enzyme	Addition	Incorporation of ^{3}H into lipids (counts/min/sample)
Rat liver microsomes	None	1387
	Diglyceride	1786
	Dolichol-P	4094
	Dolichol-P, diglyceride	6549
E. coli particulate enzyme	None	179
	Diglyceride	127
	Ficaprenol-P	3935
	Ficaprenol-P, diglyceride	6652
Sh. flexneri particulate enzyme	None	810
	Diglyceride	855
	Ficaprenol-P	23 966
	Ficaprenol-P, diglyceride	22 573

the sugar-binding lipids, the effect of UMP and UDP was rather small, although UDP had a slightly greater effect. On the other hand, in the case of bacterial preparations, with which only ficaprenol phosphate was used, UDP had a marked inhibitory effect.

Chloramphenicol had no inhibitory effect on the formation of lipid-linked sugar from UDP-[^{3}H]glucose with rat liver microsomes or bacterial preparations, either in the presence or absence of ficaprenol phosphate.

When UDP-[^{14}C]galactose was used as sugar donor, in bacterial preparations both ficaprenol phosphate and dolichol phosphate produced a large increase in lipid labelling (Table 1). On the other hand, with brain or liver microsomes no stimulation of lipid-linked sugar formation was observed either with dolichol phosphate, ficaprenol phosphate or phytol phosphate. The lack of transfer of galactose to dolichol phosphate in rat liver microsomes was also observed by Leloir (1971).

DISCUSSION

The stimulation by dolichol phosphate of formation of lipid-linked glucose from labelled UDPglucose with rat liver microsomes was first observed by Behrens & Leloir (1970). In our experiments a somewhat lower stimulation was observed with brain microsomes. A stimulation was also observed when the sugar-binding lipid was prepared from pig spleen (Jankowski & Chojnacki, unpublished results).

Column chromatography on DEAE-cellulose of alkali- and acid-stable lipids from pig liver demonstrated the presence of another more anionic sugar-binding lipid besides dolichol phosphate (Jankowski & Chojnacki, 1972). In the present experiments partial separation of dolichol phosphate from the bulk of phospholipid material was achieved (Fig. 1).

Stimulation of the formation of lipid-linked glucose in rat liver and brain microsomes and in bacterial preparations was observed both with dolichol phosphate and ficaprenol phosphate, irrespective of the known differences between the two polyisoprenols; the predominant chain length of dolichol in pig tissues is 20 isoprene residues and the *a*-terminal residue is saturated (Burgos *et al.*, 1963), whereas ficaprenol contains 11 isoprene residues, and the *a*-terminal residue is unsaturated (Stone *et al.*, 1967). The finding that with bacterial enzymes the shorter chain ficaprenol phosphate was more effective than dolichol phosphate, and the observation of the reverse phenomenon with rat enzymes might be correlated with the fact that the main polyisoprenols in bacteria are of the C_{55} type, and in mammals dolichol (C_{100}) is present (Hemming, 1969).

Differences in stimulation of mannosyl transfer from GDPmannose in pig liver microsomes were also observed by Alam *et al.* (1971) who used various polyisoprenols as sugar-binding lipids. Higashi *et al.* (1970) observed differences of the rate of phosphorylation of various polyisoprenols by enzyme preparations from *Staphylococcus aureus*. The phosphorylation with ficaprenol (C_{45} - C_{60}) and betulaprenol (C_{30} - C_{45}) was much higher than with dolichol (C_{80} - C_{105}).

The logical background for the given polyisoprenol being both the most abundant in an organism and being the optimal in enzymic assays could be the type of complementary structures that control both the function of the *cis*-polyisoprenol synthetase and the processes in which polyisoprenolic coenzymes are involved.

As pointed out by Scher & Lennarz (1969), the presence of double bond in the α-terminal isoprene residue increases the lability of the phosphate ester bond and might therefore specify the type of transfer reaction in which polyisoprenoid carriers are involved. The mechanism of the formation of both dolichol monophosphate glucose and ficaprenol monophosphate glucose as judged from the experiments with [^{32}P]UDPglucose and inhibition by UDP, irrespective of the type of lipid acceptor, was:

$$\text{UDPglucose} + \text{polyisoprenol phosphate} \rightleftharpoons$$
$$\rightleftharpoons \text{polyisoprenol phosphate glucose} + \text{UDP}$$

The formation of polyisoprenol monophosphate glucose in bacteria was described by Wright (1971). The reaction was found only in *Salmonella typhimurium* carrying the temperate phage epsilon 34 and was supposed to be directed by the phage genome. A similar observation was made by Nikaido, Nikaido & Nakae (1971).

The demonstration that both in *E. coli* and *Sh. flexneri* the formation of lipid--linked glucose from UDP-[^{3}H]glucose was stimulated by ficaprenol phosphate and dolichol phosphate will necessitate the search in these strains for a phage genome, which according to Wright (1971) could be responsible for this reaction.

The stimulation of the incorporation of galactose from UDP-[^{14}C]galactose by polyisoprenol phosphates was observed with enzyme preparations of both bacterial species used, but the mechanism of formation of lipid-linked galactose has not been studied. In Enterobacteriaceae, polyisoprenol phosphate galactose is known to be formed (Osborn, 1969), and its role is restricted to the formation of galactose--containing repeating O-antigenic sugar sequences. In *Shigella flexneri* 2a studied in the present paper, the repeating sugar sequences do not contain galactose (Simmons, 1969).

The results demonstrating a slight increase of protein labelling from UDP-[^{3}H] glucose in the presence of sugar-binding lipids are in agreement with the data of Behrens & Leloir (1970), indicating that dolichol monophosphate glucose is the sugar intermediate in glucosylation of proteins.

In our experiments, chloramphenicol showed no inhibitory effect on the formation of polyisoprenol phosphate sugars. Thus our results do not support the suggestion of Stow, Starkey, Hancock & Baddiley (1971) of a direct action of this antibiotic on the transfer of glucose from nucleotide precursor to lipid carrier.

Our results suggesting that polyisoprenol phosphate glucose might be an intermediate in the formation of other glycolipids, as indicated by the increase of lipid labelling in the presence of both polyisoprenol phosphate and diglyceride, will need further support.

The authors wish to express their gratitude to Prof. L.D. Bergelson for enabling us to perform the n.m.r. and mass spectrometric analyses at the Institute of Chemistry of Natural Products of the USSR Academy of Sciences in Moscow, to Dr. G. B. Ansell (The Medical School, Birmingham, England) for kind gift of trichloroacetonitrile, to Doc. Dr J. Kościelak (Institute of Hematology, Warsaw) for a gift of labelled UDPgalactose, to Dr Ewa Janczura (State Institute of Hygiene, Warsaw) for providing us with bacterial material, and to the staff of the PKO-Rotunda Office in Warsaw for giving us leaves of *Ficus elastica*. The excellent technical assistance of Mr. K. Prus and Mr. J. Kubiak is greatly appreciated.

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ENZYMATYCZNA SYNTEZA POLIIZOPRENOLOFOSFOCUKRÓW

Streszczenie

Dolicholofosforan i fikaprenolofosforan stymulują syntezę połączeń lipidowo-cukrowych z UDP-[6-^{3}H]glukozy w preparatach mikrosomów wątroby i mózgu szczura, przy czym efekt stymulujący dolicholofosforanu był większy. Również preparaty enzymatyczne uzyskane z *E. coli* i *Sh. flexneri* katalizowały syntezę poliizoprenolomonofosfoglukozy, przy czym fikaprenolofosforan był lepszym akceptorem reszty cukrowej niż dolicholofosforan. Mikrosomy wątroby i mózgu nie katalizowały włączania znakowanej UDPgalaktozy do lipidów, natomiast enzymy bakteryjne katalizowały włączanie radioaktywności z UDP-[^{14}C]galaktozy. Opisano zmodyfikowaną procedurę preparatyki fikaprenolofosforanu.

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SYNTHESIS OF MALONATE AND METHYLMALONATE AND THE FORMATION OF POLYENE ANTIBIOTICS

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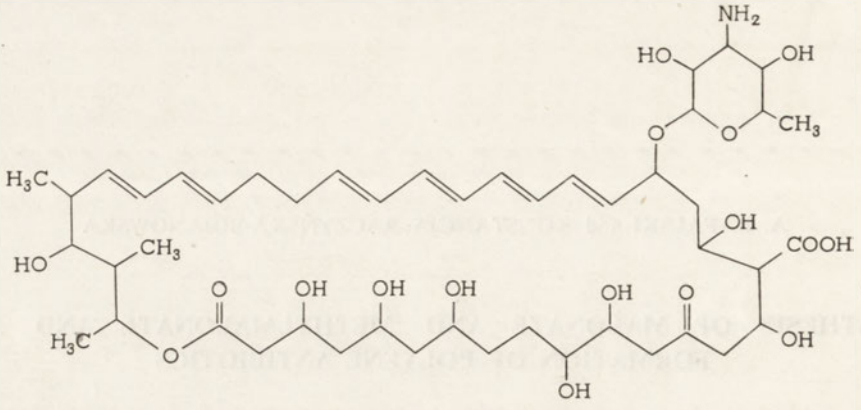
1. In *Streptomyces noursei* var. *polifungini* malonate and methylmalonate are synthesized both by carboxylation and transcarboxylation of acetyl-CoA and propionyl-CoA; the activity of methylmalonyl-CoA carboxyltransferase is by two orders of magnitude higher than that of acetyl-CoA and propionyl-CoA carboxylase. 2. Both activities are higher, parallelly to the increased lipogenesis, in the mutant giving higher yields of tetraene antibiotics of nystatin type. 3. Cooperation of transcarboxylase with phosphoenolpyruvate (PEP) carboxylase in the synthesis of building units for biosynthesis of the antibiotics is suggested. 4. Formation of antibiotics in cultures grown on carbohydrate medium is associated with a rapid decrease in concentration of C_3 acids: pyruvate and lactate and the increase of C_4 acids: oxaloacetate and malate. 5. Evidence for direct synthesis of PEP from pyruvate is presented, based on the pyruvate diphosphate kinase type of reaction. The role of this system in replenishment of PEP used in biosynthesis of antibiotics is discussed.

Acetate and propionate are the basic building units of macrolide ring of polyene antibiotics showing antifungal properties. In the formation of polyfungin A_1 (see Scheme 1), a main component of tetraene antibiotics produced by *Streptomyces noursei* var. *polifungini* (Porowska *et al.*, 1972), 16 acetate and 3 propionate units polymerize to form C_{41} macrolide ring containing the diene and tetraene functions (Birch *et al.*, 1964; Ikeda, Suzuki & Djerassi, 1967). The mechanism of polymerization is the same as in biosynthesis of fatty acids, i.e. energy is gained in carboxylation reactions, thus malonyl-CoA and methylmalonyl-CoA are responsible for elongation of acyl chain by C_2 and C_3 fragments, respectively (Wawszkiewicz & Lynen, 1964; Lynen, 1967; Manwaring, Rickards, Gaudiano & Nicolella, 1969). The close analogy between biosynthesis of fatty acids and macrolide rings of antibiotics calls attention to the role of non-specific acetyl-CoA carboxylase (acetyl-CoA : carbon dioxide ligase, EC 6.4.1.2) in the formation of antibiotics. This allosteric protein is generally known as a regulatory enzyme in the synthesis of fatty acids in microorganisms (Birnbaum, 1969; Rasmussen & Klein, 1967), plants (Burton & Stumpf, 1966) and mammals (Alberts & Vagelos, 1968; Greenspan & Lowenstein, 1968; Maragoudakis, 1970).



Scheme 1. Polyfungin A_1 identical with nystatin A_1 (after Chong & Rickards, 1970; Borowski *et al.*, 1971).

In the present work, the acetyl-CoA and propionyl-CoA carboxylase activities were studied, as well as transcarboxylation of propionyl-CoA (methylmalonyl-CoA : pyruvate carboxyl transferase, EC 2.1.3.1) in which oxaloacetate is a donor of carboxyl group. The role of the latter carboxylation system has been discussed in relation to oxaloacetate synthesis and metabolism in the investigated mutants of *S. noursei* var. *polifungini*.

MATERIALS AND METHODS

Reagents. Lactate dehydrogenase, malate dehydrogenase, myokinase, NAD, NADH, ATP, oxaloacetate, glutathione reduced, phosphoenolpyruvate, aspartate transaminase, inorganic pyrophosphatase and tris were products of Boehringer and Soehne (Mannheim, G.F.R.); coenzyme A and pyruvate kinase were from Sigma (St. Louis, Mo., U.S.A.), 2-mercaptoethanol was obtained from Koch-Light (Colnbrook, Bucks., England), ethyleneglycolmonomethyl ether (methoxyethanol) from Schuchardt (München, G.F.R.) and propionic anhydride from Union Chemique Belgique (Bruxelles, Belgium). Dithionitrobenzoic acid (DTNB) was from Calbiochem (Los Angeles, Calif., U.S.A.) and [U-^{14}C]aspartate from the Radiochemical Centre (Amersham, England); $NaH^{14}CO_3$ was from Biuro Dystrybucji Izotopów (Świerk, Poland). Other reagents were from Fabryka Odczynników Chemicznych (Gliwice, Poland). Propionyl-CoA and acetyl-CoA were synthesized by the method of Simon & Shemin (1953) from corresponding anhydrides.

Strains and culture conditions. Streptomyces noursei var. *polifungini* (ATCC 21581): u.v. mutants, high-productive 213/41; and low-productive 853/26; 820/25 wereobtained from Mrs. D. Kotiuszko, M.Sc., from the Division of Microbiology of this Institute. Mutants were grown in 500 ml Erlenmeyer flasks on a rotary shaker (240 strokes/min) at 28°C in a medium containing per 100 ml: corn steep liquor

1 g, $CaCO_3$ 0.8 g, $(NH_4)_2SO_4$ 0.4 g, sonicated brewer's yeast 0.3 g and soya-bean oil 3 g (lipid medium) or soluble starch 3 g and glucose 4 g (carbohydrate medium). Mycelial mass was practically the same in the 120-h cultures of all mutants. The yield of antibiotics obtained with the low-productive 853/26 mutant, compared with the yield obtained with the high-productive 213/41 mutant, was 10% on carbohydrate medium and 1% on lipid medium (Roszkowski *et al.*, 1971). Mutant 820/25 produced traces of tetraene antibiotics, non-detectable microbiologically, on both applied media.

Preparation of extracts for enzyme assays. Contents of 2 - 4 fermentation flasks were pooled, the mycelium was spun down at 8000 rev./min for 15 min at 0 - 3°C, washed twice with water, suspended in 0.05 M-tris buffer, pH 8.0, containing 10 mM--2-mercaptoethanol, and sonicated. The ultrasonic extracts for determination of acetyl-CoA, propionyl CoA and PEP carboxylases were incubated with lysozyme (1 mg/ml) for 30 min at 30°C. The extracts for the assay of carboxyltransferase and PEP-synthesizing system were dialysed on Sephadex G-25 coarse after centrifugation. Localization of the enzymes in the cell wall fraction and spheroplasts, separated on lysozyme treatment, was performed as described by Ruczaj, Sawnor--Korszyńska, Paśś & Raczyńska-Bojanowska (1969).

Preparation of extracts for metabolite determination. Two volumes of 10% (v/v) perchloric acid was added to the washed mycelium and homogenized in a glass homogenizer for 3 min. The extract was neutralized with 20% KOH and separated from potassium perchlorate after a 30 min standing in ice.

Acetyl-CoA and propionyl-CoA carboxylase (EC 6.4.1.2) was determined by measuring the acyl-CoA dependent formation of acid-stable radioactivity derived from $NaH^{14}CO_3$. The incubation mixture contained in a volume of 0.5 ml: tris buffer, pH 8.0, 100 µmol; ATP, 2.5 µmol; $MgCl_2$, 5 µmol; NaF, 10 µmol; $NaH^{14}CO_3$, 5 µCi (sp. act. 6 µCi/µmol), the enzymic extract containing 1 - 2 mg of protein, and acetyl-CoA or propionyl-CoA, 0.5 µmol (Wawszkiewicz & Lynen, 1964). After 60 min incubation at 30°C the reaction was stopped by the addition of 0.5 ml of 2 N-HCl, the mixture was centrifuged, 0.4 ml of supernatant was dried, dissolved in 1 ml of anhydride ethanol-methoxyethanol mixture (1:1, v/v), 8 ml of scintillation solution was added and radioactivity was measured; the activity was expressed in counts of radioactivity per 100 sec or calculated in nmol of radioactive compounds formed/h/mg protein. The control was run without acyl-CoA.

Methylmalonyl-CoA carboxyltransferase (EC 2.1.3.1.) activity was measured according to Wood & Stjernholm (1961) by determining pyruvate formed from oxaloacetate. The reaction mixture contained in 1 ml: tris buffer, pH 7.6, 100 µmol; glutathione reduced, 1 µmol; oxaloacetate, 2 µmol; propionyl-CoA, 0.5 µmol and enzymic protein 3 - 5 mg. In control mixture propionyl-CoA was omitted. After 15 min incubation at 30°C the reaction was terminated by the addition of 1 ml of 10% $HClO_4$, the mixture was centrifuged, and the neutralized supernatant was used for determination of pyruvate with lactate dehydrogenase. The activity was expressed in nmol/h/mg protein.

Citrate synthetase (EC 4.1.3.7) was determined spectrophotometrically according to Weitzmann (1969).

Malate dehydrogenase (EC 1.1.1.37) was measured after Yoshida (1969).

Aspartate aminotransferase (EC 2.6.1.1.) was assayed by the method of Bergmeyer & Berndt (1962).

Phosphoenolpyruvate carboxylase (EC 4.1.1.31) was assayed by the method based on incorporation of radioactivity from $NaH^{14}CO_3$ into malate in the modified system of Maruyama, Easterday, Chang & Lane (1966). The incubation mixture contained: tris buffer, pH 8.0, 100 µmol; $NaH^{14}CO_3$, 5 µCi (sp. act. 6 µCi/µmol); $MgCl_2$, 5 µmol; glutathione reduced, 2 µmol; NADH, 1 µmol; malate dehydrogenase (5 units) and 0.2 ml of extract (1 - 2 mg protein) in the total volume of 0.5 ml. The mixture was incubated for 30 min at 30°C, the reaction was stopped by the addition of 1 ml 2 N-HCl and radioactivity measured in the scintillation counter. The activity was expressed in nmol of radioactive acid-stable fraction formed/h/mg protein.

The phosphoenolpyruvate synthesizing system was assayed according to Benziman & Palgi (1970) and either the decrease in pyruvate concentration or the increase of AMP and ADP was measured; in the former case the reaction mixture without ATP was used as a control, in the latter a control was run without pyruvate. The reaction mixture contained in 1 ml: tris-HCl buffer, pH 8.0, 100 µmol; $MgCl_2$, 10 µmol; pyruvate, 2 µmol; ATP, 5 µmol; pyrophosphatase and dialysed enzymic extract, 0.5 ml (3 - 5 mg protein). The assay mixture was incubated for 30 min at 30°C.

Synthesis of PEP[1] from pyruvate was also measured on the basis of apparent carboxylation of pyruvate in the system containing PEP carboxylase which is present in excess in the enzymic extract, and provided additionally with aspartate transaminase, glutamate, $NaH^{14}CO_3$ and acetyl-CoA. The complete reaction mixture contained in a total volume of 0.6 ml: tris-HCl buffer, pH 8.0, 100 µmol; $MgCl_2$, 5 µmol; pyruvate, 1 µmol; acetyl-CoA, 0.1 µmol; glutamate, 2 µmol; $NaH^{14}CO_3$, 5 µCi; pyrophosphatase, aspartate transaminase and 0.2 ml of dialysed enzymic extract (1 - 2 mg protein). The reaction mixture was incubated for 30 min at 30°C. Acetyl-CoA was added to stimulate PEP carboxylase activity. Synthesis of PEP was measured in terms of radioactivity (counts per 100 sec) of the acid-stable aspartate fraction.

Concentration of P_i in both reaction mixtures prior to incubation was 1.2 mM, and the replacement of tris by phosphate buffer had no effect on apparent carboxylation of pyruvate.

Determination of lactate, malate, oxaloacetate, pyruvate, PEP, ADP and AMP. L-Lactate, malate and oxaloacetate were assayed enzymically according to Hohorst (1962), pyruvate after Bücher, Czok, Lamprecht & Latzko (1962), PEP with pyruvate kinase, and ADP and AMP with pyruvate kinase and adenylate kinase after Adam (1962).

[1] Abbreviation: PEP, phosphoenolpyruvate.

Determination of antibiotics. Concentration of tetraene antibiotics was measured spectrophotometrically at 304 and 312 nm after Korchagin & Savushkina (1963).

Determination of protein. This was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Chromatography. The deproteinized reaction mixtures were used for chromatographic analysis. ATP, ADP, AMP, PEP, PP_i and P_i were separated on Whatman no. 1 paper using isopropyl ether - *n*-butanol - 90% formic acid (30:30:20, by vol.) system of Wood (1961). Phosphate compounds were detected by spraying with 1% ammonium molybdate followed by 10% ascorbic acid (Wood, 1961) and visual examination under u.v. lamp.

In the isotopic experiments on transcarboxylation, oxaloacetate and methylmalonate were separated after alkaline hydrolysis of CoA esters in 2 N-NH_4OH for 30 min (Simon & Shemin, 1953), by thin-layer chromatography on silica gel in the amyl acetate - acetic acid - water (60:20:1, by vol.) system, twice in the same direction. Acids were visualized with 1% bromophenol red in 50% ethanol, eluted from silica gel with the mixture used in the isotopic assay of carboxylase activity and radioactivity was determined.

Radioactivity measurements were carried out in USB-2 scintillation counter (Biuro Urządzeń Techniki Jądrowej, Warszawa, Poland), the scintillation solution consisting of 0.3% PPO and 0.03% POPOP (Reanal, Budapest, Hungary) in toluene.

RESULTS

Distribution of carboxylating activities in mycelium

Preliminary experiments on efficiency of extracting acetyl-CoA carboxylase showed binding of this enzyme to the cell structures in *S. noursei* var. *polifungini*. Lysozyme treatment of sonicated mycelium increased the activity of acetyl-CoA carboxylase in the extract (Fig. 1); the effect of lysozyme was practically undetect-

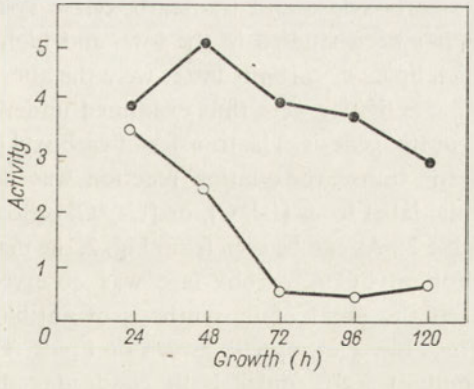


Fig. 1. The release of acetyl-CoA carboxylase upon lysozyme treatment of mycelium. Sonicated mycelium of mutant 213/41 was treated with lysozyme (1 mg/ml) for 30 min at 30°C, centrifuged and the supernatant used for the enzymic assay as described in Methods. The activity expressed in counts/100 sec/mg protein $\times 10^{-3}$. ●, Lysozyme treated, and ○, non-treated mycelium.

able in the 24-h mycelium and increased with the age of cultures. During intensive synthesis of antibiotics in the stationary phase of growth about 80% of the activity was bound to the mycelial structures and was released by lysozyme. Determination of the enzymic activities in the spheroplasts and the cell wall fraction obtained on lysozyme treatment of the 48-h mycelium in the hypertonic sucrose solution showed (Table 1) that almost total activity of both carboxylases: acetyl-CoA carboxylase and PEP carboxylase was found in the cell wall fraction including periplasmic enzymes, while as much as 60% of the activity of the PEP-synthesizing system was present in spheroplasts. The lability of transcarboxylating system under conditions of lysozyme treatment made impossible localization of this enzyme by the used method.

Table 1

Localization of acetyl-CoA carboxylase, PEP carboxylase and PEP-synthesizing system in mycelium

Washed 48-h mycelium of mutant 213/41 was treated with lysozyme (1 mg/ml) and EDTA (0.025 M. in 20% sucrose containing 10 mM-2-mercaptoethanol. The solubilized cell wall fraction was sepa) rated by centrifugation at 16 000 rev./min. The enzymic activities were assayed as in Methods-

Preparation	Acetyl-CoA carboxylase (counts/100 sec)	PEP carboxylase (counts/100 sec)	PEP synthesizing system (nmol/15 min)
Whole lysozyme-treated cells	52 850	1 350 000	600
Soluble fraction	58 500 (95%)	1 625 000 (99%)	230 (39%)
Spheroplasts	3 045 (5%)	20 850 (1%)	365 (61%)

Synthesis of malonate and methylmalonate by carboxylation and transcarboxylation

Participation of the carboxylase and transcarboxylase systems in the synthesis of tetraene antibiotics has been studied in the low- and high-productive mutants, grown on media in which lipids or carbohydrates were the alternative carbon source. The investigated enzymic activities were thus examined under conditions favouring either gluconeogenesis or lipogenesis. The transfer of carboxyl group of oxaloacetate to propionyl-CoA in the transcarboxylation reaction was proved in the isotopic experiments in which the label from [1-^{14}C], or [U-^{14}C]oxaloacetate was recovered in methylmalonate (Table 2). As can be seen from Fig. 2, the maximum of the activity of acetyl-CoA and propionyl-CoA carboxylase was observed on both media in the 48-h cultures, i.e. at the onset of biosynthesis of antibiotics. Both these activities were by about 75% lower in mycelia grown on lipids. The activities found in the high-productive mutant were under both conditions distinctly higher. The same time-course of the activity of acetyl-CoA carboxylase and propionyl-CoA carboxylase under various culture conditions and practically the same activity ratio towards both acyl-CoA derivatives throughout the whole growth period suggest

Table 2

Isotopic evidence for transcarboxylation of propionyl-CoA in S. noursei *var.* polifungini

Extracts of sonicated mycelium of mutant 213/41 dialysed on Sephadex G-25 were used in reaction mixture containing [^{14}C]oxaloacetate labelled uniformly or in carboxyl group. The uniform labelling of oxaloacetate was obtained by introducing aspartate transaminase (5 units) and uniformly labelled aspartate in the same concentration as oxaloacetate in the mixture for the assay of methylmalonyl-CoA carboxyltransferase described in Methods. Incorporation of ^{14}C label into carboxyl group of oxaloacetate was based on the reaction catalysed by PEP carboxylase in the presence of PEP and $NaH^{14}CO_3$; propionyl-CoA (0.5 μmol) was added to the reaction mixture used for the assay of PEP carboxylase. After 30 min incubation at 30°C, the mixture was deproteinized and the radioactivities of methylmalonate and oxaloacetate were counted after separation by thin-layer chromatography. Details in Methods.

Carboxyl group donor	Radioactivity (counts/100 sec)		Conversion of oxaloacetate (%)
	Oxaloacetate recovered	Methylmalonate formed	
[U-^{14}C]Oxaloacetate	15 705	1445	27
[1-^{14}C]Oxaloacetate	4 422	1070	19

the occurrence of one, non-specific enzymic protein showing lower affinity to propionyl-CoA.

The activity of methylmalonyl-CoA carboxyltransferase was by about two orders of magnitude higher than that of carboxylase and was similarly as that of

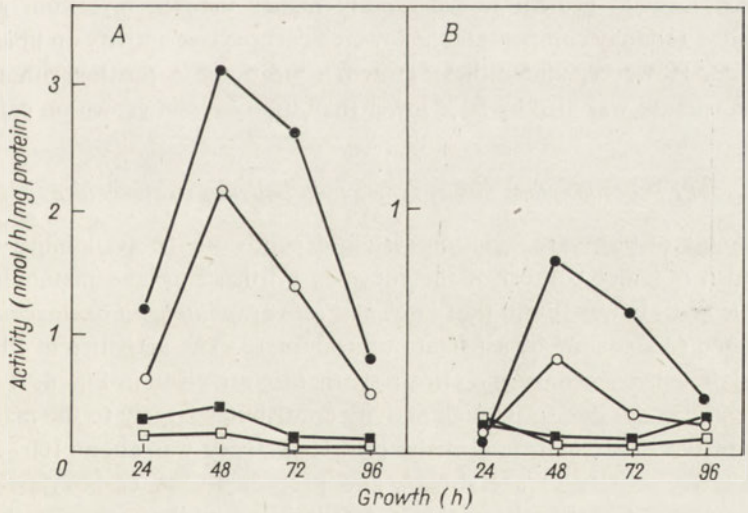


Fig. 2. Time-course of the acetyl-CoA and propionyl-CoA carboxylation in the mutants of *S. noursei* var. *polifungini* grown on carbohydrate (*A*) and lipid (*B*) media. Acetyl-CoA carboxylase in ●, high (213/41) and ■, low (853/26) antibiotic productive mutants; propionyl-CoA carboxylase in ○, high- and □, low-productive mutants. Media and conditions of assay as in Methods.

carboxylase much lower in the low-productive mutant (Fig. 3). It is noteworthy that on lipid medium the time-course of this enzymic activity was different from that in mycelium grown on carbohydrates. A sharp decline of the carboxyltransferase activity was observed beginning from the 24 h of growth. The activity at this early

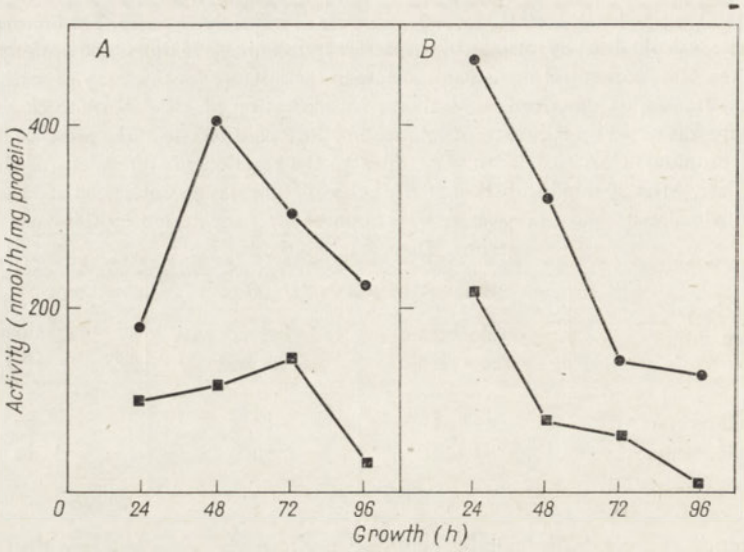


Fig. 3. Time-course of methylmalonyl-CoA carboxyltransferase in the mutants of *S. noursei* var *polifungini* grown on carbohydrate (*A*) and lipid (*B*) media. ●, High-productive mutant; ■, low--productive mutant. Media and conditions of assay as in Methods.

logarithmic phase of growth was distinctly higher than in mycelium grown on carbohydrates and may compensate the lowered carboxylase activity on lipid medium at this stage. However, under these growth conditions in postlogarithmic phase, carboxyltransferase was also by 50% lower than in mycelium grown on carbohydrates.

PEP carboxylase and enzymes metabolizing oxaloacetate

Functioning of transcarboxylation system depends on the availability of oxaloacetate which is under control of the enzymes synthesizing and metabolizing this dicarboxylic acid. It was found that in *S. noursei* var. *polifungini* oxaloacetate is not transaminated to aspartate by aspartate transaminase. The activities of PEP carboxylase, malate dehydrogenase and citrate synthetase are given in Fig. 4. It is evident that PEP carboxylase due to its high activity contributes largely to the maintenance of concentration of oxaloacetate in the cell. Its activity was about 100 - 200 times higher than the activity of acetyl-CoA and propionyl-CoA carboxylase and corresponded to that of carboxyltransferase. Similarly as in the case of carboxylation of acetyl-CoA and propionyl-CoA the activity of PEP carboxylase was distinctly higher in the high-productive mutant and was reduced in mycelium grown on lipids (Fig. 4B).

The activity of citrate synthetase was practically the same in both investigated mutants on both media, while the activity of malate dehydrogenase was reversely related to the ability of the mutants to synthesize tetraene antibiotics. A different

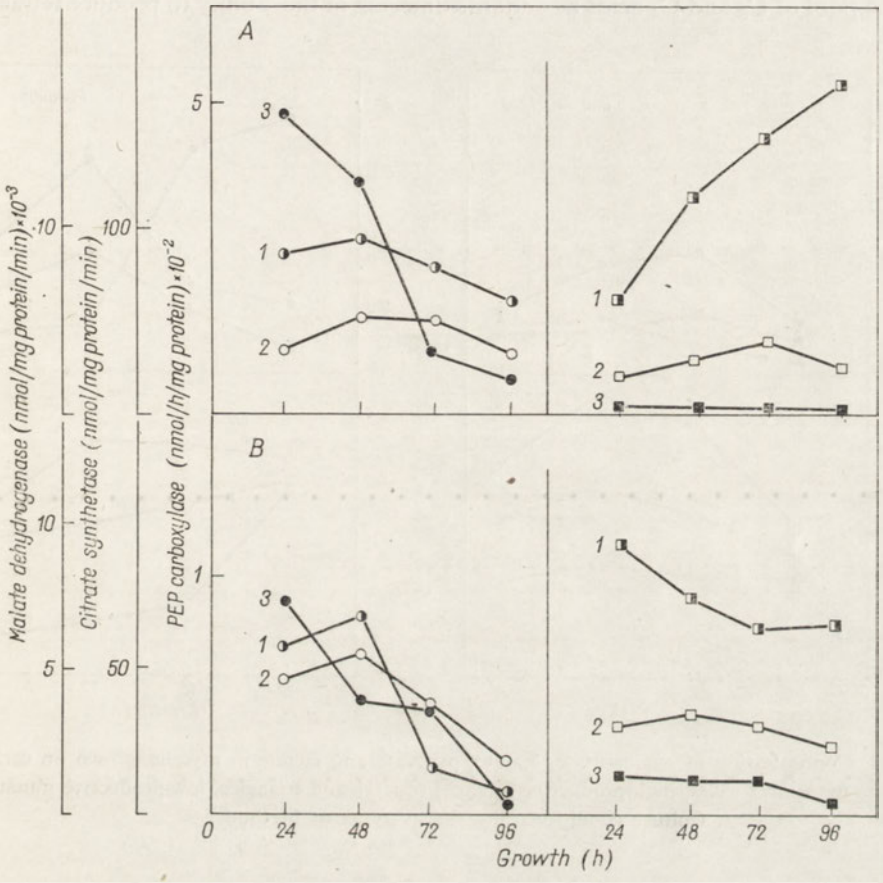


Fig. 4. The activitjes of malate dehydrogenase, citrate synthetase and PEP carboxylase in the mutants of *S. noursei* var. *polifungini* grown on carbohydrate (*A*) and lipid (*B*) media. Circles, high-productive mutant; squares, low-productive mutant. *1*, Malate dehydrogenase; *2*, citrate synthetase; *3*, PEP carboxylase. The enzyme assays as Methods.

response of the latter enzyme to the carbon source of the medium was also observed in both mutants. In the high-productive mutant growing on carbohydrates the activity of malate dehydrogenase was slightly decreased during growth (Fig. 4A), this decrease was, however, very pronounced in the stationary phase of growth in mycelium grown on lipids (Fig. 4B). In the low-productive mutant grown on carbohydrates the initial low activity of this dehydrogenase rose rapidly in the stationary phase of growth whereas it was slightly decreased in mycelium grown on lipid medium.

The profiles of concentration of metabolites during growth of both mutants (Fig. 5, 6) showed the prevalence of reduced forms of the investigated C_3 and C_4 acids: i.e. lactate and malate over pyruvate and oxaloacetate both in logarithmic and post-logarithmic phases of growth. However, the distinct differences have been found in the pool of C_3 and C_4 acids in mutants differing in the ability to produce tetraene

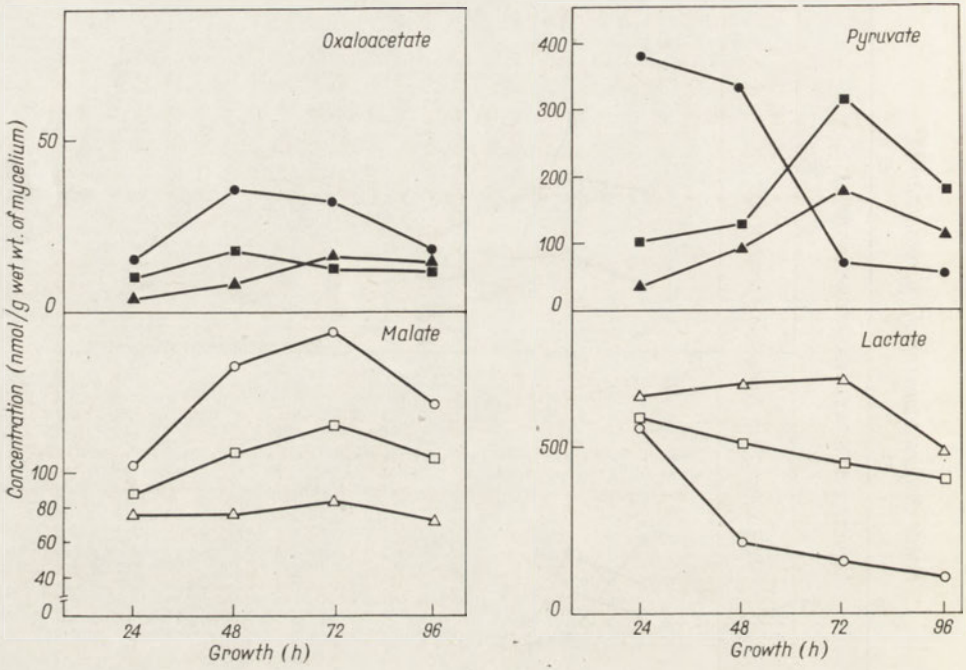


Fig. 5. Concentration of oxaloacetate, malate, pyruvate and lactate in mycelia grown on carbohydrate medium. Circles, high-productive mutant; squares and triangles, low-productive mutants. Culture conditions and the assay as in Methods.

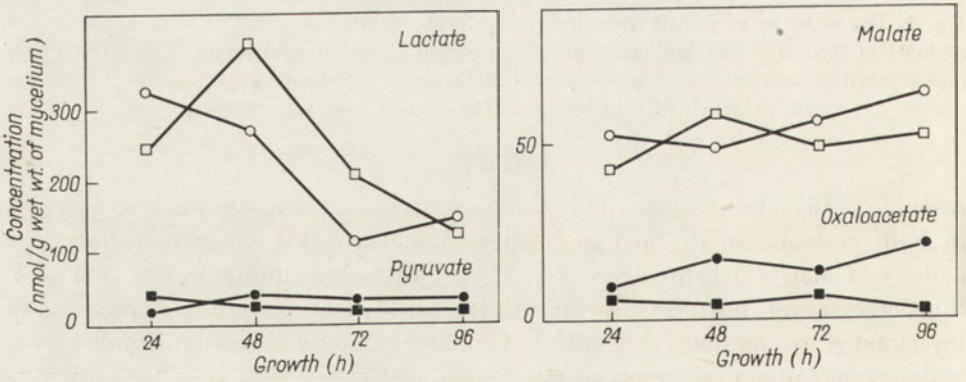


Fig. 6. Concentration of oxaloacetate, malate, pyruvate and lactate in mycelia grown on lipid medium. Circles, high-productive mutant; squares, low-productive mutant.

antibiotics. Intensive synthesis of antibiotics on carbohydrates was correlated with a rapid decrease in concentration of pyruvate and lactate, while in both low-productive mutants the concentration of pyruvate was increased in the 48 and 72-h cultures, i.e. during formation of antibiotics. This suggests a limited use of C_3 fragments in the process by these mutants. On the contrary, the pool of C_4 acids, oxaloacetate and malate, was higher in the high-productive mutant parallelly to the higher PEP carboxylase activity and lower activity of malate dehydrogenase. On lipid medium, the concentration of the investigated metabolites was lower than in mycelium grown on carbohydrates, the prevalence of reduced forms was maintained, but the differences in the mutants were less pronounced. Accumulation of C_3 in the stationary phase of growth of the low-productive mutant on lipid medium was not observed, probably because of the direct use of C_2 fragments for the synthesis of antibiotics. The amount of oxaloacetate was higher, similarly as on carbohydrate medium, in the mutant synthesizing antibiotics at higher yield.

Functioning of PEP carboxylase and carboxyltransferase and a constant withdrawal of methylmalonyl-CoA for the synthesis of antibiotics, would rely on the supply of PEP. The possibility of the replenishment of PEP by direct synthesis from pyruvate by PEP synthase or pyruvate diphosphate kinase was envisaged. These two systems, recently discovered in microorganisms (Cooper & Kornberg, 1967; Evans & Wood, 1971; Benziman & Palgi, 1970; Benziman & Eizen, 1971), plants (Hatch & Slack, 1968) and protozoa (Reeves, Menzies & Hsu, 1968) utilize two energy bonds of ATP overcoming thus energy barriers associated with reversal of the pyruvate kinase reaction.

Evidence for direct conversion of pyruvate to PEP

Since crude extracts of *S. noursei* var. *polifungini* were rich in PEP carboxylase, formation of PEP from pyruvate was detected by measuring the apparent carboxylation of pyruvate in the presence of $NaH^{14}CO_3$ and ATP. Oxaloacetate formed was converted by aspartate transaminase to the acid-stable aspartate and formation of PEP was expressed in radioactivity counts recovered in this fraction. Data given in Table 3 clearly indicate that formation of aspartate from pyruvate *via* PEP and oxaloacetate depended on ATP and to a large extent on pyrophosphatase. Participation of the latter enzyme in conversion of pyruvate to PEP in the used system suggests a pyruvate diphosphate kinase type of reaction described by Benziman & Palgi (1970) for *Acetobacter xylinum* and by Reeves *et al.* (1968) for protozoa. When the mycelial cells were transferred for 3 h to a medium containing pyruvate instead of glucose, it was found that on incubation of the dialysed extract with pyruvate and ATP both the conversion of pyruvate and formation of ADP and AMP were increased (Table 4). The decrease of pyruvate was referred to the control without ATP, and changes in nucleotides to the control without pyruvate. Both AMP and ADP were determined because of the presence of adenylate kinase in crude extracts.

Since spheroplasts contain the PEP-synthesizing system and are practically devoid of PEP carboxylase (Table 1), this fraction was used for direct phosphorylation

Table 3

The apparent carboxylation of pyruvate

The complete assay mixture (0.6 ml) was that of PEP carboxylase: 0.2 ml of dialysed extract from mycelium of 213/41 mutant (1 - 2 mg protein); $NaH^{14}CO_3$, 5 μCi; $MgCl_2$, 5 μmol; glutathione reduced, 2 μmol, supplemented with pyruvate, 1 μmol; acetyl-CoA, 0.1 μmol; ATP, 5 μmol; pyrophosphatase and the aspartate transaminase - glutamate system converting oxaloacetate to the acid-stable aspartate. Concentration of P_i in the mixture prior to incubation was 1.2 mM. Incubation period 30 min at 30°C. Details see Methods.

System	Counts/100 sec
Complete	3670
ATP omitted	476
Pyruvate omitted	546
Pyrophosphatase omitted	832

Table 4

Effect of carbon source on PEP-synthesizing system

The cells from 48-h cultures of mutant 213/41 were spun down, suspended in solution containing 0.1% $(NH_4)_2SO_4$ and 2% glucose or 2% Na-pyruvate alternatively, and separated after 3-h cultivation period and sonicated. The assay reaction mixture contained in 1 ml: tris-HCl buffer, 100 μmol; $MgCl_2$, 10 μmol; pyruvate, 2 μmol; ATP, 5 μmol; pyrophosphatase, and the dialysed ultrasonic extract 0.5 ml (3 - 5 mg of protein). Incubation period 30 min at 30°C. Concentration of P_i in the assay mixture prior to incubation, 1.2 mM.

Carbon source	Changes in concn. (μmol/ml)	
	Pyruvate decreased	AMP+1/2 ADP formed
Glucose	0.220	0.368
Pyruvate	0.472	0.656

Table 5

ATP-dependent phosphorylation of pyruvate to PEP in spheroplasts

Complete system contained in 1 ml: tris-HCl buffer, pH 8.0, 100 μmol; $MgCl_2$, 10 μmol; ATP, 5 μmol; pyruvate, 2 μmol; pyrophosphatase, and 0.5 ml of dialysed extract of spheroplasts. Concentration of P_i prior to incubation, 1.2 mM. The mixture was incubated for 15 min at 30°C, deproteinized with $HClO_4$, neutralized and used for the assay.

System	Changes in concn. (nmol/ml)		
	Pyruvate decreased	PEP formed	AMP+1/2 ADP formed
Complete	360	81	444
ATP omitted	174	0	0
Pyruvate omitted	—	—	276
Net change	186	81	168

of pyruvate. The results of this experiment presented in Table 5 show close stoicheiometric relation between the amount of pyruvate converted in the presence of ATP and the amount of AMP and ADP formed during phosphorylation of pyruvate. The amount of PEP formed in the mixture was lower by a half as compared with the amounts of pyruvate, probably due to the action of trace activity of PEP carboxylase in the system.

DISCUSSION

Carboxylation of acetyl-CoA and propionyl-CoA and transcarboxylation of propionyl-CoA involving oxaloacetate as the carboxyl group donor are both operating in *S. noursei* var. *polifungini* synthesizing macrolide ring of tetraene antibiotics of nystatin type. The obtained data on carboxylation of acetyl-CoA and propionyl-CoA suggest the occurrence of one non-specific protein showing lower affinity towards propionyl-CoA. Specificity of the methylmalonyl-CoA carboxyltransferase has not been determined.

Both the carboxylating and transcarboxylating activities are higher in the high-productive mutant. It was also found (Roszkowski, Kotiuszko, Rafalski, Morawska & Raczyńska-Bojanowska, 1972) that the enhanced activity of acetyl-CoA and propionyl-CoA carboxylation is associated with the increase not only in formation of tetraene antibiotics but also in lipogenesis: the latter was manifested by accumulation of lipid esters and long-chain fatty acids in mutants which showed different antibiotic-synthesizing abilities and were grown on carbohydrate medium. Thus channeling of carboxylated acyl-CoA units for biosynthesis of lipids and tetraene antibiotics involves a common pool complemented by both kinds of carboxylation reactions.

The activity of methylmalonyl-CoA carboxyltransferase in *S. noursei* var. *polifungini* is by two orders of magnitude higher than that of carboxylase. As shown by Ochoa & Kaziro (1965) carboxyltransferase in propionibacteria is also far more active than the non-specific carboxylase, and it is the most active enzymic constituent of the methylmalonyl-CoA cycle in which propionate is synthesized by this organism. A similar transcarboxylation system involving ribulose diphosphate as the acceptor of carboxyl group of oxaloacetate, constitutes an integral part of CO_2 fixation in plants *via* the Hatch-Slack-Kortchak pathway (Hatch & Stumpf, 1961; Hatch & Slack, 1966).

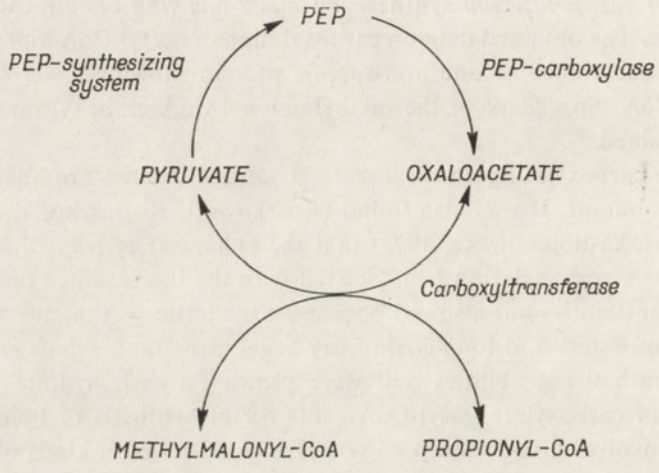
Our results suggest that transcarboxylation in *S. noursei* var. *polifungini* is coupled with the synthesis of oxaloacetate by a very active PEP carboxylase, which probably plays a role of anaplerotic enzyme maintaining the level of the intermediates of Krebs cycle, which are used for biosynthetic processes (Kornberg, 1966). The evidence presented in the paper implies also direct synthesis of PEP from pyruvate in *S. noursei* var. *polifungini*. The requirement for pyrophosphatase in the used assay system suggests participation of pyruvate diphosphate kinase (Reeves *et al.*, 1968; Benziman & Palgi, 1970; Evans & Wood, 1971) in the following reaction:

$$\text{Pyruvate} + P_i + \text{ATP} \rightleftharpoons \text{PEP} + \text{AMP} + \text{PP}$$

rather than phosphorylation by pyruvate synthase, discovered by Cooper & Kornberg (1967):

$$\text{Pyruvate} + \text{ATP} \rightleftharpoons \text{PEP} + \text{AMP} + P_i$$

PEP synthesis from pyruvate may suggest cooperation of PEP carboxylase, carboxyltransferase and pyruvate diphosphate kinase in a cyclic process (Scheme 2) in which constant withdrawal of methylmalonyl-CoA (and possibly malonyl-CoA) for biosynthesis of lipids or antibiotics is compensated by replenishment of PEP, operation of the system being dependent on the other hand on the abundant supply of propionyl-CoA (and possibly acetyl-CoA).



Scheme 2.

The postulated cooperation of the discussed enzymes depends both on enzymic activities and concentration of the respective substrates under various conditions of growth and antibiotic synthesis. On carbohydrate medium the maximum activity of carboxylation and transcarboxylation was observed at the initial stages of antibiotic synthesis while the activity of PEP carboxylase decreased gradually from the 24 h of growth onward. A similar decrease in the activity of acetyl-CoA carboxylase during biosynthesis of chlorotetracycline was observed by Běhal & Vaněk (1970). This time-course induced the authors to suggest participation of a different enzyme system: oxidative oxaloacetate decarboxylase (Gatenbeck & Mahlén, 1968) in the formation of malonate in *S. aureofaciens*. However, this system is also based on the synthesis of oxaloacetate by PEP carboxylase, which as an anaplerotic enzyme in *S. aureofaciens* shows the maximum activity in the early logarithmic phase of growth. Therefore one should rather postulate that in *S. noursei* var. *polifungini* anaplerotic sequences coupled with transcarboxylation operate in biosynthetic processes both in primary and secondary metabolism. The intensity of these processes and the need for carbon units are much smaller in secondary metabolism during stationary phase of growth. Differences in metabolism of C_3 and C_4 acids

in relation to antibiotic synthesis are clearly demonstrated in cultures grown on carbohydrate medium.

In cultures grown on lipid medium, i.e. under conditions repressing lipogenesis, carboxylating and transcarboxylating activities are significantly decreased during biosynthesis of antibiotics probably due to the known inhibiting effect of fatty acids or their CoA derivatives (Martin & Vagelos, 1962; Rasmussen & Klein, 1967; Greenspan & Lowenstein, 1968). The activity of PEP carboxylase is also much lower when lipids serve as the main carbon source, and, at variance with the results of Izui, Yoshinaga, Morikawa & Katsuki (1970) this activity was not stimulated by fatty acids. As demonstrated by Roszkowski *et al.* (1971), the role of PEP carboxylase as an anaplerotic enzyme under these conditions may be supported or taken over by malate dehydrogenase (decarboxylating). Since the yield of antibiotics in the high-productive mutant is the same on both media one may conclude that formation of carboxylated acyl-CoA units suffices for antibiotic production when the synthesis of fatty acids is limited. The system responsible for the synthesis of polyacyl secondary metabolite was separated from that responsible for the synthesis of fatty acids (Dimroth, Walter & Lynen, 1970).

The role of malate dehydrogenase in regulation of oxaloacetate content in the mutants of *S. noursei* var. *polifungini* is not well understood and more information is needed concerning the efficiency of PEP-synthesizing system under various conditions of antibiotic synthesis.

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SYNTEZA MALONIANU I METYLOMALONIANU A POWSTAWANIE POLIENOWYCH ANTYBIOTYKÓW

Streszczenie

1. U *S. noursei* var. *polifungini* malonian i metylomalonian są syntetyzowane zarówno w reakcji karboksylacji jak transkarboksylacji acetylo CoA i propionylo CoA: aktywność metylomalonylo CoA karboksytransferazy jest wyższa o dwa rzędy wielkości od czynności karboksylazy acetylo CoA i propionylo CoA.

2. Obydwie aktywności są wyższe, równolegle ze zwiększoną lipogenezą, u mutanta charakteryzującego się większą wydajnością tetraenowego antybiotyku typu nystatyny.

3. Wysunięto przypuszczenie, że synteza jednostek budulcowych w syntezie antybiotyku oparta jest na współdziałaniu transkarboksylacji i karboksylacji fosfoenolopirogronianu (PEP).

4. Syntezie antybiotyku w kulturach rosnących na podłożu węglowodanowym towarzyszy gwałtowny spadek stężenia kwasów C_3: pirogronowego i mlekowego oraz zwiększone stężenie kwasów C_4: szczawiooctowego i jabłkowego.

5. Przedstawiono dowody na występowanie układu syntetyzującego PEP bezpośrednio z pirogronianu w reakcji katalizowanej przez układ typu kinazy pirogroniano-dwufosforanowej. Przedyskutowano rolę tego układu w uzupełnianiu PEP, wykorzystywanego w syntezieantybiotyku.

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HYDROGEN BONDING SCHEME INVOLVING RIBOSE 2′-HYDROXYLS IN POLYRIBOURIDYLIC ACID

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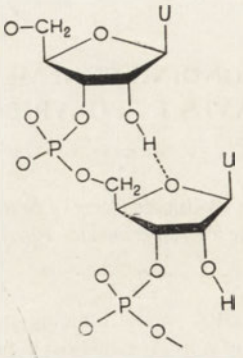
It is shown, by means of CPK model building studies, that both the single-stranded and twin-helical forms of poly rU may exist in forms in which the 2′-hydroxyl of one residue is hydrogen-bonded to the ribose ring oxygen of a neighbouring residue.

During the course of a recent investigation, by optical methods, of the conformation of mononucleosides, dinucleoside monophosphates and homopolynucleotides of uracil and thymine, in which the carbohydrate moieties were, in turn, deoxyribose, ribose and 2′-*O*-methylribose, particular attention was directed to the problem of intramolecular hydrogen bonding involving the 2′-OH. This is of obvious importance in attempts to interpret the differences in conformational behaviour between corresponding ribo and deoxyribo model systems, e.g. RNA and DNA, and in relation to the role of 2′-*O*-methyl nucleosides in tRNA and rRNA. The suggestion was advanced that the single-stranded, so-called random coil form, of poly rU, as well as the twin-stranded helical form, may exist in conformations in which the 2′-OH of a uridine residue hydrogen bonds to the ring $O_{(4')}$ of the ribose of a neighbouring residue, as shown in Scheme 1 (Rabczenko & Shugar, 1970, 1971). A similar proposal was independently advanced by Abraham (1971) in the formulation of a three-dimensional model for tRNA.

Hydrogen bonding involving the 2′-OH has been variously invoked, on the basis of studies with model systems, to interpret the differences between RNA and DNA. Ts'o, Rapaport & Bollum (1966) postulated the existence of such a bond to the 2-carbonyl of a pyrimidine or the ring $N_{(3)}$ of a purine in the same residue. Maurizot, Brahms & Eckstein (1969) demonstrated that the differences between corresponding ribo and deoxyribo dinucleoside monophosphates are dependent on the nature of the carbohydrate residue between the bases, and proposed the existence of a hydrogen bond between the 2′-OH and a phosphate oxygen at the 3′-position. Neither of the foregoing proposals is, however, consistent with the

results of model building studies. Diffraction data, while not conclusive, argue against such hydrogen bonding schemes (Arnott, Hutchinson, Spencer, Wilkins, Fuller & Langridge, 1966). However, in at least two instances, X-ray data and model building studies (Arnott, Dover & Wonacott, 1969; O'Brien & MacEwan, 1970) pointed to the *possibility* of a hydrogen bond between the ribose 2′-OH of one helix and a phosphate oxygen in a neighbouring helix.



Scheme 1.

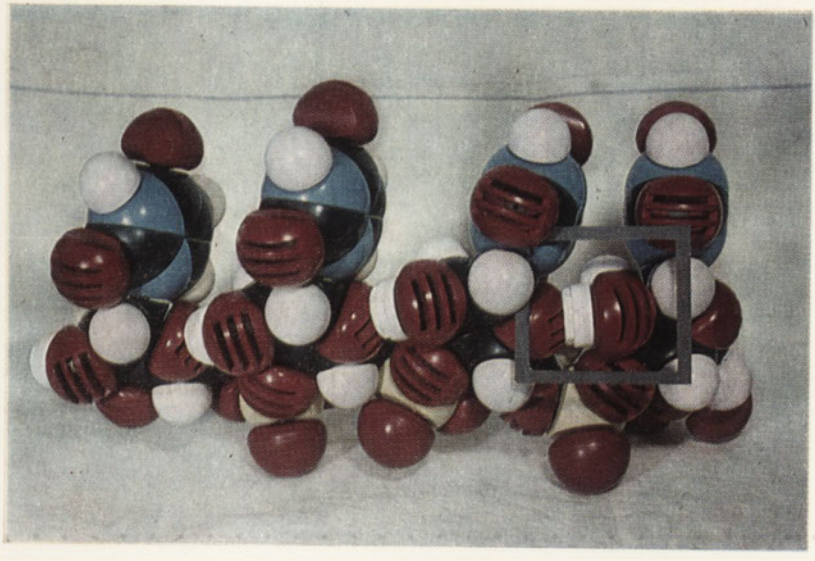
It is rather striking that poly dU (and poly dT) is incapable of forming a helical structure under conditions where poly rU (and poly rT) does (see Żmudzka, Bollum & Shugar, 1969, for pertinent data). It is clearly tempting to interpret this difference in behaviour in terms of an intramolecular hydrogen bond involving the 2′-OH as a donor.

However, poly 2′-OMeU, in which such hydrogen bonding is excluded, forms a helical structure even more stable than poly rU (Żmudzka & Shugar, 1970, 1971). In fact, this increase in stability appears at the level of dinucleotides (Rabczenko & Shugar, 1971; A. F. Drake, personal communication). Consequently hydrogen bonding of the 2′-OH in poly rU is not a *prerequisite* for formation of a helical structure. Unfortunately, replacement of the 2′-OH by 2′-OMe, while eliminating hydrogen bonding involving the former, introduces an apolar group which may increase stabilizing forces by hydrophobic interactions, or modify them as a result of steric hindrance. Consequently the behaviour of poly 2′-OMeU does not exclude the possibility of 2′-OH intramolecular bonds in poly rU.

It appears to us, on the other hand, somewhat difficult to ascribe the ability of poly rU to form a helical structure through hydrogen bonding of the 2′-OH *within* the same residue. Bonding between neighbouring residues seems a more likely possibility, as illustrated in Scheme 1, and supported by model building studies with CPK models (Rabczenko & Shugar, 1970; 1971) (Fig. 1a).

Indirect support for the foregoing hydrogen bonding scheme, at least in the case of the single-stranded form of poly rU, is forthcoming from the findings of Inners & Felsenfeld (1970), who demonstrated by means of hydrodynamic methods

A



B

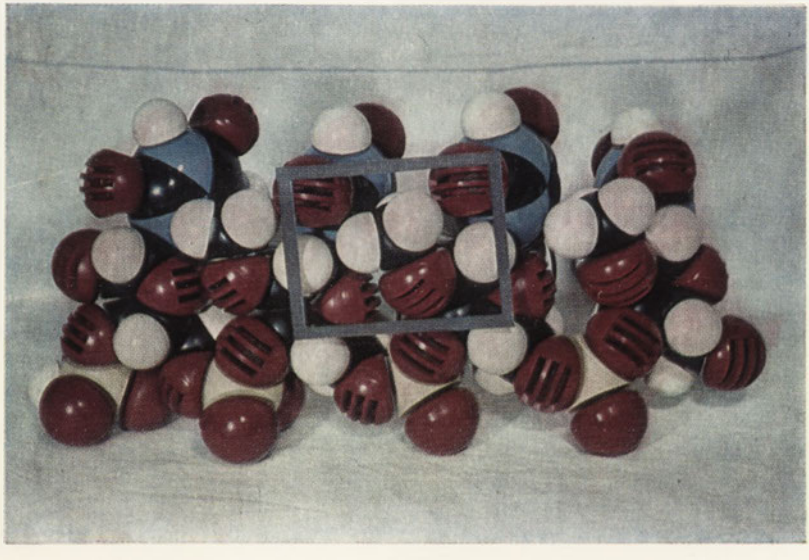


Plate I. A, Conformation of poly rU with the proposed hydrogen bonding (in frame) of 2′-OH of one ribose residue to $O_{(4')}$ of adjacent residue. B, The probable conformation of poly Um with the methyl groups (in frame) between the nitrogen bases, increasing the hydrophobic interactions.

that poly rU exhibits a considerably greater degree of rigidity than that anticipated theoretically for a random coil. The hydrogen bonding scheme illustrated in Scheme 1 can account for this behaviour, not only qualitatively, but also semi-quantitatively. The plate in Fig. 1 illustrates, with the aid of a CPK model, the appearance of such a hydrogen-bonded single poly rU strand. For comparison purposes the same figure shows the appearance of a poly 2′-OMeU strand, with the *O*-methyl groups located *cis* with respect to the 2′-H. The existence of such hydrogen bonds in a poly rU strand imparts pronounced rigidity to the chain. It does not, of course, necessarily follow that all residues are linked by such bonds. It would undoubtedly be of interest to examine the hydrodynamic properties of poly dU (or poly dT) in relation to those reported for poly rU (Inners & Felsenfeld, 1970).

Even if all the residues are hydrogen-bonded as in Scheme 1, model building studies show that it is still possible to form a normal twin-stranded helix, either between two such strands with the same polarity, or within the same strand by formation of a hairpin structure. In the latter case, the permissible type of base-pairing will be different, as elsewhere discussed (Rabczenko & Shugar, 1971).

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UDZIAŁ 2′-PODSTAWNIKÓW RYBOZY W STABILIZACJI STRUKTURY DRUGORZĘDOWEJ KWASU POLIURYDYLOWEGO

Streszczenie

Pokazano z pomocą modeli atomowych CPK, że pojedynczo-niciowa struktura oraz dwuniciowe spiralne struktury poli rU mogą istnieć w konformacji, w której 2′-hydroksyl jednej jednostki wiąże, poprzez wiązanie wodorowe do tlenu pierścienia rybozy, następną jednostkę nukleotydową.

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RECENZJE KSIĄŻEK

OLFACTION AND TASTE (T. Hayashi, ed.) Wenner-Gren Center International Symposium Series, Vol. 8. Pergamon Press, Symposium Publication Division, Oxford 1967; str. 835, cena £10.

Szczegółowe badania węchu i smaku, w porównaniu z innymi zmysłami, były dotychczas dość skąpe i nie wyjaśniały szeregu spraw związanych z funkcjonowaniem tych zmysłów. Przedstawione w omawianej książce materiały wypełniają tę dość dotkliwą lukę i zdają się zapowiadać dalszy rozwój badań tych dwóch zmysłów. Omawiana książka jest zbiorem 53 referatów wygłoszonych na drugim międzynarodowym sympozjum, które odbyło się w Tokio we wrześniu 1965 r. Obok referatów ogólnych przedstawiono referaty poświęcone bardzo szczegółowym zagadnieniom, przy czym wszyscy autorzy prezentują własne wyniki badań wraz z opisem stosowanych metod.

Pierwsza grupa trzynastu referatów poświęcona jest głównie węchowi u ssaków i ptaków. W tej grupie szczególną uwagę czytelnika przyciągają referaty: J. E. Amoore i D. Venstrom (*Correlation between stereochemical assessments and organoleptic analysis of odorous compounds*) będący rozszerzeniem i uzupełnieniem stereochemicznej teorii zapachu Amoore'a, następnie referat J. R. Hughes i D. E. Hendrix (*The frequency component hypothesis in relation to the coding mechanism in the olfactory bulb*), zajmujący się głównie kodowaniem wrażeń węchowych, czy wreszcie referat A. Demerdache i R. H. Wright (*Low frequency molecular vibration in relation to odor*). Pozostałe referaty tej grupy poświęcone są bardziej szczegółowym tematom.

Następna grupa referatów obejmuje 24 prace, poświęcone zmysłowi smaku u ssaków. W tej grupie nie ma żadnego referatu omawiającego jakąś ogólną koncepcję czy teorię recepcji smakowej i działania zmysłu smaku. Referaty koncentrują się na szczegółowych badaniach recepcji smakowej i reakcji fizjologicznych, czy behawiorystycznych, na bodźce smakowe. Do najciekawszych zaliczyłbym pracę C. Pfaffmann, G. L. Fisher i M.K. Frank (*The sensory and behavioral factors in taste preferences*) wskazującą na uzupełnianie się badań fizjologicznych i behawiorystycznych przy doświadczeniach na zmyśle smaku szczura, oraz pracę M. Ichioka (*Gustatory impulses — their number and temporal patterns*) przeprowadzoną na ludziach, psach i szczurach. Wykorzystano tu stymulację elektryczną nerwów i stymulację naturalną języka celem uzyskania niektórych danych, które pozwoliłyby na choćby częściowe zrozumienie centralnego mechanizmu w odczytywaniu kodu zmysłowego. Trzecim szczególnie interesującym referatem jest praca N. Ishiko, M. Amatsu i V. Sato (*Thalamic representation of taste qualities and temperature change in the cat*).

Trzecia grupa, złożona z pięciu referatów, poświęcona jest chemorecepcji u ryb. Najciekawszą w tej grupie jest praca J. Bardach, M. Fujiya i A. Holl (*Investigations of external chemoreceptors of fishes*), którzy na trzech gatunkach ryb badali wrażliwość chemoreceptorów na szereg substancji chemicznych, szczególnie aminokwasów.

Wreszcie ostatnia grupa ośmiu referatów poświęcona jest chemorecepcji u owadów, przy czym aż trzy prace dotyczą muchy plujki. Szczególnie interesująca jest praca K. N. Saxen (*Some factors gover. i ng olfactory and gustatory responses of insects*), w której przedstawione zostały wyniki badań reakcji smakowo-węchowej owadów w różnych warunkach doświadczalnych, jak też w różnych fazach rozwojowych.

Poza tymi grupami znajduje się jeszcze kilka prac; wśród nich niewątpliwą ciekawostkę stanow praca N. Tangolac, który przedstawił aparat nazwany przez autora „elektro-odo-komórką" przeznaczony do badania i oceniania mięsa i ryb.

Omawiana książka jest zbiorem bardzo ciekawych referatów, które niewątpliwie będą pomocne w zakresie badań nad zmysłami węchu i smaku. Książka stanowi bardzo cenną pozycję dla biochemików, fizjologów i innych, którzy zajmują się badaniem narządów zmysłów.

Juliusz Popowicz

PREVENTION OF Rh SENSITIZATION. WHO Technical Report Series no. 468; str. 36.

Konflikt grupowy w zakresie układu grupowego Rh stanowi poważny problem kliniczny. Wagę tego problemu podkreśla fakt, że częstotliwość występowania fenotypu Rh- u ludzi jest rzędu 15%. Z tego też względu Światowa Organizacja Zdrowia powołała międzynarodowy komitet ekspertów, których zadaniem było przedstawienie różnych zagadnień związanych z zapobieganiem konfliktowi Rh. Sprawozdanie ekspertów zawiera dokładne wskazania dla lekarzy klinicystów odnośnie stosowania globulin anty-Rh w przypadkach konfliktu grupowego. Omówione są również problemy kontroli przygotowanych globulin oraz ich testowania.

Immunoglobuliny klasy IgG zawierające przeciwciała anty-Rh otrzymywane są z osocza ochotników szczepionych krwinkami Rh+. Preparaty, otrzymywane na ogół alkoholową metodą Cohna, powinny zawierać przynajmniej 10 g białka na 100 ml dla zapewnienia odpowiedniego stężenia przeciwciał anty-Rh. Szczepienie kobiet, u których występuje konflikt grupowy, przeprowadza się albo po zabiegu przerwania ciąży albo też po pierwszym porodzie, podając jednorazowo 100 mg globulin anty-Rh.

Przedstawione w sprawozdaniu wyniki wskazują, że stosowanie immunoglobulin anty-Rh pozwoliło na zmniejszenie śmiertelności noworodków z 1,3 do 0,13 na 1000 porodów.

Sprawozdanie WHO nr 468 jest bardzo cennym i użytecznym dokumentem zarówno dla lekarzy praktyków, jak i dla zakładów teoretycznych pracujących nad zagadnieniem konfliktu grupowego Rh.

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