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J. SZOPA

EFFECT OF TOTAL HISTONE AND PARTICULAR HISTONE FRACTIONS ON THE STRUCTURE OF DEOXYRIBONUCLEIC ACID

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The u.v. difference spectra and hydrodynamic properties of reassociated soluble histone-DNA complexes were studied. The difference spectra of complexes of DNA with total histone and particular histone fractions (except the DNA-*f1* complex) showed minima at about 260 nm and maxima in the region of 280 nm, the position of the latter being dependent on the histone fraction and histone/DNA ratio. The minima are interpreted as pointing to stabilization of the double-helical DNA, and maxima as pointing to its destabilization. Differences were observed in the effect on DNA structure between histones and NaCl.

Henson & Walker (1970) and Wilhelm *et al.* (1970) demonstrated that DNA in nucleohistone has a more compact structure than free DNA. The decrease in asymmetry of DNA is related to histones *f2b*, *f2a2* and *f3*, whereas fraction *f1* has no effect on the conformation of DNA in the complex. Henson & Walker (1971) suggested that if a single histone fraction is involved in maintaining the supercoiled structure of DNA, it would be fraction *f2a2*. According to Šponar *et al.* (1970), the supercoil formation is determined by the amount of bound histone rather than its nature.

It is rather difficult to investigate the role of histone fractions by removing them from the nucleohistone as only fraction *f1* dissociates selectively. Therefore in the presented work, reassociated, soluble complexes of DNA with histone fractions were used. Changes occurring in conformation of DNA were evaluated on the basis of difference absorption spectra, and of changes in specific and intrinsic viscosity.

MATERIALS AND METHODS

Sodium salt of DNA was obtained from calf thymus according to Kay *et al.* (1952) and additionally purified by repeating several-fold the last step of the procedure. The purified preparation had the A_{258}/A_{230} ratio of 2.15, A_{258}/A_{280} ratio 1.7, ε_p 6580, and contained 0.38% of protein. In all experiments, the concentration of free DNA or DNA in complex was 12 μg/ml.

Histones. Total histone was prepared from calf thymus according to Davison *et al.* (1954) in the modification of Phillips & Johns (1959). Lysine-rich histone (*f1*), moderately lysine-rich histones (*f2a* and *f2b*) and arginine-rich histone (*f3*) were obtained by procedure II of Johns (1964). Purity of the preparations was checked by starch-gel electrophoresis according to Hnilica *et al.* (1966), and the obtained patterns corresponded to those of Johns (1964). The preparations were homogeneous on polyacrylamide-gel electrophoresis according to Reisfeld *et al.* (1962) except *f2a* which was resolved into two bands. Fractions *f2b* and *f3* had the same mobility.

Reassociation of histone-DNA complex. DNA, 3 mg, was dissolved in 1 ml of 0.15 M-NaCl - 0.015 M-sodium citrate and diluted 100-fold with bidistilled water, dialysed for 12 h against three changes of the 100-fold diluted above salt solution (1.5 μM-NaCl - 0.15 μM-sodium citrate, further referred to as dilute salt solution), and diluted with the same solution to a concentration of 24 μg of DNA/ml (pH 6.5). Histones were dissolved in the dilute salt solution to a concentration of 24 or 12 μg/ml (pH 6.5). To the DNA solution, the same volume of the histone solution was slowly added with constant stirring. The samples were filtered through sintered glass filter G-4. The extinction at 340 and 400 nm of the obtained nucleohistone solutions did not exceed the extinction of free DNA by more than 0.005.

Viscosity measurements were carried out in an Ubbelohde viscometer. Specific viscosity (η_{sp}), intrinsic viscosity ([η]) and the second virial coefficient (A_2) were determined.

Difference spectra were determined in a VSU2-P spectrophotometer (K. Zeiss, Jena, G.D.R.). For examination of DNA-histone complexes, two pairs of 1-cm cells were used; one contained the DNA-histone solution (proper sample), and the second pair contained in one cell DNA and in another the histone solution. Over the studied range of wavelengths, when all four cuvettes were filled with water or DNA solution, the deviation of extinction from zero value was ± 0.001.

Determination of protein and nucleic acids. Histones were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard; DNA was measured spectrophotometrically by the method of De Deken-Grenson & De Deken (1959) applying the coefficient of 32.94.

RESULTS

Difference spectra

The difference spectra of partly and completely denatured DNA *versus* native DNA presented in Fig. 1 show that denaturation of DNA resulted in shifting of the maxima toward shorter wavelengths and a positive shift of the whole curves. Figure 2 illustrates the effect of increasing NaCl concentration on difference spectra of DNA. At 258 nm there appeared a minimum which was more pronounced at higher salt concentration.

The difference spectra of DNA-histone complexes at a histone/DNA ratio of 0.5, are shown in Fig. 3A. All complexes exhibited at 258 nm minima which differed in magnitude, and maxima situated at different wavelengths. Complexes of DNA

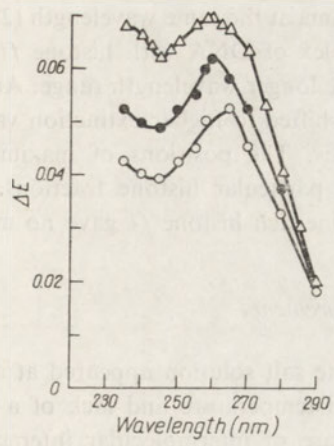

Fig. 1

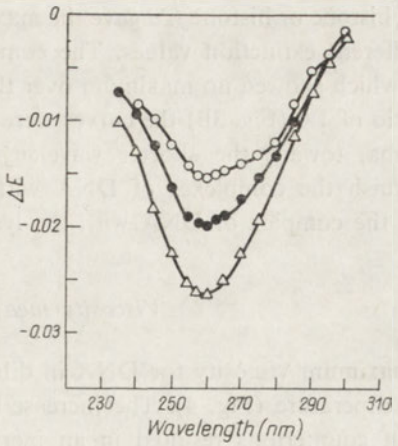

Fig. 2

Fig. 1. Difference spectra of partly and completely denatured DNA, in dilute salt solution, pH 6.5; ○, at 23% hyperchromism; ●, at 27% hyperchromism; △, at 30% hyperchromism.

Fig. 2. Difference spectra of DNA in solutions of increasing salt concentration: ○, 0.42 M-NaCl; ●, 0.70 M-NaCl; △, 0.98 M-NaCl.

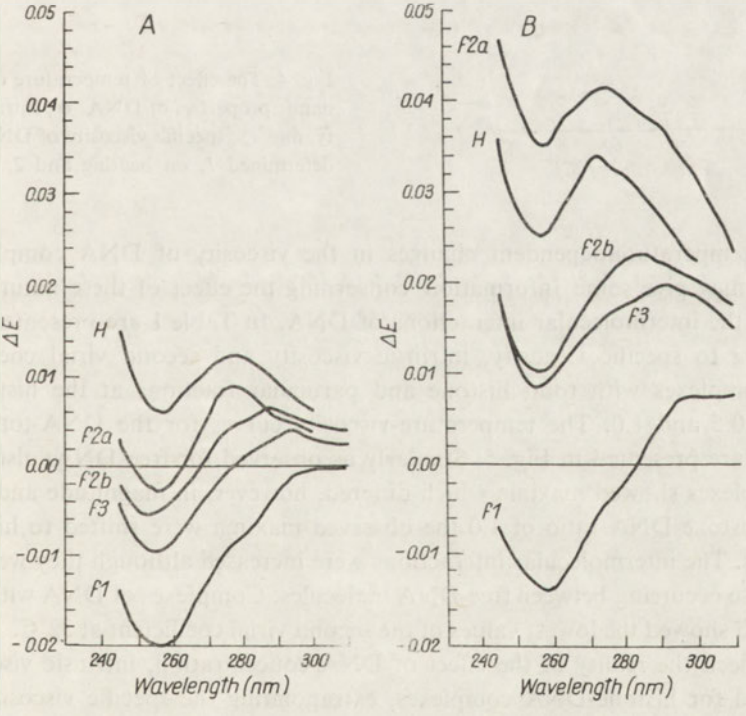

Fig. 3. Difference spectra of the complexes of DNA with total histone (*H*) and the indicated histone fractions at the histone/DNA ratio of: *A*, 0.5 and *B*, 1.0.

with total histone or histone *f2a* gave the maxima at the same wavelength (277.5 nm), but at different extinction values. The complex of DNA with histone *f1* was the only one which showed no maximum over the longer wavelength range. At histone/ /DNA ratio of 1.0 (Fig. 3B) the curves were shifted to higher extinction values and the maxima, toward the shorter wavelengths. The positions of maxima permit to distinguish the complexes of DNA with particular histone fractions. Also in this case, the complex of DNA with the lysine-rich histone *f1* gave no maximum.

Viscosity measurements

The maximum viscosity for DNA in dilute salt solution appeared at about the melting temperature (Fig. 4). The increase in temperature and lack of a sufficient amount of counterions resulted in an increase of intermolecular interactions. In concentrated salt solution (0.15 M-NaCl - 0.015 M-sodium citrate) no maxima of viscosity were observed.

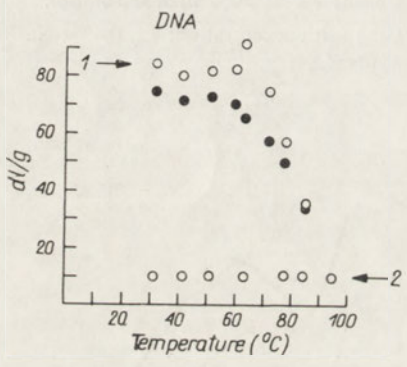

Fig. 4. The effect of temperature on hydrodynamic properties of DNA. ●, Intrinsic viscosity, and ○, specific viscosity of DNA solutions determined *1*, on heating and *2*, on cooling.

The temperature-dependent changes in the viscosity of DNA complexes with histones may give some information concerning the effect of these natural polycations on the intermolecular interactions of DNA. In Table 1 are presented the data pertaining to specific viscosity, intrinsic viscosity and second virial coefficient of DNA complexes with total histone and particular fractions at the histone/DNA ratio of 0.5 and 1.0. The temperature-viscosity curves for the DNA-total histone complex are presented in Fig. 5. Similarly as observed for free DNA, also DNA in the complexes showed maxima which differed, however, in magnitude and position. At the histone/DNA ratio of 1.0 the observed maxima were shifted to higher temperatures. The intermolecular interactions were increased although they were weaker than those occurring between free DNA molecules. Complexes of DNA with histones *f2a* and *f3* showed the lowest values of the second virial coefficient at 32°C.

To check the reality of the effect of DNA concentration, intrinsic viscosity was calculated for histone-DNA complexes, extrapolating the specific viscosity to zero gradient of velocity and zero concentration. No maxima were obtained over the whole range of temperature studied. At the histone/DNA ratio of 0.5, the complexes

of DNA with *f1* and *f3* showed the lowest limiting value of viscosity, and the complex with *f2b*, the highest value. At the histone/DNA ratio of 1.0, the complex with *f2b* showed the lowest intrinsic viscosity, and with *f1*, the highest.

Table 1

Effect of temperature on the hydrodynamic properties of the reassociated histone-DNA complexes

$\eta_{32°}$, specific viscosity; $[\eta]_{32°}$, intrinsic viscosity; $A_232°$, the second virial coefficient at a temperature of 32°C. η_{max}, $[\eta]$ and A_2max, the corresponding values at the temperature at which the specific viscosity exhibits the highest value ($T\eta_{max}$).

Histone /DNA ratio	Reassociated complex	Specific viscosity			Intrinsic viscosity		Second virial coefficient	
		$\eta_{32°}$ (dl/g)	$T\eta_{max}$ (°C)	η_{max} (dl/g)	$[\eta]_{32°}$ (dl/g)	$[\eta]$ (dl/g)	$A_232°$	A_2max
	Free DNA	82.0	68.0	92.0	75.0	67.0	0.20	0.515
0.5	DNA-*f1*	53.0	80.0	60.0	51.5	51.0	0.10	0.170
	DNA-*f3*	52.0	72.0	54.0	50.0	40.0	0.07	0.200
	DNA-*f2a*	52.5	72.5	56.5	52.0	48.5	0.06	0.150
	DNA-*f2b*	65.0	73.5	71.0	61.0	58.0	0.14	0.200
	DNA-total histone	60.0	74.5	64.5	55.0	49.0	0.10	0.250
1.0	DNA-*f1*	35.0	83.0	42.0	28.0	27.0	0.15	0.300
	DNA-*f3*	31.0	74.5	34.0	27.0	19.0	0.11	0.330
	DNA-*f2a*	31.0	75.5	36.0	26.5	21.0	0.09	0.280
	DNA-*f2b*	31.0	77.5	37.0	24.0	21.0	0.16	0.310
	DNA-total histone	32.0	79.0	36.0	26.0	20.0	0.14	0.350

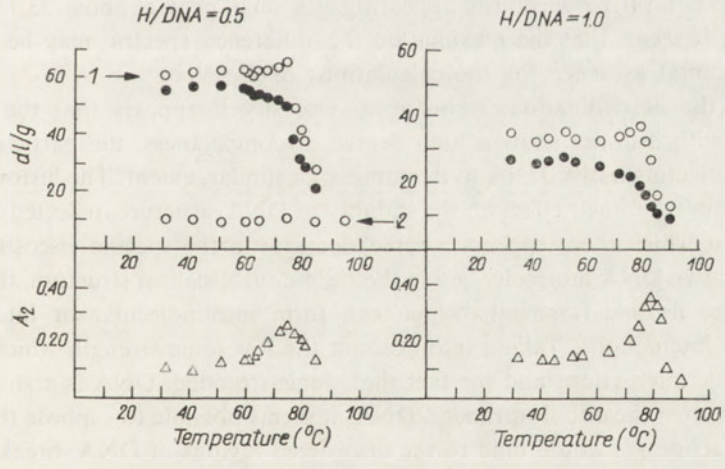

Fig. 5. Effect of temperature on hydrodynamic properties of DNA. ○, Specific viscosity; ●, intrinsic viscosity and △, the second virial coefficient of DNA complexes with total histone: *1*, on heating and *2*, on cooling, at the indicated histone/DNA ratios.

The temperature at which appears the maximum of intermolecular interactions reflects the stability of particular complexes. Thus the complex of DNA with the lysine-rich histone *f1* showed the highest stability, and that with the arginine-rich histone *f3*, the lowest.

DISCUSSION

From the difference spectra of DNA solutions it appears that the hypochromic effect becomes more pronounced with increasing salt concentration. The minima at 258 nm may be explained by screening of phosphate residues, especially in the regions of disturbed rigidity. This screening permits the bases to approach to such a distance at which hydrogen bonds may be formed, which leads to ordering of the DNA structure in the involved region.

Denatured DNA, native DNA in the presence of salts, and reassociated histone--DNA complexes exhibit in the difference spectra minima at 258 nm which cannot serve as basis for differentiation of the effect of the particular factors on the structure of DNA. On the other hand, it is possible to obtain some data concerning the mode of action of histone on DNA conformation from the maxima for particular histone--DNA complexes, which differ from each other. However, the observed maxima for the complexes of DNA with whole histone or particular histone fractions (except *f2a*) are not very characteristic, as at higher histone concentration they are shifted toward shorter wavelengths. These maxima may be interpreted as being due to non-ordered DNA structure since above 240 nm the spectrum of nucleohistone is dependent only on the absorbance of DNA. The displacement of maxima toward shorter wavelengths at higher histone concentration and the concomitant positive shift of the curves may indicate (similarly as in the case of thermal denaturation) a stronger change of the DNA structure. Thus the particular histone fractions would differ in their ability to affect DNA structure. De Voe (1969) calculated for poly ATGC the point hyperchromism appearing as a small peak at about 33.7×10^3 cm^{-1} (297 nm). It seems that the maxima on the difference spectra may be interpreted as experimental evidence for the calculations of De Voe.

From the determinations of intrinsic viscosity it appears that the complexes of DNA with histones show a high degree of compactness, the particular histone fractions affecting native DNA to the same, or a similar, extent. The histone fractions differ, however, in their effect on the stability of DNA structure, reflected by the temperature at which there appears a rapid decrease in the specific viscosity.

In a native DNA molecule, beside the rigid double-helical structure, there appear some more flexible fragments which can form intramolecular or intermolecular base-base interactions. Taking into account the low ionic strength which facilitates electrostatic interactions and the fact that single-stranded DNA is a stronger polyelectrolyte than the double-stranded DNA, it seems possible to suppose that histones (except fraction *f1*) would bind to the disordered regions of DNA, breaking the hydrogen bonds. This may explain the appearance of maxima at 275 - 285 nm. This concept is in agreement with the assumption of Spytkowski (1963) that DNA in nucleoprotein is in a quasi-single helical form, and with the suggestion of Ramm

et al. (1971) who, on the basis of circular dichroism measurements concluded that DNA in nucleoprotein is in a partly denatured state.

The results obtained in the present work point to arrangement of fraction *f1* mainly along the DNA helix. Such an arrangement of the molecules of lysine-rich histone is supported by the results of Juhasz *et al.* (1971) who demonstrated that nucleohistone deprived of histone *f1* had the same template activity as free DNA.

The specific viscosity of DNA in dilute salt solution showed a temperature--dependent maximum. Lack of such a maximum in the concentrated salt solution permits to suppose that molecular interactions are increased in solution of low counterion concentration and at elevated temperature. Addition of polycations shifted the maximum towards higher temperatures by lowering these interactions. This effect also may be interpreted by the increase in intermolecular interactions or it should be alternatively accepted that the increase in viscosity is due to deproteinization of the DNA-histone complex preceding its melting. The presence of the maximum for free DNA precludes the latter interpretation. As the intrinsic viscosity showed no temperature-dependent maxima, it appears that the extrema do not concern the particular molecules but their aggregates.

The results of the present experiments indicate that the effect of histones on DNA structure is not confined only to their ionic nature.

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WPŁYW HISTONÓW I ICH FRAKCJI NA STRUKTURĘ KWASU DEZOKSYRYBONUKLEINOWEGO

Streszczenie

Badano widma różnicowe w UV oraz własności hydrodynamiczne roztworów reasocjowanych rozpuszczalnych kompleksów histonów z DNA. Widma różnicowe kompleksów DNA z histonem całkowitym i jego frakcjami, z wyjątkiem kompleksu DNA-*f1*, wykazują minima przy około 260 nm i maksima około 280 nm; położenie maksimów zależy od frakcji histonów i stosunku histon/DNA. Minima interpretuje się jako stabilizację, natomiast maksima jako destabilizację podwójnej spirali DNA. Stwierdzono różnicę pomiędzy wpływem histonów i NaCl na strukturę DNA.

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THE EFFECT OF 1-NITRO-9-AMINOACRIDINE ON RESPIRATION AND GLYCOLYSIS IN EHRLICH ASCITES TUMOUR CELLS

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1-Nitro-9-(3′-dimethylaminopropylamine)-acridine (C-283) added during incubation of Ehrlich ascites tumour cells *in vitro* and *in vivo* inhibits their endogenous respiration and respiration in the presence of glucose, as well as aerobic lactate production and glucose uptake. C-283 diminishes also the activity of key glycolytic enzymes and brings about a decrease in the content of cellular ATP. The effect of C-283 can be explained partly as the result of inhibition of enzyme biosynthesis and of changed $NAD/NADH_2$ ratio.

1-Nitro-9-(3′-dimethylaminopropylamine)-acridine (compound C-283) inhibits *in vitro* and *in vivo* protein synthesis, and has a considerable cytostatic effect (Radzikowski *et al.*, 1967, 1969; Kwaśniewska-Rokicińska & Winkler, 1969; Chotkowska & Konopa, 1972). The relatively low toxicity and distinct antimitotic activity of C-283 made possible some attempts at treating tumours in man (Kwaśniewska-Rokicińska, 1971; Kwaśniewska-Rokicińska & Święcki, 1971).

The mechanism of biological action of C-283 is so-far little known. The correlation between glycolytic activity and the growth rate of tumours, as well as cell migratory ability was observed by several authors (Weber, 1966; 1968; Lea *et al.*, 1968; Korohoda, 1970). Nemec *et al.* (1962) demonstrated that the inhibition *in vitro* of cell division in Ehrlich ascites tumour by certain chemical compounds was accompanied by inhibition of glycolysis. Acridine derivatives (acridine orange, acriflavine and proflavine) which caused photosensitized inhibition of anaerobic glycolysis (Hunter *et al.*, 1965), also caused photoinactivation of tumour cells (Bellin *et al.*, 1961). This might suggest that the decreased growth rate was associated with the inhibition of energy production in the glycolytic pathway.

In the present work, the effect of C-283 on respiration, glycolysis, activity of some glycolytic enzymes and the content of ATP in ascites tumour cells, was studied.

MATERIALS AND METHODS

Ehrlich ascites tumour cells were propagated in Porton and CBA mouse strains; the experiments, both *in vivo* and *in vitro*, were performed on the 7th day after in-

oculation using a commercial preparation of C-283. The preparation, lyophilized in ampoules, was dissolved in sterilized physiological saline solution.

In experiments *in vitro*, the ascites tumour cells were aseptically withdrawn, centrifuged, washed with sterilized Krebs-Ringer phosphate buffer, pH 7.4, and incubated with C-283 at 37°C in the above buffer containing 10 mM-glucose. The concentration of C-283 was 0.2, 2, 20 and 100 μg per 1 ml of the tumour cell suspension, containing 0.2 - 0.3 ml of packed cells. After the indicated time period the cells were centrifuged and used for assays.

In experiments *in vivo*, the C-283 solution was injected intraperitoneally in doses of 1, 2 and 10 mg per 1 kg of mouse weight; after 24 or 36 h the cells were aseptically withdrawn, centrifuged and used for assays.

Respiration was measured at 38°C by the Warburg method (Umbreit *et al.*, 1951) in Krebs-Ringer phosphate buffer, pH 7.4, fortified with 50 mM-tris-HCl buffer, in the presence or absence of 10 mM-glucose. Lactate production was determined at 15-min intervals, after deproteinization of samples with 0.6 M-perchloric acid, using the enzymic method of Horn & Bruns (1956). At the same time glucose utilization was measured by the method of Hugget & Nixon (1957) in samples deproteinized with 0.33 M-perchloric acid.

For determinations of enzyme activities, the cells after incubation with C-283 and centrifugation were washed with 200 mM-tris-HCl buffer, pH 7.4, containing 115 mM-KCl, 10 mM-$MgCl_2$ and 2 mM-EDTA, then resuspended in the same buffer solution and homogenized in partly frozen state in Potter-Elvehjem glass homogenizer. Supernatants were obtained as described by Gumińska *et al.* (1969). Enzyme activities were measured spectrophotometrically at 340 nm at 25°C under conditions assuring pseudo-zero order kinetics (during 3 min period at 1 min intervals).

Hexokinase was assayed according to Racker (1947) in 100 mM-tris-HCl buffer, pH 8.0, containing 10 mM-$MgCl_2$, 2 mM-EDTA, 0.4 mM-ATP, 1.2 mM-glucose, 0.6 mM-NADP and 0.02 mg of glucose-6-phosphate dehydrogenase per 1 ml of the reaction mixture. The reaction was started by the addition of glucose.

Phosphofructokinase was assayed as described by Racker (1947) in 200 mM--tris-HCl buffer, pH 8.0, containing 10 mM-$MgCl_2$, 2 mM-EDTA, 1.6 mM-ATP, 1.2 mM-fructose-6-phosphate, 0.4 mM-$NADH_2$, and aldolase, phosphotriose isomerase and glycerol-3-phosphate dehydrogenase (8, 1600 and 16 Bücher units, respectively, per 1 ml of the reaction mixture). The reaction was started by adding fructose-6-phosphate.

Pyruvate kinase was assayed after Bücher & Pfleiderer (1955) in 200 mM-tris-HCl buffer, pH 7.4, containing 100 mM-KCl, 20 mM-$MgSO_4$, 2 mM-EDTA, 4 mM-2--phosphoenolpyruvate, 2 mM-ADP, 0.4 mM-$NADH_2$ and 0.05 mg of lactate dehydrogenase per 1 ml of the reaction mixture. The reaction was started by adding ADP.

Lactate dehydrogenase was estimated according to the method of Kubowitz & Ott (1943) in 0.4 mM-tris-HCl buffer, pH 7.4, containing 7 mM-$MgCl_2$, 2 mM-EDTA and 1 mM-pyruvate. The reaction was started by adding pyruvate.

A part of the cell pellet after incubation with C-283 was homogenized with cold 0.6 M-perchloric acid. In the extract, ATP was determined by the method of Adam (1965) and fructose-1,6-diphosphate according to Bücher & Hohorst (1965).

The results were calculated per 1 mg of protein in homogenates, suspensions or supernatants, and for statistical analysis Student's test was used.

Protein was assayed by the method of Lowry *et al.* (1951).

Substrates and enzymes were obtained from Boehringer & Soehne Co. (Mannheim, West Germany); C-283 synthesized according to Ledóchowski (1966) was a product of Polfa (Warszawa, Poland).

RESULTS

The direct effect of C-283 on respiration of Ehrlich ascites tumour cells (Table 1) was manifested as a slight, but not statistically significant, stimulation of oxygen

Table 1

Effect of C-283 on respiration of Ehrlich ascites tumour cells in the presence of glucose

The respiration was measured at 38°C in Krebs-Ringer phosphate buffer containing 50 mM-tris-HCl, 10 mM-glucose, and 2.5 - 4.5 mg cell protein/ml. Mean values ±S.D. are given, the number of determinations being indicated in parentheses.

Incubation with C-283			Respiration	
	time (h)	concn.	μmol O_2/mg protein/h	%
In vitro		μg/ml		
	0	0 (control)	0.53±0.15 (14)	(100.0)
		2.0	0.48±0.18 (11)	90.5
		20.0	0.69 (1)	130.1
		100.0	0.61±0.17 (14)	115.1
	1	0 (control)	0.78±0.15 (6)	(100.0)
		2.0	0.77±0.22 (6)	98.8
	3	0 (control)	0.30±0.15 (14)	(100.0)
		0.2	0.26±0.11 (14)	86.7
		2.0	0.15±0.06* (11)	50.0
		100.0	0.14±0.04* (6)	46.7
	18	0 (control)	0.48±0.16 (4)	(100.0)
		0.2	0.35±0.11 (4)	72.9
		2.0	0.22±0.16 (4)	45.8
		100.0	0.08±0.04** (4)	16.7
In vivo		mg/kg wt.		
	24	0 (control)	0.72±0.05 (4)	(100.0)
		2.0	0.52±0.15 (4)	72.1
		10.0	0.48±0.20 (4)	66.6
	36	0 (control)	0.53±0.15 (18)	(100.0)
		1.0	0.30±0.15**(18)	56.6

* $p=0.05$. ** $p=0.01$.

consumption. After a longer time of incubation with C-283 and at its higher concentrations, oxygen consumption was inhibited. The inhibition was also observed after 24-h action of C-283 on the ascites cells in the peritoneal cavity.

The inhibition of respiration by high concentrations of C-283 was greater in the presence of glucose than the inhibition of endogenous respiration, the Crabtree effect being enhanced (Table 2).

Table 2

Effect of C-283 on endogenous respiration and respiration in the presence of glucose, of Ehrlich ascites tumour cells

For experimental conditions see Table 1. Mean values ±S.D. are given, the number of determinations being indicated in parentheses.

Incubation with C-283			Respiration (μmol O_2/mg protein/h)		Crabtree effect (%)
	time (h)	concn.	endogenous	with 10 mM-glucose	
In vitro		μg/ml			
	18	0 (control)	0.63±0.19 (5)	0.48±0.16 (3)	—23.8
		0.2	0.42±0.25 (5)	0.35±0.11 (3)	—16.6
		20.0	0.31±0.27 (5)	0.22±0.16 (3)	—29.0
		100.0	0.24±0.21*(4)	0.08±0.04*(3)	—66.6
In vivo		mg/kg wt.			
	24	0 (control)	0.96±0.04 (4)	0.72±0.05 (4)	—25.0
		2.0	0.96±0.09 (4)	0.52±0.15 (4)	—45.7
		10.0	0.73±0.24 (4)	0.48±0.20 (4)	—34.2

* $p=0.05$.

In vitro, in the presence of C-283, especially at its lower concentration, lactate production and glucose uptake were at first somewhat stimulated. After prolonged incubation with C-283, or at its higher concentrations, glycolysis was inhibited as manifested by a decrease in lactate production and glucose uptake (Table 3). The inhibition of glycolysis was also observed after injection of C-283 into the peritoneal cavity of tumour-bearing animals.

The effect of C-283 on glycolytic enzymes is presented in Table 4. Lactate dehydrogenase activity was not inhibited, and at higher C-283 concentrations it was even increased. On the other hand, the activities of hexokinase, phosphofructokinase and pyruvate kinase were decreased, the decrease for hexokinase being the least pronounced.

Incubation with C-283 caused a decrease in the amount of ATP in Ehrlich ascites tumour cells, especially at higher concentrations of C-283 (Table 5).

Since acridines are rapidly oxidized by cellular NAD, after administration of C-283 an excessive formation of $NADH_2$ might be expected, which also could act as a factor inhibiting glycolysis. When to the buffer fortified with 1 mM-ATP and

Table 3

Effect of C-283 on aerobic glycosis of Ehrlich ascites tumour cells

For incubation, Krebs-Ringer phosphate buffer, pH 7.4, containing 50 mM-tris-HCl, 10 mM-glucose and 2.5 - 4.5 cell protein/ml, was used. Mean values ±S.D. are given, the number of determinations being indicated in parentheses. Due to very high scatter of control values, when two experiments were performed the results of both are presented.

Incubation with C-283			Lactate production		Glucose uptake	
	time (h)	concn.	µmol/mg protein/h	%	µmol/mg protein/h	%
In vitro		µg/ml				
	0	0 (control)	0.62±0.14 (5)	100.0	0.45±0.08 (4)	100.0
		2.0	0.85±0.17 (4)	137.0	0.63±0.23 (4)	140.0
		20.0	0.79 (1)	127.4	—	—
		100.0	0.40±0.25 (5)	64.5	0.41±0.17**(4)	91.1
	1	0 (control)	0.79 (1)	100.0	0.81 (1)	100.0
		2.0	0.79 (1)	100.0	0.68 (1)	83.9
	3	0 (control)	0.39 (1)	100.0	0.30 (1)	100.0
		0.2	0.32 (1)	82.0	0.28 (1)	93.3
		2.0	0.28 (1)	71.8	0.10 (1)	33.3
		100.0	0.11 (1)	28.2	0.06 (1)	20.0
	3	0 (control)	0.55 (1)	100.0	0.65 (1)	100.0
		0.2	0.38 (1)	69.1	0.71 (1)	109.2
		100.0	0.21 (1)	38.2	0.18 (1)	27.7
	18	0 (control)	0.28±0.11 (4)	100.0	0.42±0.30 (4)	100.0
		0.2	0.19±0.07 (4)	67.7	0.29±0.18 (4)	69.0
		2.0	0.14 (2)	50.0	0.14±0.05 (4)	33.3
		100.0	0.11±0.03*(4)	39.2	0.13±0.12 (4)	30.9
In vivo		mg/kg wt.				
	24	0 (control)	1.00 (1)	100.0	1.13 (1)	100.0
		2.0	0.81 (1)	81.0	0.72 (1)	63.7
		10.0	0.72 (1)	72.0	0.98 (1)	86.7
	24	0 (control)	0.96 (1)	100.0	0.79 (1)	100.0
		2.0	0.69 (1)	71.9	0.47 (1)	59.5
		10.0	0.54 (1)	56.2	0.48 (1)	60.7
	36	0 (control)	0.73±0.25 (6)	100.0	0.53±0.14 (6)	100.0
		1.0	0.49±0.18 (6)	67.1	0.35±0.14 (6)	66.0

* $p=0.05$. ** $p=0.01$.

NAD, $NADH_2$ was added to a NAD/$NADH_2$ ratio of 0.2, glycolysis of cell homogenates or cell suspensions was inhibited (Table 6). The inhibition was partly abolished by increasing the NAD/$NADH_2$ ratio. However, even a twofold excess of NAD failed to remove entirely the inhibitory effect of $NADH_2$. The inhibition of lactate formation was accompanied by accumulation of fructose-1,6-diphosphate.

Table 4

Effect of C-283 on the activities of some glycolytic enzymes in supernatants of Ehrlich ascites tumour cells

For determination of lactate dehydrogenase and pyruvate kinase activities, the reaction mixture (2.5 ml) contained 3.5 - 5 μg of supernatant protein, and for determination of phosphofructokinase and hexokinase, 350 - 500 μg of supernatant protein. Mean values ± S.D. are given, the number of determinations being indicated in parentheses; when two experiments were performed the results of both are presented.

Incubation with C-283			Lactate dehydrogenase		Pyruvate kinase		Phosphofructokinase		Hexokinase	
	time (h)	concn.	mIU/mg protein	%	mIU/mg protein	%	mIU/mg protein	%	mIU/mg protein	%
In vitro	3	μg/ml								
		0 (control)	2087±248 (4)	(100.0)	1420±412 (5)	(100.0)				
		0.2	2180 (2)	104.4	1012±300 (3)	71.2				
		2.0	3056±818 (4)	146.5	1026±252 (5)	72.2				
		100.0	3900 (2)	186.8	1278±632 (3)	90.0				
	12	0 (control)	2180 (1)	(100.0)	1300 (1)	(100.0)	8.6 (1)	(100.0)	4.7 (1)	(100.0)
		0.2	2140 (1)	98.2	1080 (1)	83.0	6.8 (1)	79.1	4.1 (1)	87.2
		2.0	3020 (1)	138.5	870 (1)	66.9	6.4 (1)	74.4	3.1 (1)	65.9
		100.0	4200 (1)	192.7	379 (1)	29.2	2.6 (1)	30.2	2.9 (1)	61.7
	12	0 (control)	3185 (1)	(100.0)	1725 (1)	(100.0)	14.8 (1)	(100.0)	11.7 (1)	(100.0)
		0.2	3865 (1)	121.3	1225 (1)	71.0	13.2 (1)	89.2	12.2 (1)	104.2
		2.0	4020 (1)	126.2	1470 (1)	85.2	13.8 (1)	93.2	8.9 (1)	76.1
		100.0	3905 (1)	122.6	1035 (1)	60.0	6.5 (1)	43.9	7.8 (1)	66.7
	18	0 (control)	3122±800 (5)	(100.0)	1794±770 (5)	(100.0)	10.3±6.0 (5)	(100.0)	10.7±2.0 (5)	(100.0)
		0.2	3816±765 (5)	122.2	1286±329 (5)	71.6	4.1±3.9 (4)	39.8	9.3±2.3 (4)	86.8
		20.0	3878±735 (5)	124.2	995±329 (5)	55.4	3.0±2.3 (4)	29.1	9.5±2.0 (4)	88.7
		100.0	4326±575* (5)	138.5	883±292* (5)	49.2	3.6±2.3 (5)	34.9	6.8±1.8*(5)	63.5
	24	0 (control)	3222±952 (8)	(100.0)	1724±948 (5)	(100.0)				
		0.2	4046±993** (8)	125.5	660±222 (3)	38.4				
In vivo	24	mg/kg wt.								
		0 (control)	2810±632 (10)	(100.0)	1518±306 (15)	(100.0)				
		1	3116±980 (10)	110.8	661±396**(10)	43.5				
		2	3174 (1)	112.9	519 (2)	34.2				
		10	4872±990* (3)	173.3	366± 48** (3)	24.1				

* $p=0.05$. ** $p=0.01$.

Table 5

Effect of C-283 on the amount of ATP in Ehrlich ascites tumour cells

The incubation medium consisted of Krebs-Ringer phosphate buffer, pH 7.4, containing 50 mM-tris-HCl, 10 mM-glucose, C-283 and 4 - 6 mg cell protein/ml; temperature 37°C.

Expt. no.	ATP control values (mμmol/mg protein)	Incubation with C-283 (μg/ml)			
		time (h)	0.2	20.0	100.0
			Content of ATP (% of control)		
I	2.85	12	99	68	56
II	4.30	12	98	84	38
I	9.46	18	104	92	68
II	7.60	18	90	57	56
III	3.31	18	82	81	60
IV	4.02	18	75	52	9
V	8.35	18	112	70	55

Table 6

Effect of nicotinamide-adenine dinucleotide on lactate production and fructose-1,6-diphosphate accumulation in homogenates of Ehrlich ascites tumour cells

The incubation medium contained 50 mM-tris-HCl buffer, pH 7.4, 25 mM-$KHCO_3$, 2.4 mM-K_2HPO_4, 6.7 mM-$MgCl_2$, 70 mM-KCl, 10 mM-glucose, 1 mM-ATP and 2 - 3 mg of homogenate protein per 1 ml of the medium. During 1 h incubation at 37°C, samples were taken for analysis at 15 min intervals.

Expt. no.	Additions		Lactate		Fructose-1,6-diphosphate	
	NAD (mM)	NADH (mM)	μmol/mg protein/h	%	μmol/mg protein/h	increasing factor
I	0.3	0 (control)	0.95	(100)	0.017	
	0.3	1.5	0.24	25	0.176	10
II	0.3	0 (control)	0.41	(100)	0.027	
	0.3	1.5	0.15	37	0.124	4.6
I	1.0	0 (control)	0.70	(100)	—	
	1.0	1.5	0.41	58		
I	3.0	0 (control)	0.89	(100)	0.023	
	3.0	1.5	0.83	93	0.207	9
II	3.0	0 (control)	0.45	(100)	0.019	
	3.0	1.5	0.28	62	0.135	7

DISCUSSION

In rapidly growing tumour cells capable of active migration and endocytosis, energy production is greatly enhanced, also in the glycolytic pathway. In most normal cells, respiration reduces the rate of glycolysis, in tumour cells, however,

lactate accumulates also under aerobic conditions. The ATP generated on respiration does not inhibit efficiently the glycolytic pathway. It has been demonstrated that in tumours the concentration of key glycolytic enzymes is much higher than in normal cells (Weber, 1966; Gumińska *et al.*, 1969; Gumińska, 1971), and there is some evidence that these enzymes in tumours are less sensitive to their natural inhibitors (Hofman, 1966).

After a longer action, C-283 inhibits both respiration and glycolysis of tumour cells. Measurements of activities of glycolytic enzymes under conditions permitting evaluation of the enzyme concentration in biological material, revealed a decrease in the activities of hexokinase, phosphofructokinase and pyruvate kinase. According to Weber (1966), these key glycolytic enzymes belong to the same functional gene unit, and their biosynthesis is regulated in a coordinated fashion (Weber *et al.*, 1965; Weber, 1968).

Acridine displays the ability to complex with DNA. Lerman (1964) demonstrated that aminoacridine intercalated between two base pairs of DNA molecule, and the effect of proflavine (Hurwitz *et al.*, 1962; Scholtissek & Rott, 1964; Bose *et al.*, 1966) and acriflavine (Bose *et al.*, 1966) was found to be similar to that of actinomycin D.

C-283, similarly as other acridine derivatives, was demonstrated by Chorąży and coworkers (Chorąży *et al.*, 1967; Mendecki *et al.*, 1969; Filipski *et al.*, 1970) to complex with DNA, thus blocking its function as template for RNA synthesis. Therefore it seems possible to suppose that the coordinated decrease in the activity of the key enzymes of glycolysis is due to selective inhibition of their synthesis, especially as the inhibition appeared only after preincubation of the cells with C-283. Reduced biosynthesis of those proteins the content of which in untreated tumour cells is high, may affect the mechanism of energy production, as indicated by the diminished amount of ATP.

The influence of C-283 on glycolysis seems, however, to involve a more complex mechanism. The inhibition of glycolysis after longer incubation cannot be explained only by suppression of key enzyme synthesis. Hunter *et al.* (1964, 1965) observed that, depending on conditions, a brief stimulation of glycolysis by acridine could precede the inhibitory phase, and that inhibition of glycolysis after incubation with acridine could be overcome by addition of NAD or pyruvate. The low redox potential of some acridine derivatives may lead to their oxidation by NAD and accumulation of $NADH_2$, which inhibits glycolysis (Weber *et al.*, 1967) leading to accumulation of fructose-1,6-diphosphate.

The decrease in the amount of ATP in the cell resulting from the inhibition of respiration and glycolysis by C-283 might be one of the main factors in the cytostatic effect of C-283 in tumour cells which, owing to their rapid growth and uncontrolled division, are particularly sensitive to deficient energy supply.

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WPŁYW 1-NITRO-9-AMINOAKRYDYNY NA ODDYCHANIE I GLIKOLIZĘ KOMÓREK RAKA WYSIĘKOWEGO EHRLICHA

Streszczenie

1-Nitro-9-(3′-dwumetyloaminopropylamino)-akrydyna (C-283) dodana podczas inkubacji *in vivo* i *in vitro* komórek raka wysiękowego Ehrlicha hamuje oddychanie endogenne i oddychanie w obecności glukozy, a także produkcję mleczanu i zużycie glukozy w warunkach tlenowych. C-283 powoduje także spadek aktywności kluczowych enzymów glikozy oraz spadek stężenia ATP w komórce.

Obserwowane zmiany metabolizmu mogą być częściowo wynikiem zahamowania pod wpływem C-283 biosyntezy białek enzymatycznych, częściowo zaś mogą być wynikiem zakłócenia normalnego stosunku $NAD/NADH_2$.

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LONG-CHAIN POLYPRENOLS OF TROPICAL AND SUBTROPICAL PLANTS*

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A high content of free *cis/trans* polyprenols was found in leaves of several tropical and subtropical plants (mainly Moraceae and Euphorbiaceae). The polyprenols occur as a mixture of prenologues, the polyprenol-9, -10, -11 or -12 being the main component, depending on the plant species.

Since the discovery of the function of C_{55} polyprenol in the biosynthesis of bacterial cell wall polymers (see review by Lennarz & Scher, 1972) a more general importance of the occurrence of similar polyprenols in plant material (Hemming, 1969) became evident. The largest amounts of free long-chain polyprenols (C_{55} and C_{60}) with three internal *trans* double bonds were found in leaves of *Ficus elastica* and *Hevea brasiliensis* (Stone *et al.*, 1967; Dunphy *et al.*, 1967). It has also been demonstrated that ficaprenol (C_{55} polyprenol) from *F. elastica* may effectively replace the bacterial polyprenol, at least in some reactions of the formation of peptidoglycan (Higashi *et al.*, 1970) and O-antigen (Jankowski *et al.*, 1972). Polyprenols are convenient tools for studying biosynthetic pathways of formation of polysaccharides in bacteria.

In this paper we report on the occurrence and characteristics of polyprenols in tropical and subtropical plants, with the aim both to extend the list of plants giving high yields of these substances, and to obtain more chemotaxonomic data for elucidation of the phenomenon of accumulation of free polyprenols in plant photosynthesizing tissues.

EXPERIMENTAL

Plant material. Leaves of tropical and subtropical plants (listed in Table 1) growing in the Botanical Garden in Moscow were taken from the middle parts of the branches.

Chemicals. [1,1′-^{14}C]Acetic anhydride (sp. act. 0.03 mCi/mmol) was from the Institute for Nuclear Research (Świerk, Poland). Aluminum oxide, acid, Brockman

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activity IV, was from M. Woelm (Eschwege, G.F.R.). Kieselguhr G and Kieselgel G for thin-layer chromatography were from Merck (Darmstadt, G.F.R.), and solanesol was from Hoffman la Roche (Basle, Switzerland).

Isolation of free polyprenols. The procedure employed was based on that used by Stone *et al.* (1967). Specimens of fresh leaves, 2 g, were cut into small pieces and ground in a mortar with glass powder and 10 ml of acetone. The acetone extract was diluted with an equal volume of water and extracted with three 5 ml portions of ethyl ether. The ethereal extract was evaporated in a stream of nitrogen. The residue was dissolved in 1 ml of light petroleum (b.p. 40 - 60°C) and applied to a column (1.3 × 10 cm) of aluminum oxide. The column was eluted successively with 50 ml portions of light petroleum and 2%, 10% and 15% ethyl ether in light petroleum. The fractions containing the substances corresponding to free polyprenols (as judged from t.l.c. in solvent I and II, see below) usually appeared in the first fractions of the eluent containing 10% ethyl ether. They were pooled and dried in a stream of nitrogen.

Formation of polyprenyl acetates. The dry residue containing free polyprenols dissolved in 0.5 ml of toluene, was treated with 50 µl of [^{14}C]acetic anhydride and one drop of pyridine, and the mixture left for 16 h at room temperature. It was then washed with 1 ml of cold 2 N-HCl and extracted with three 5 ml portions of ethyl ether. The ethereal extract was back-washed with water, evaporated in a stream of nitrogen and dissolved in 1 ml of toluene.

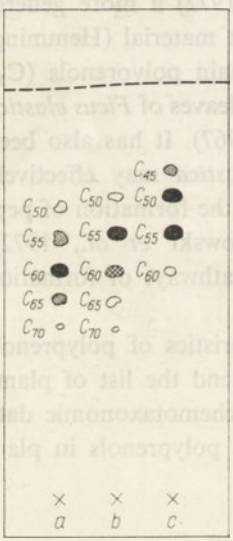

Fig. 1. Reversed-phase thin-layer chromatography in solvent III of prenyl acetates from: *a*, *Anaxagorea brevipens*; *b*, *Hura crepitans*; *c*, *Ficus subrepanda*. The spots were detected with ethanolic solution of fluorescein (0.01%, w/v).

Thin-layer chromatography. For chromatography on Kieselgel G plates (0.25 mm thick), chloroform was used as solvent (I). Reversed-phase thin-layer chromatography was performed on 0.25 mm plates of Kieselguhr G impregnated with 5% paraffin in light petroleum, using paraffin-saturated mixtures of water and acetone (10:90, v/v, solvent II and 2:98, v/v, solvent III). Spots were detected by spraying

with 10% ethanolic phosphomolybdic acid and subsequent heating, or with 0.01% ethanolic solution of fluorescein (Dunphy *et al.*, 1967). The pattern of chromatographic separation of various polyprenyl acetates in solvent III is presented in Fig. 1. The number of carbon atoms are known from the spectrum of n.m.r., mass spectrometry, gas chromatography, and comparison on reversed-phase thin-layer chromatography with the mixture of polyprenol acetates from *F. elastica.*

Estimation of radioactivity. The radioactivity on thin-layer plates was localized by autoradiography using Foton 44CUK X-ray plates. Material scraped from the plates was counted in a Packard Tri-Carb scintillation spectrometer using Bray's scintillation fluid (Bray, 1960).

Mass spectrometry was conducted with an LKB-9000 mass spectrometer (ionization energy 70 e.v., source temperature 220°C).

Nuclear magnetic resonance spectrometry was performed with a JNM-4 H-100 Jeol instrument. The spectra of 30 mg samples in CCl_4 were recorded.

Gas chromatography was performed in a Pye 104 Gas Chromatograph fitted with a flame ionization detector, using 3 ft, 1/16″ stainless steel column containing 0.3% SE 30 on silanized chromosorb 30/60 mesh.

RESULTS

The content of various polyprenols in the studied plants, presented in Table 1, was calculated from the amount of polyprenyl [^{14}C]acetates estimated after reversed-phase thin-layer chromatography in solvent III. The figures in column 9 represent the recovery of total counts of acetylated polyprenol fraction obtained by preparative thin-layer chromatography in solvent I. In some of the plants, especially those with a low content of polyprenols, the recovery was poor. This was due to the presence of other, shorter chain acetylated alcohols moving with the front in solvent III. These alcohols may have not been separated from long-chain polyprenols in the course of column chromatography on aluminum oxide. The content of polyprenols in leaves of each plant (column 10), was calculated from the sum of counts recovered in the spots of separated individual polyprenyl acetates.

In the case of all plants presented in Table 1, except *Begonia hederaefolia*, the polyprenols appeared as distinct spots already when crude acetone extracts were chromatographed in solvent I, and also on chromatographing the fraction of free polyprenols (eluted from aluminum oxide) in solvent II. Quantitative estimation demonstrated a high content of polyprenols in the majority of studied plants belonging to Moraceae, Euphorbiaceae and also to Annonaceae. It should be added that only a small proportion of polyprenols was found in the esterified form in plant leaves when the lipid fraction eluted from aluminum oxide with light petroleum and 2% ethyl ether in light petroleum was checked for their presence after alkaline hydrolysis (Wellburn *et al.*, 1967).

The relative abundance of the individual prenologues varies with the plant species. Thus in *Exoecaria bussei* the C_{45}-polyprenol was the most abundant, and in *Ficus subrepanda* the C_{50}-polyprenol. In a number of plants the C_{55}-polyprenol

Table 1

Quantitative estimation of various polyprenols from the studied plants

The figures represent the amount of radioactive [^{14}C]acetates formed by acetylation of the polyprenyl-containing fraction from aluminum--oxide chromatography and subsequent thin-layer chromatography.

Familia	Plant	c.p.m. $\times 10^{-3}$									
		1	2	3	4	5	6	7	8	9	10
		Total from Kieselgel	C_{40}	C_{45}	C_{50}	C_{55}	C_{60}	C_{65}	C_{70}	Recovery (%)	Total content (mmol/kg)
Apocynaceae	*Plumeria rubra*	20.6			1.4	13.5	3.6			90	0.33
Annonaceae	*Anaxagorea brevipens*	108			8.0	12.5	38.5	18.6	5.5	77	1.48
	Annona reticulata	44.6		1.6	18.0	8.1	5.7			75	0.59
Begoniaceae	*Begonia hederaefolia*	N.D.	—	—	—	—	—	—	—	—	—
Eucommiaceae	*Eucommia ulmoides*	29.4	1.3	5.2	3.2	2.2				40	0.21
Euphorbiaceae	*Hura crepitans*	196			10.0	92.0	58.2	7.5	1.6	86	3.01
	Mallotus barbatus	146		7.7	49.8	33.6	9.8			69	1.8
	Exoecaria bussei	131	8.1	42.5	30.8	3.0	2.4			66	1.5
	Codiaeum variegatum	88			12.0	36.0	9.1	3.1		65	1.08
	Euphorbia tirucalli	48.6			13.6	1.2				30	0.39
	Euphorbia splendens	26.2			2.6	2.8	2.3			30	0.14
	Putranjiva roxburghii	16.2		1.2	2.0	2.4	1.3			40	0.12
Guttiferae	*Mammea americana*	73.4	3.5	8.7	10.4	5.9				39	0.51
Moraceae	*Ficus subrepanda*	260		33.6	90.2	82.9	13.5			84	3.93
	Ficus craterostoma	208		9.9	47.5	79.7	27.1	6.6		82	3.04
	Ficus triangularis	176		6.2	60.4	66.7	18.8			86	2.6
	Ficus altissima	181		6.8	34.5	61.2	40.5			79	2.55
	Ficus retusa	142		3.0	16.6	79.6	18.9	1.9		85	2.14
	Ficus elastica	148		3.3	12.0	69.2	30.8	1.2		79	2.08
	Ficus bengalensis	152		6.0	35.0	54.8	20.2			76	2.07
	Ficus lyrata	78		4.1	20.0	27.1	10.7			79	1.1
	Ficus religiosa	21.4		1.2	3.1	1.6	1.6			35	0.13

was the main component. The C_{60}-polyprenol was the main component in the polyprenol mixture isolated from *Anaxagorea brevipens*.

In Fig. 2 is shown the n.m.r. spectrum of the crude polyprenol fraction from *F. subrepanda* obtained from an aluminum oxide column. It is evident that the proportion of the protons in the *cis* methyl groups to the olefinic hydrogen ($\tau = 8.34$) was higher than that of the methyl groups in *trans* configuration ($\tau = 8.42$). The characteristic peaks at τ about 5 and 6 (Stone *et al.*, 1967) are also visible.

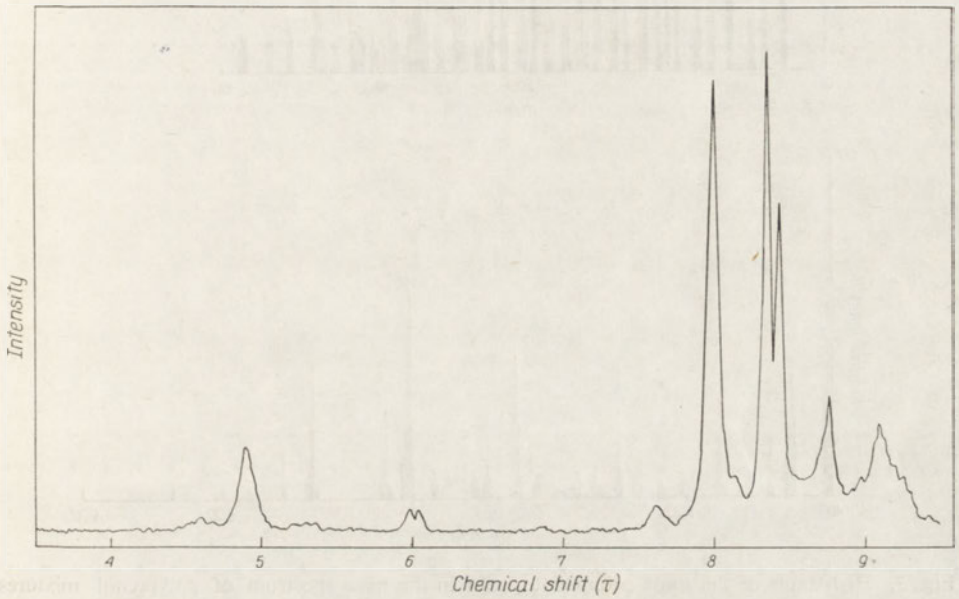

Fig. 2. Nuclear magnetic resonance spectra of polyprenol mixtures from *Ficus subrepanda*. The preparation from aluminum oxide column was used.

The results of mass spectrometry of the mixture of polyprenols from *F. subrepanda* are shown in Fig. 3. The even-numbered molecular ions were as expected from the results obtained by reversed-phase chromatography. The relatively high proportion of the molecular ions 698 of a C_{50}-polyprenol and the characteristic cracking pattern, i.e. loss of water (18) and of isoprene residues (69 and 68 mol.wt.) is evident. The polyprenol preparation from *Hura crepitans* gave a similar mass spectrum as the mixture of polyprenols from *F. subrepanda*. The mass spectrum of the preparation obtained from *A. brevipens* was also similar except that the relative proportion of the heaviest molecular ion, 912, was higher than in the former preparation and some other unidentified fragments were present.

Gas chromatography of the polyprenol preparations from *F. subrepanda* is shown in Fig. 4. Gas chromatography of the preparations from *H. crepitans* and *A. brevipens*, and also of solanesol (C_{45}, all-*trans*) and ficaprenol isolated previously in our laboratory (Jankowski & Chojnacki, 1972) was also performed and the results are summarized in Table 2 in the form of retention times relative to that

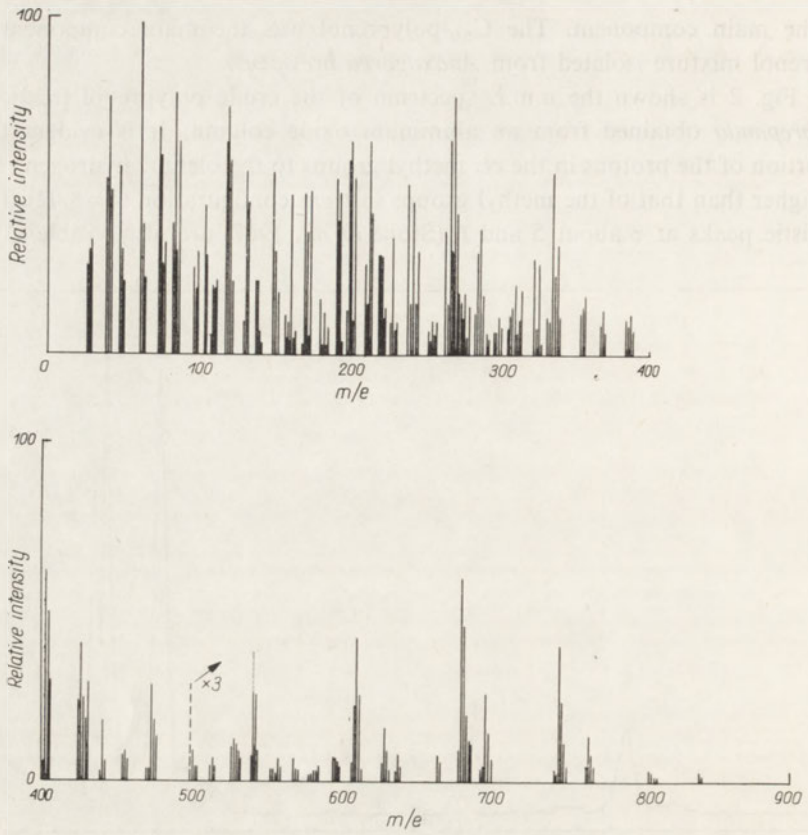

Fig. 3. Histogram of the most prominent peaks in the mass spectrum of polyprenol mixtures from *Ficus subrepanda*.

Table 2

Gas chromatography retention times of components of mixtures of free polyprenols isolated from some plants, relative to solanesol C_{45} all-trans

Plant	Probable prenologue				
	C_{45}	C_{50}	C_{55}	C_{60}	C_{65}
Anaxagorea brevipens	—	—	4.72	6.09	7.65
Hura crepitans	1.0	1.91*	4.4	6.09	—
Ficus subrepanda	0.81	2.39	4.5	6.1	—
Ficus elastica	0.99	2.6	4.5	6.0	—

* Flat and asymmetric.

for solanesol. The correspondence of gas chromatographic peaks of each of the studied polyprenol preparations and of solanesol with the data of reversed-phase thin-layer chromatography of acetates is visible. It is not known, however, whether the emerging peaks are due to the actual alcohols or to the resulting respective hydrocarbons.

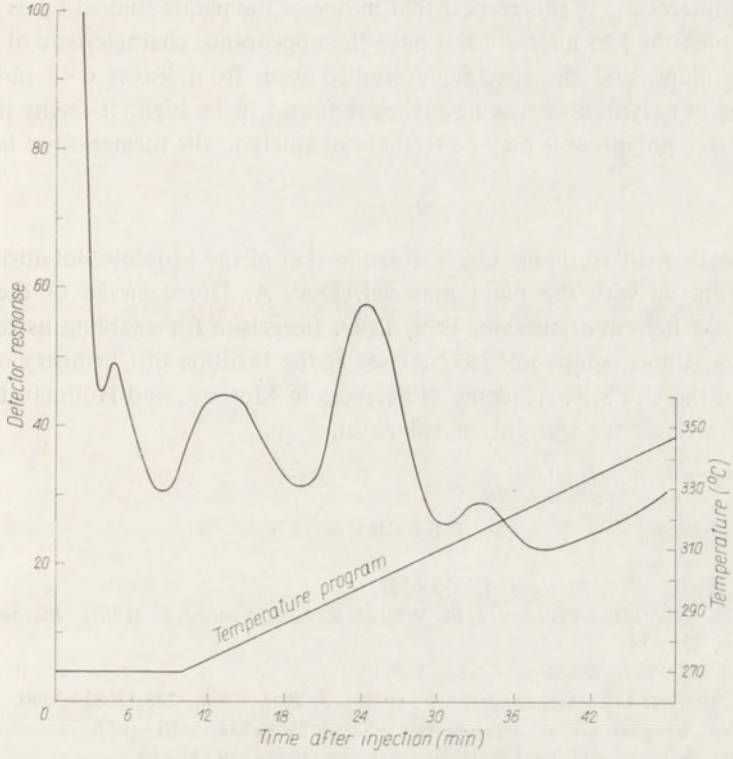

Fig. 4. Gas chromatography of the polyprenol mixtures from *Ficus subrepanda*.

DISCUSSION

The results of our studies point to the occurrence of large amounts of free polyprenols in various plants of tropical and subtropical regions. The highest contents of polyprenols in plant leaves were reported by Stone *et al.* (1967) and Dunphy *et al.* (1967). According to the unpublished studies of Dr. J. Pyrek (Dept. of Organic Chemistry, Warsaw University) large quantities of castaprenols (1% of dry weight) are present in leaves of *Trichosanthes palmata* L (Cucurbitaceae) of Indian origin. In the majority of plants studied by Wellburn & Hemming (1966) the content of polyprenols was very low. According to our previous unpublished estimations, the polyprenol content in old, yellowish leaves of *Deilephila euphorbiae*, *Leontondon taraxacum* and *Taraxacum kok-saghyz* was also very low. In a number of available tropical and subtropical plants, polyprenol families with either C_{55} or C_{60} dominant prenologue were found, similarly as in the case of *F. elastica* (Stone *et al.*, 1967) and *Hevea brasiliensis* (Dunphy *et al.*, 1967). Some plants, however, were found to contain mainly C_{50} and C_{45} polyprenols. The high content of free polyprenols in leaves was considered to be related to the production of natural rubber (Dunphy *et al.*, 1967), and with the process of senescence of the leaf (Wellburn & Hemming,

1966). It is interesting in this respect that in one of the plants studied by us (*H. crepitans*), in which the leaf juice did not have the appearance characteristic of a rubber-producing plant, and the specimens studied were from leaves 6 - 7 months old, the content of polyprenols was nevertheless found to be high. It seems that a high content of free polyprenols may be related not solely to the formation of latex.

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DŁUGOŁAŃCUCHOWE POLIPRENOLE W ROŚLINACH TROPIKALNYCH I SUBTROPIKALNYCH

Streszczenie

Znaleziono duże ilości wolnych poliprenoli w liściach badanych roślin tropikalnych i subtropikalnych (głównie Moraceae i Euphorbiaceae). W zależności od gatunku rośliny w mieszaninie prenologów może dominować poliprenol-9, -10, -11, -12.

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EFFECT OF ACETYLATION OF HISTONES ON THEIR INTERACTION WITH DNA*

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The ability of acetylated histones to precipitate DNA and the stability of the constituted complexes with DNA was found to decrease with increased blocking of NH_2 groups. These changes were, however, smaller than could be expected on the assumption that only ionic interactions are involved in formation of the histone-DNA complexes.

The interaction of histones with DNA received considerable attention in recent years (see review by Stellwagen & Cole, 1969). The formation and properties of histone-DNA complexes were studied under different conditions of ionic strength, pH, kind of ions, etc. However, there are no data whether and in what manner partial blocking of free NH_2 groups of histones may modify their affinity to DNA.

In this paper we present the results of experiments on the effect of acetylation of NH_2 groups of calf thymus histone on its ability to precipitate DNA from solution and on stability of the formed complexes.

MATERIALS AND METHODS

Preparation of histones. Total histone was obtained by the method of Fambrough & Bonner (1969) from calf thymus nucleohistone, isolated according to Hnilica (1966).

Preparation of acetylated histones. This was performed by the method of Fraenkel-Conrat (1957) using different amounts of acetic anhydride. Acetylated histones were precipitated with trichloroacetic acid (final concn. 20%), centrifuged at 3000 *g* for 30 min, washed once with acidified acetone (acetone - conc. HCl, 200:0.1, v/v) and then twice with acetone, and dried under vacuum. The degree of acetylation was calculated from the difference between the amount of free NH_2 groups before and after acetylation, determined by the ninhydrin method of Yemm & Cocking

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(1955). Using 5, 10, 15 and 100 µl of acetic anhydride per 200 mg of protein, the histone was acetylated in 10, 25, 31 and 45%, respectively.

Precipitation of DNA with histones was performed as described by Butler & Johns (1964). The amount of precipitated DNA was calculated from the extinction at 260 nm in the supernatant obtained by centrifugation at 3000 *g* for 30 min.

The effect of NaCl *concentration on solubility of DNA-histone complexes.* To samples containing 0.5 mg of DNA in 5 ml of 0.14 M-NaCl, pH 7.0, 1 ml of solution containing histone preparations in amounts sufficient for complete precipitation of DNA was added, then 4 M-NaCl was added to the final NaCl concentration of 0.14 - 1.0 M. After 30 min stirring at room temperature, the samples were centrifuged at 3000 *g* for 30 min and in the clear supernatant the extinction at 260 nm was measured.

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Reagents. Calf thymus DNA was from Worthington Biochem. Corp. (Freehold, N.J., U.S.A.); bovine serum albumin from Michrome (London, England); acetic anhydride (freshly distilled, fraction 129 - 132°C) and other reagents were from P.O.Ch. (Gliwice, Poland).

RESULTS AND DISCUSSION

The course of precipitation of DNA by native histone and histones acetylated to varying degrees, from 0.14 M-NaCl solution at pH 7.0 is presented in Fig. 1A, B. The amount of precipitated DNA increased proportionally to the amount of the added histone, and this permitted to determine the minimum histone/DNA weight ratio required for complete precipitation of DNA. At the minimum ratio all histone was bound to DNA.

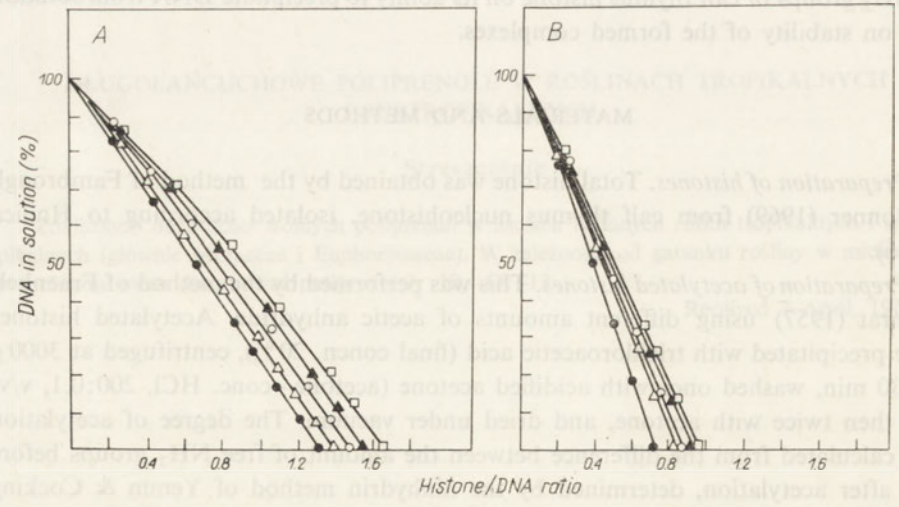

Fig. 1. Precipitation of DNA dissolved in *A*, 0.14 M-NaCl, pH 7.0, and *B*, 0.05 M-$MgCl_2$, pH 7.0, by histone: ●, native; and acetylated in: △, 10%; ○, 25%; ▲, 31%; □, 45%.

Table 1 contains values of these ratios determined in 0.14 M-NaCl at varying pH values and at two $MgCl_2$ concentrations at pH 7.0. Under all conditions tested, the higher was the degree of acetylation, the greater amount of histone was required to precipitate DNA. Since at the lowest histone/DNA ratio sufficient for complete precipitation of DNA all added histone interacts with DNA, it may be supposed that the differences in the histone/DNA ratios reflect changes in the properties of the formed complexes, related to partial blocking of the NH_2 groups of histone.

Table 1

Effect of acetylation of histone on its ability to precipitate DNA

The results are expressed as minimum histone/DNA weight ratio required for complete precipitation of DNA.

Acetylation (%)	pH in 0.14 M-NaCl				Molarity of $MgCl_2$, pH 7.0	
	5.0	6.0	7.0	8.0	0.01 M	0.05 M
0	1.20	1.26	1.32	1.35	0.90	0.72
10	1.35	1.38	1.40	1.41	1.00	0.84
25	1.36	1.46	1.48	1.51	1.08	0.88
31	1.40	1.48	1.54	1.54	1.15	0.92
45	1.48	1.56	1.65	1.62	1.23	0.95

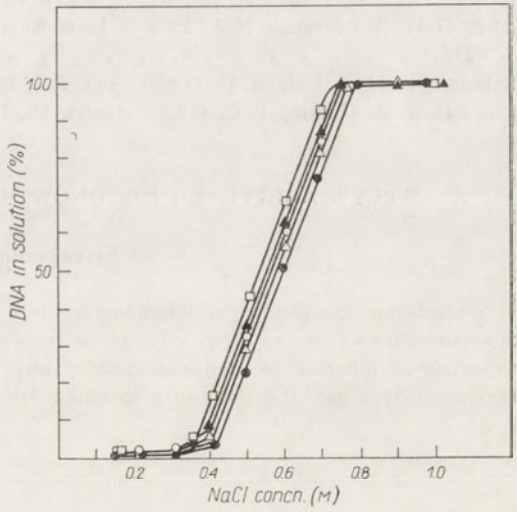

Fig. 2. Effect of histone acetylation on the solubility of histone-DNA complexes. Histone: ●, native; and acetylated in: △, 10%, ○, 25%; ▲, 31%; □, 45%.

The pH-dependent changes of the histone/DNA ratio were practically the same for native histone and the histones acetylated to varying degrees.

The stability of DNA complexes with the acetylated histones was studied on complexes formed in 0.14 M-NaCl at pH 7.0. As it may be seen from Fig. 2, all

studied complexes were insoluble in 0.4M-NaCl; at higher NaCl concentrations the complexes began to dissolve, the degree of solubility at a given NaCl concentration being the higher, the higher was the degree of acetylation of histone; at NaCl concentrations exceeding 0.8 M all complexes were completely soluble.

The obtained results indicate that the blocking of NH_2 groups of histones changes their affinity to DNA. These changes are, however, significantly smaller than one can expect assuming that only ionic interactions between histones and DNA take place. The presented data are consistent with the suggestion of Bradbury *et al.* (1967) that interactions other than ionic ones play an important role in the binding of DNA and histones in nucleohistone.

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WPŁYW ACETYLACJI HISTONÓW NA ICH INTERAKCJĘ Z DNA

Streszczenie

Stwierdzono, że częściowe zablokowanie wolnych grup NH_2 histonów resztą acetylową obniża ich zdolność do wytrącania DNA z badanych roztworów, jak również zwiększa rozpuszczalność wytworzonych kompleksów. Jednakże zmiany wywołane acetylacją są mniejsze, niżby należało oczekiwać przyjmując, że histony są połączone z DNA wyłącznie wiązaniami jonowymi.

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RIBONUCLEASE AND POLYPEPTIDE TRYPSIN INHIBITORS FROM BOVINE AND PORCINE PANCREAS

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Ribonuclease and trypsin inhibitors were isolated from pancreas trichloroacetic acid extract. The preparations of ribonuclease and inhibitors of the Kazal and Kunitz type from bovine pancreas were homogeneous on polyacrylamide-gel electrophoresis, whereas the preparations of ribonuclease and inhibitor of the Kazal type from porcine pancreas were heterogeneous. It is supposed that heterogeneity of the porcine preparations results from intracellular autolysis related to the lack of the polyvalent trypsin inhibitor of the Kunitz type in hog pancreas.

Bovine pancreas is a rich source of ribonuclease and proteolysis inhibitors. Anti-proteolytic activity is due to the presence of two trypsin inhibitors, namely the basic, polyvalent inhibitor of trypsin, chymotrypsin and kallikrein of the Kunitz type, and of the trypsin-specific inhibitor of the Kazal type. The two inhibitors have similar molecular weight (Kunitz inhibitor 6500, Kazal inhibitor 6100), and differ in their physico-chemical and biological properties. Ribonuclease, the basic low-molecular--weight protein, is similarly as the trypsin inhibitors, soluble in trichloroacetic acid. From trichloroacetic acid extract of bovine pancreas Wilimowska-Pelc (1971) isolated the Kazal inhibitor, Malicka (1972), the ribonuclease and Wilusz (1971) and Wilusz *et al.* (1973) obtained the Kunitz inhibitor from several bovine tissues.

In the present work an attempt has been made to isolate both the two trypsin inhibitors and ribonuclease from a trichloroacetic acid extract of bovine and porcine pancreas.

MATERIALS AND METHODS

Trichloroacetic acid extracts. Bovine or porcine pancreas obtained from the slaughterhouse not later than 2 h after killing of the animal, was placed in ice and transported to the laboratory. Fat was removed and the pancreas immersed in cold 5% trichloroacetic acid (TCA) and left for 30 min at about 5°C; then it was passed through a meat-grinder and homogenized in a Waring-Blendor homogenizer with

2 vol. of cold 5% TCA. The homogenate was centrifuged at 1200 *g*, the supernatant filtered through filter paper and adjusted to pH 5 against bromocresol blue. Then ammonium sulphate was added to 0.8 saturation, the mixture left at 5°C for 2 h and centrifuged. The sedimented protein was dissolved in two volumes of water and dialysed for a period not exceeding 24 h against 0.01 M-acetate buffer, pH 5.

CM-cellulose chromatography. The dialysed protein solution was subjected to chromatography on a CM-cellulose column (2.5 × 30 cm), the elution being performed with two convex concentration gradients of acetate buffer, pH 5, from 0.01 to 0.16 M and from 0.16 to 1.0 M.

Bio-Gel P-10 chromatography. The fractions eluted from CM-cellulose which showed the highest antiproteolytic activity, were pooled and ammonium sulphate was added to 0.8 saturation. The precipitated protein was dissolved in water, applied to the column of Bio-Gel P-10, and eluted with water.

Polyacrylamide-gel electrophoresis was carried out according to Reisfeld *et al.* (1962) at pH 4.4.

Protein determinations were performed by the tannin method of Mejbaum-Katzenellenbogen (1955) with gelatin as standard. In the eluates and the purified inhibitor preparations the protein was determined at 280 nm in a Zeiss (Jena) VSU 2-P spectrophotometer. For the Kunitz inhibitor the coefficient $E^{1\%}_{1cm}=8.9$ (Kassell *et al.*, 1963) was taken, for the Kazal inhibitor from bovine pancreas $E^{1\%}_{1cm}=6.5$ (Kazal *et al.*, 1948), and from porcine pancreas, 5.18 (Greene *et al.*, 1968).

Sugar determination was made by the phenol method of Dubois *et al.* (1956), using glucose as standard.

Ribonuclease activity was determined by the method of Anfinsen *et al.* (1954) in 0.1 M-acetate buffer, pH 5; the time of incubation was 10 min at 37°C. As one unit of activity was accepted that amount of enzyme which under these conditions gave an increase in E_{260} of 0.1.

Antiproteolytic activity. The antitrypsin and antichymotrypsin activities were determined by the method of Kunitz (1947), applying a 10-min preincubation of the enzyme. One unit of antiproteolytic activity was defined as that amount of inhibitor which inhibited in 50% the activity of 2 μg of trypsin.

Stability of the trypsin-inhibitor complexes was studied by the method of Laskowski & Wu (1953).

Reagents. Trypsin and chymotrypsin (2× crystallized) were prepared according to Northrop & Kunitz as described by Laskowski (1955). The trypsin preparation was inactive towards tyrosine ethyl ester (Serva, Heidelberg, G.F.R.). Pepsin, 3× cryst., was a product of Nutr. Biochem. Corp. (Cleveland, Ohio, U.S.A.). Casein, white, soluble, was a product of British Drug Houses (Poole, Dorset, England), carboxymethylcellulose Whatman CM-70 of Balston Ltd (Maidstone, Kent, England), Bio-Gel P-10 of Calbiochem (Los Angeles, Calif., U.S.A.), ribonucleic acid of Fluka A.G. (Buchs, Switzerland). Acrylamide, *N,N'*-methylenebisacrylamide (crystalline) and *N,N,N',N'*-tetramethyldiamine (TEMED) were from Koch-Light Lab. (Colnbrook, Bucks, England). Tannin was of French origin distributed by P.O.Ch. All other reagents were analytical grade products of P.O.Ch. (Gliwice, Poland).

RESULTS

The course of purification of ribonuclease from TCA-extracts is presented in Table 1, and that of trypsin inhibitors, in Table 2. The TCA-extract from bovine pancreas was found to have twice as much of protein as the extract from porcine pancreas, and tenfold higher ribonuclease activity. On the other hand, the total antitrypsin activity in the TCA-extract from bovine pancreas was lower than in the extract from porcine pancreas.

Table 1

Isolation of ribonuclease from trichloroacetic acid extracts from bovine and porcine pancreas

The values were calculated per 1 kg of fresh tissue weight.

Step	Protein (mg)		Total activity ($10^3 \times$ units)		Yield (%)		Specific activity ($10^3 \times$ units/mg protein)		Purification factor	
	ox	pig	ox	pig	ox	pig	ox	pig	ox	pig
TCA-extract	1500	750	1473	148	100	100	0.980	0.190	1.0	1.0
Ppt. at 0.8 $(NH_4)_2SO_4$ sat.	1200	600	1360	120	92	81	1.130	0.200	1.0	1.0
Dialysis	900	450	1239	90	84	60	1.370	0.200	1.4	1.0
CM-cellulose chromatography	320	100	611.8	45	41	30	1.900	0.450	1.9	2.3
Crystallization	240	—	528	—	35	—	2.200	—	2.2	—

Table 2

Isolation of the basic polyvalent trypsin inhibitor of the Kunitz type and of the trypsin-specific inhibitor of the Kazal type, from trichloroacetic acid extracts of bovine and porcine pancreas

The values were calculated per 1 kg of fresh tissue weight.

Step	Protein (mg)		Total activity ($10^3 \times$ units)		Yield (%)		Specific activity ($10^3 \times$ units/mg protein)		Purification factor	
	ox	pig	ox	pig	ox	pig	ox	pig	ox	pig
TCA-extract	1500	750	70	102	100	100	0.046	0.14	1.0	1.0
Ppt. at 0.8 $(NH_4)_2SO_4$ sat.	1200	600	63	91	90	90	0.052	0.15	1.1	1.1
Dialysis	900	450	47	69	67	67	0.052	0.15	1.1	1.1
Kazal inhibitor										
CM-cellulose chromatography	8.8	33.0	17.0	52	25	51	1.90	1.60	41.0	11.2
Bio-Gel P-10 chromatography	3.3	7.0	11.9	36.4	17	35	3.60	5.20	76.0	37.0
Kunitz inhibitor										
CM-cellulose chromatography	29.0	0	14.7	0	21	0	0.50	0	10.8	
Bio-Gel P-10 chromatography	3.0	0	10.3	0	14.7	0	3.50	0	76.0	

In the first two steps of preparation consisting of ammonium sulphate fractionation and dialysis, 40% of the protein material was lost, with concomitant loss of ribonuclease and antitrypsin activities. The loss of activity of bovine ribonuclease (20%) was lower than that of porcine ribonuclease (40%). Antitrypsin activity in both cases corresponded to 67% of the initial activity. Chromatography on CM--cellulose gave very similar protein elution profiles for the preparations from bovine and porcine pancreas (Fig. 1a, b). In both cases one ribonuclease activity peak,

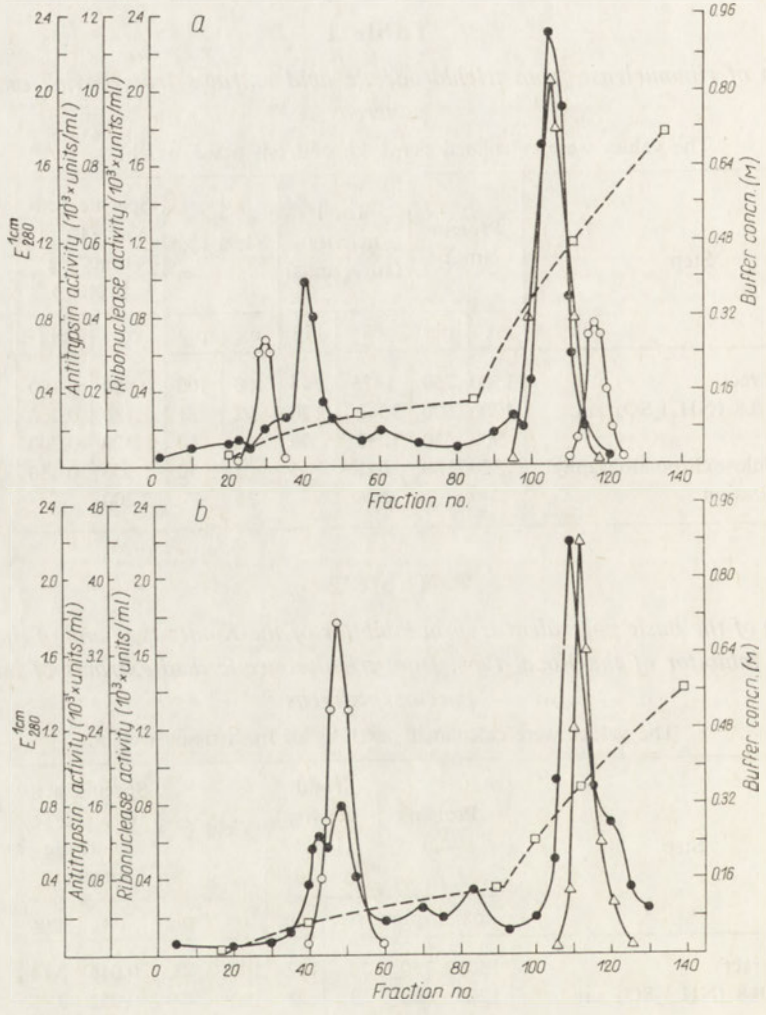

Fig. 1. CM-cellulose column chromatography of trichloroacetic acid-soluble proteins of *a*, bovine and *b*, porcine pancreas. The proteins were eluted from the column (2.5×30 cm) with a concentration gradient of acetate buffer, pH 7.5. ●, $E_{280}^{1\,cm}$; ○, antitrypsin activity; △, ribonuclease activity; □, concn. of acetate buffer. The amount of protein applied to the column, volume of fractions and elution rate were: for bovine pancreas 305 mg, 7 ml and 60 ml/h, and for porcine pancreas 951 mg, 8 ml and 80 ml/h, respectively.

eluted at the buffer concentration of about 0.4 M, was obtained. On the other hand, the antitrypsin activity of bovine pancreas appeared in two peaks, one eluted at low, and the second at high ionic strength. In the preparations from porcine pancreas, this second antitrypsin activity was absent.

Ribonuclease activity. The CM-cellulose fractions no. 100 - 110 showing the highest activity and corresponding to about 50% of the activity applied to the column, were pooled and ammonium sulphate was added up to distinct turbidity (about 0.6 saturation). After 24 h at room temperature, the ribonuclease from bovine pancreas crystallized as sheaves of slender needles. From 1 kg of pancreas, about 240 mg of crystalline ribonuclease was obtained, with an activity of 2.2×10^3 units/mg protein (Table 1). The preparation contained 0.5% of sugars, and showed no antiproteolytic or proteolytic activity as determined against casein at pH 7.6. On polyacrylamide-gel electrophoresis at pH 4.4 it gave a single protein band (Fig. 2a). To obtain salt-free ribonuclease, the crystalline preparation was dissolved in water, dialysed against water and crystallized from 50% ethanol.

The ribonuclease from porcine pancreas did not crystallize under the conditions applied for the bovine enzyme. The fractions from CM-cellulose column showing the highest activity (no. 100 - 116) were pooled and freeze-dried. From 1 kg of pancreas, 100 mg of protein was obtained with specific activity of 0.450×10^3 units/mg protein (Table 1); the preparation was free from antiproteolytic activity and contained 14% of sugars. On polyacrylamide-gel electrophoresis at pH 4.4 it was resolved into four portein bands (Fig. 2b).

Trypsin inhibitor of the Kazal type. The CM-cellulose column fractions showing the highest antitrypsin activity, eluted with low-ionic-strength acetate buffer, were subjected to chromatography on Bio-Gel P-10. In the case of bovine pancreas,

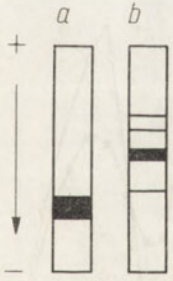

Fig. 2

Fig. 3

Fig. 2. Polyacrylamide-gel electrophoresis of: *a*, crystalline ribonuclease from bovine pancreas and *b*, lyophilized ribonuclease from porcine pancreas. The electrophoresis was carried out according to Reisfeld *et al.* (1962) at pH 4.4; a current of 2.5 mA per tube was applied for 15 min and then 5 mA for another 55 min. To the gel, 100 μg of protein was applied. For staining, a 1% solution of Amido Black in 7% acetic acid was used, and excess of the dye was removed with 7% acetic acid.

Fig. 3. Polyacrylamide-gel electrophoresis of pancreatic trypsin inhibitors: *a*, bovine, Kazal type; *b*, porcine, Kazal type; *c*, bovine, Kunitz type. Pooled active fractions no. 21 - 25 from Bio-Gel P-10 chromatography (see Figs. 4 and 5) were applied to the gel. For details of electrophoresis see legend to Fig. 2.

three protein peaks were obtained, the last one containing the whole antitrypsin activity (Fig. 4). Fractions no. 21 - 25, corresponding to 70% of the activity subjected to chromatography, on electrophoresis in polyacrylamide gel gave one protein band (Fig. 3a). In the Kunitz test, 1 μg of the inhibitor inhibited 3.5 μg of trypsin.

The inhibitor from porcine pancreas subjected to separation on Bio-Gel P-10 gave two protein peaks, the whole activity being recovered in the second peak (Fig. 5). The pooled active fractions containing about 68% of the activity applied to the column,

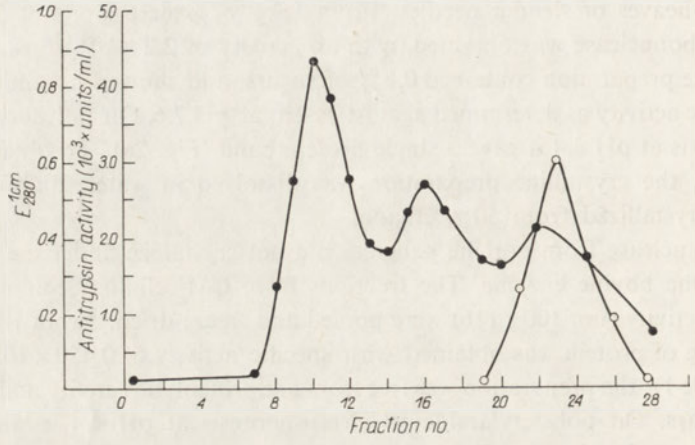

Fig. 4. Bio-Gel P-10 chromatography of the Kazal-type inhibitor from bovine pancreas. To the column (1.5×80 cm) 4 mg of protein (fractions no. 27 - 31 from CM-cellulose chromatography, cf. Fig. 1a) was applied and eluted with water, fractions of 2 ml being collected at a rate of 25 ml/h. ●, $E^{1\,cm}_{280}$; ○, antitrypsin activity.

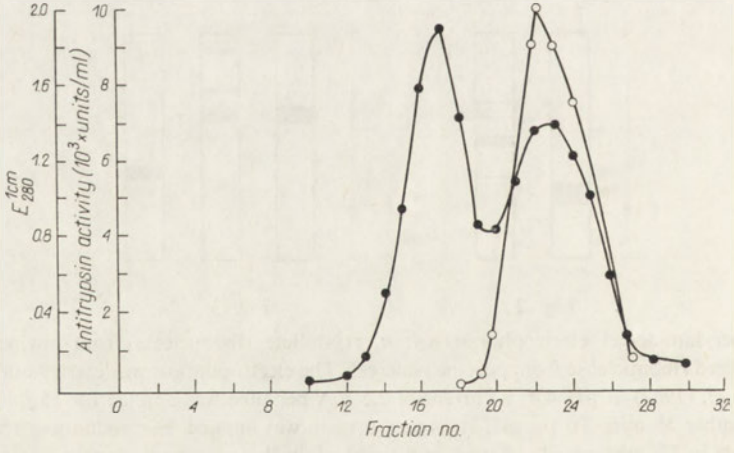

Fig. 5. Bio-Gel P-10 chromatography of the Kazal-type inhibitor from porcine pancreas. To the column (1.5×90 cm) 14 mg of protein (fractions no. 45 - 49 from CM-cellulose chromatography, cf. Fig. 1b) was applied and eluted with water, fractions of 2 ml being collected at a rate of 30 ml/h. ●, $E^{1\,cm}_{280}$; ○, antitrypsin activity.

resolved on polyacrylamide-gel electrophoresis into three portein bands (Fig. 3b). In the Kunitz test, 1 μg of the inhibitor inhibited 5.2 μg of trypsin.

Neither of the trypsin inhibitors showed any antichymotrypsin activity, both formed unstable complexes with trypsin and were digested by pepsin, thus corresponding in their characteristics to the acid trypsin-specific inhibitor of the Kazal type.

The yield and specific activity of the inhibitor from bovine pancreas were lower by a half and the degree of its purification was twice as high, as compared with the porcine inhibitor; on polyacrylamide gel, the bovine preparation was homogeneous whereas the porcine one was heterogeneous.

Basic polyvalent trypsin inhibitor of the Kunitz type. Bovine pancreas contained, in addition to the inhibitor of the Kazal type, a second inhibitor activity which on elution from CM-cellulose overlapped the descending arm of the ribonuclease peak (Fig. 1a). On Bio-Gel P-10 (Fig. 6) a distinct peak of antiproteolytic activity

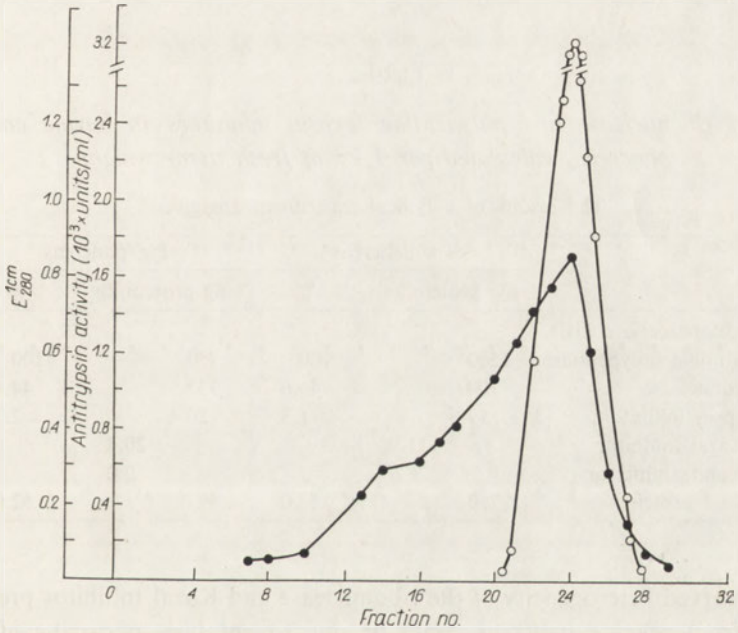

Fig. 6. Bio-Gel P-10 chromatography of the Kunitz-type inhibitor from bovine pancreas. To the column (1.5×85 cm) 13.6 mg of protein (fractions no. 115 - 122 from CM-cellulose column, cf. Fig. 1a) were applied and eluted with water, fractions of 2 ml being collected at a rate of 25 ml/h. ●, $E_{280}^{1\,cm}$; ○, antitrypsin activity.

was obtained. Fractions no. 22 - 27 showing the highest antitrypsin and antichymotrypsin activities were pooled and crystallized by adding ammonium sulphate to slight turbidity. The inhibitor crystallized as hexagonal columns. From 1 kg of pancreas, 3 mg of the crystalline preparation, still containing trace amounts of ribonuclease, was obtained. Recrystallization gave a preparation free from ribonuclease activity and homogeneous on polyacrylamide-gel electrophoresis (Fig. 3c).

One mg of the purified preparation inhibited 3.5 μg of trypsin and 2.5 μg of chymotrypsin. This inhibitor, in contrast to the Kazal inhibitor, was resistant to tryptic digestion and formed stable complexes with trypsin and chymotrypsin. These properties and the crystalline form are characteristic of the polyvalent trypsin inhibitor of the Kunitz type.

DISCUSSION

On the basis of the presented results, an attempt was made to calculate the content of ribonuclease and inhibitors in bovine and porcine pancreas (Table 3). Concomitantly with the greater amount of protein material extracted with TCA from ox pancreas, the amount of ribonuclease calculated per 1 kg of the tissue was twice as large as in pig pancreas. The amount of the trypsin inhibitor was the same (about 20 mg/kg) but porcine pancreas contained only the trypsin-specific inhibitor whereas in bovine pancreas both the Kazal and Kunitz inhibitors were present.

Table 3

Content of ribonuclease and polypeptide trypsin inhibitors in bovine and porcine pancreas, calculated per 1 kg of fresh tissue weight

The results of a typical experiment are given.

	Ox pancreas		Pig pancreas	
	mg protein/kg	%	mg protein/kg	%
Trichloroacetic acid--soluble polypeptides	1500	100	750	100
Ribonuclease	700	46.6	333	44.4
Trypsin inhibitors	19.6	1.3	20.4	2.7
Kazal inhibitor	11.0		20.4	
Kunitz inhibitor	8.6		0.0	
Ballast protein	780	52.0	397	52.9

The observed heterogeneity of the ribonuclease and Kazal inhibitor preparations obtained from porcine pancreas could be due to autolysis occurring after death of the animal, prior to TCA-extraction. The resulting partial degradation of the studied proteins by lysosomal proteases did not affect, however, their active sites.

In bovine pancreas the action of lysosomal proteases may have been prevented by the polyvalent inhibitor of the Kunitz type, thus making possible isolation of undegraded ribonuclease and the two trypsin inhibitors, as demonstrated by homogeneity of the respective preparations on polyacrylamide-gel electrophoresis.

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RYBONUKLEAZA I POLIPEPTYDOWE INHIBITORY TRYPSYNY Z TRZUSTKI WOŁOWEJ I WIEPRZOWEJ

Streszczenie

Z wyciągów trójchlorooctowych trzustek wołowych otrzymano homogenne na żelu poliakryloamidowym preparaty rybonukleazy, inhibitora trypsyny typu Kazala i inhibitora typu Kunitza. Z trzustek wieprzowych otrzymano rybonukleazę i tylko jeden inhibitor (typu Kazala), przy czym preparaty te były heterogenne na żelu poliakryloamidowym. Wysunięto przypuszczenie, że heterogenność preparatów z trzustek wieprzowych spowodowana jest wewnątrzkomórkową autolizą, związaną z brakiem poliwalentnego inhibitora typu Kunitza.

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ALKYLATION OF CYTIDINE-5′-PHOSPHATE: MECHANISMS OF ALKYLATION, INFLUENCE OF *O*′-ALKYLATION ON SUSCEPTIBILITY OF PYRIMIDINE NUCLEOTIDES TO SOME NUCLEOLYTIC ENZYMES, AND SYNTHESIS OF 2′-*O*-ALKYL POLYNUCLEOTIDES*

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1) Dialkylsulphate alkylation of 5′-CMP in alkaline medium gave 2′(3′)-*O*-alkyl-5′--CMP and 2′,3′-di-*O*-alkyl-5′-CMP as major products, and three minor products identified as alkylphosphate esters of the foregoing, and of 5′-CMP itself. Under controlled conditions there was virtually no alkylation of the cytosine ring N_3, nor of the exocyclic amino group. 2) The mechanism of the alkylation reaction was investigated. The differences in reactivity of the ribose hydroxyls, the phosphate group and the cytosine ring N_3 were ascribed to the differences in tendency of these three nucleophilic centres to react according to the mechanism S_N1 or S_N2. 3) The isolated 2′(3′)-*O*-methyl-5′-CMP, or 2′(3′)-*O*-ethyl-5′-CMP, consisted of 85 - 90% of the biologically important 2′-isomer. The 2′(3′)-*O*-ethyl-5′-CMP was converted by nitrite deamination to 2′(3′)-*O*-ethyl-5′-UMP. All three 5′-monophosphates were converted to the 5′-pyrophosphates *via* the morpholidates. 4) The three foregoing 5′-pyrophosphates proved to be substrates for polynucleotide phosphorylase. The 10 - 15% of the 3′-isomers only slightly inhibited polymerization; furthermore, they did not act as chain terminators. 5) 2′(3′)-*O*-Methyl-5′-CMP and 2′(3′)-*O*-ethyl-5′-CMP were dephosphorylated by snake venom 5′-nucleotidase, but at much slower rates than 5′-CMP; while 2′,3′-di-*O*-methyl-5′-CMP was relatively resistant. The methyl esters of *O*′-methyl derivatives of 5′-CMP were also substrates for the venom phosphodiesterase, albeit poorer ones than the methyl ester of 5′-CMP. 6) The relative levels of 5′-nucleotidase activity of six different snake venoms were qualitatively estimated against 5′-CMP and 2′(3′)-*O*-methyl-5′-CMP as substrates.

Considerable progress has now been achieved in the development of methods for the preparation of *O*′-alkylated nucleosides (for review with leading references, see Kuśmierek *et al.*, 1973a). It is frequently desirable to convert such nucleosides to the corresponding nucleotides and this is usually done with the aid of one of the

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standard methods for chemical phosphorylation or, alternatively, by enzymic phosphorylation (Janion *et al.*, 1970). Such *O'*-alkylated nucleotides are of considerable biological interest, inasmuch as 2'-*O*-methyl nucleotides are found in various tRNA species (Hall, 1971), and 2'-*O*-ethyl nucleotides are found in the tRNA's of L-ethionine-induced hepatic carcinoma (Ortwerth & Novelli, 1969). *O'*-Alkyl nucleotides are also of general interest for studies of nucleotide conformation, the role of the 2'-hydroxyl in RNA conformation, enzyme specificity (Kole *et al.*, 1971), and enzymic synthesis of polynucleotides (Rottman & Henlein, 1968; Janion *et al.*, 1970; Żmudzka & Shugar, 1971; Gerard *et al.*, 1971; Khurshid *et al.*, 1972).

We report here in some detail on the products of alkylation of 5'-CMP[1], the preparation by deamination of the corresponding 5'-UMP analogues, the presumed mechanism of the alkylation reaction, and the utilization of the products for studies on the specificity of some enzymes, as well as the preparation of 2'-*O*-alkyl model polynucleotides.

During the course of this investigation, it was reported by Tazawa *et al.* (1972) that 2'-*O*-alkyl nucleoside-5'-phosphates could be prepared by alkylation of the nucleoside-3',5'-cyclic phosphates; the resulting 2'-*O*-alkyl nucleoside-3',5'-cyclic phosphate was then treated with cyclic phosphodiesterase to give the 2'-*O*-alkyl nucleoside-5'-phosphate. This procedure makes use of commercially available nucleoside-3',5'-cyclic phosphates, which are relatively expensive. In principle it is applicable to both purine and pyrimidine nucleoside-3',5'-cyclic phosphates and, from this point of view, is relatively advantageous. In practise, however, its application to pyrimidine nucleoside cyclic phosphates is extremely limited since, following alkylation of the 2'-hydroxyl, the 3',5'-cyclic phosphate ring is resistant to 3',5'-cyclic phosphodiesterase, while chemical hydrolysis gives, as the major product, the nucleoside-3'-phosphate.

RESULTS AND DISCUSSION

Alkylation of 5'-CMP. Treatment of 0.015 M-5'-CMP with dimethylsulphate in strongly alkaline medium led to formation principally of *O'*-methylated products. Paper chromatography (solvent *A*, see Table 1) of the reaction mixture (see Experimental) demonstrated the presence of 6 u.v. absorbing spots: A_m, 25%; B_m, 35%; C_m, 31%; D_m, 3%; E_m, 3%; F_m, 3%. All of these exhibited u.v. spectra at various pH values virtually identical with that for 5'-CMP, indicating the absence of methylation of the ring N_3. The products were identified, by means of chromatography and by enzymic tests (see below), as: A_m, unreacted 5'-CMP; B_m, 2'(3')-*O*-Me-5'-CMP, with the ratio of the 2' isomer to the 3' of 88:12; C_m, 2',3'-di-*O*-Me-5'-CMP. D_m, E_m and F_m proved to be the methyl esters of A_m, B_m and C_m, respectively[2].

[1] The following are illustrative of the abbreviations employed: 2'-*O*-MeC, 2'-*O*-methylcytidine; 2'(3')-*O*-Me-5'-CMP, 2'(3')-*O*-methylcytidine-5'-phosphate; 2',3'-di-*O*-Me-5'-CMP, 2',3'-di-*O*-methylcytidine-5'-phosphate; 2'(3')-*O*-Et-5'-UDP, 2'(3')-*O*-ethyluridine-5'-pyrophosphate; poly(2'-OMeC), poly 2'-*O*-methylcytidylic acid.

[2] The corresponding ethylated products are denoted as A_e, B_e, etc.

Table 1

Paper chromatography of relevant compounds

Ascending chromatography was used with Whatman paper no. 1 and the following solvent systems (all proportions v/v): (*A*) ethanol - 0.5 M-ammonium acetate, 5:2; (*B*) isopropanol - water - conc. NH_4OH, 7:2:1; (*C*) water - saturated *n*-butanol.

Compound	R_F value with solvent		
	A	*B*	*C*
Cytidine	0.60	0.43	0.10
2′-*O*-MeC	0.70	0.61	0.27
2′,3′-Di-*O*-MeC	0.82	0.79	0.48
2′(3′)-*O*-EtC	0.78	0.78	0.43
5′-CMP (A_m, A_e)	0.08	0.08	0.00
2′(3′)-CMP	0.11	0.13	0.00
2′(3′)-*O*-Me-5′-CMP (B_m)	0.16	0.11	0.00
2′,3′-Di-*O*-Me-5′-CMP (C_m)	0.27	0.15	0.00
5′-CMP, methyl ester (D_m)	0.37	0.21	0.04
2′(3′)-*O*-Me-5′-CMP, methyl ester (E_m)	0.54	0.35	0.04
2′,3′-Di-*O*-Me-5′-CMP, methyl ester (F_m)	0.69	0.56	0.04
2′(3′)-*O*-Et-5′-CMP (B_e)	0.18	0.17	—
2′,3′-Di-*O*-Et-5′-CMP (C_e)	0.30	—	—
5′-CMP, ethyl ester (D_e)	0.44	—	—
2′(3′)-*O*-Et-5′-CMP, ethyl ester (E_e)	0.60	—	—
2′,3′-Di-*O*-Et-5′-CMP, ethyl ester (F_e)	0.78	—	—
2′(3′)-*O*-Et-5′-UMP	0.31	—	—
2′(3′)-*O*-Me-5′-CMP, morpholidate	0.59	—	—
2′(3′)-*O*-Et-5′-CMP, morpholidate	0.64	—	—
2′(3′)-*O*-Et-5′-UMP, morpholidate	0.80	—	—
2′(3′)-*O*-Me-5′-CDP	0.08	—	—
2′(3′)-*O*-Et-5′-CDP	0.10	—	—
2′(3′)-*O*-Et-5′-UDP	0.13	—	—

Fully analogous results were obtained by treatment of 5′-CMP with diethylsulphate under the same conditions. Formation of triesters under these conditions is excluded because of the known alkali lability of phosphate triesters.

The problem of the relative resistance of the cytosine ring N_3 in 1-substituted cytosines to alkylation by dialkylsulphate in strongly alkaline medium has been previously discussed (Kuśmierek & Shugar, 1971). The alkylation of the pentose hydroxyls of nucleosides under these conditions is due to ionization of the hydroxyls, facilitating their attack by the alkylating reagent, and has been more extensively discussed in connection with alkylation of derivatives of cytidine (Kuśmierek *et al.*, 1973a) and 1-β-D-arabinofuranosylcytosine (Darżynkiewicz *et al.*, 1972; Giziewicz & Shugar, 1973).

Somewhat surprising in the present instance was the low yield of methyl esters (~9%) amongst the products of 5′-CMP alkylation. Previously reported methylation of 5′-CMP with dimethylsulphate at pH 5 - 7 gave a more than 30% yield of methyl esters (Haines *et al.*, 1964).

Methylation of the phosphate group of nucleotides by dimethylsulphate has been discussed by Griffin & Reese (1963). Methylation of 5′-AMP at pH~7 led to formation of about 50% products which were methyl esters, whereas at pH 4.5 only traces of such products were detectable. The marked decrease at the lower pH was interpreted by the authors as due to the decreased nucleophilicity of the phosphate monoanion as compared to the dianion, since the pK values of the phosphate of nucleotides are about 1 and 6, so that at pH 7 the phosphate is in the form of the dianion and at pH 4 as the monoanion.

In the case of 5′-CMP methylation under our conditions (pH > 13), the phosphate group is fully ionized and, in the light of the conclusions of Griffin & Reese (1963), one would have anticipated a much higher yield of methyl phosphate esters than the 9% actually observed. The foregoing unexpected finding led to the design of several additional experiments.

Possible lability under our strongly alkaline conditions of phosphate esters may be excluded, since monoalkyl esters of 5′-nucleotides, like diesters of phosphoric acid, are known to be stable in alkali (Kosolapoff, 1950; Szer & Shugar, 1961). Alkaline hydrolysis does occur in the case of esters of 2′(3′)-nucleotides due to transphosphorylation with concomitant formation of a triester (Brown *et al.*, 1955). Furthermore, we have found that the methyl ester of 2′,3′-di-*O*-Me-5′-CMP is stable in strongly alkaline medium.

Consideration was then given to the possibility that the observed phenomenon is the result of competition for the alkylating reagent by other, stronger nucleophilic centres such as the ionized ribose hydroxyls. A qualitatively similar interpretation may be drawn from an analysis of the data of Lawley (1957) on the methylation of deoxynucleotides with dimethylsulphate at pH 7. The greater the susceptibility to alkylation of the nitrogenous base, the lower the degree of reactivity of the phosphate group, e.g. with dGMP, where the aglycon was the most reactive amongst the nucleotides investigated, no esterification of the phosphate group was observed.

Koskikallio (1972) investigated the nucleophilic reactivity of the anions of acids of medium and weak strengths in aqueous medium relative to the methyl esters of strong acids (such as $MeClO_4$, $MeNO_3$, $MeSO_3Ph$). A linear correlation was observed between the logarithm of the reaction rate constant and the pK_a of the conjugate acid with a given anion, i.e. $\log k_N = a \cdot pK_a + b$. The coefficients a and b are both constant for a group of nucleophiles with the same atom in the nucleophilic centre and, for all practical purposes, are independent of the alkylating agents studied. On the assumption that dimethylsulphate, as the methyl ester of a strong acid, does not fundamentally differ from the esters investigated by Koskikallio (1972), the reactivity of the ionized *cis*-hydroxyl groups of ribose, with a pK_a about 12.5 (Fox & Shugar, 1952), should be at least 10-fold higher than that of the phosphate group, pK_a~6.

With a view to testing the proposal regarding the competitive reactivity of the ribose hydroxyls, a model compound was selected in which the hydroxyl groups were blocked, 2′,3′-di-*O*-Me-5′-CMP. Unexpectedly the yield of methyl ester in

this case did not exceed 13%, hence not significantly different from the 9% observed for 5′-CMP alone.

Some additional information about the mechanism of 5′-CMP alkylation was then sought by increasing the substrate concentration 10-fold, i.e. 0.15 M-5′-CMP. Treatment of such a solution with a 10-fold lower quantity of dimethylsulphate than in the case of 0.015 M-5′-CMP resulted in 100% conversion of the substrate to products. Chromatography (solvent system *A*) demonstrated formation of a larger number of products than in the case of alkylation of 0.015 M-5′-CMP. The intensities of the spots corresponding to D_m, E_m and F_m were comparable to those of B_m and C_m. Spectral analyses pointed to the presence of ring N_3 alkylated products both in isolated spots and as contaminants in spots consisting largely of products not substituted on N_3. A more accurate analysis was therefore conducted on a sample reaction mixture for which the extent of conversion of 5′-CMP to products was similar to that for reaction of 0.015 M-5′-CMP, i.e. about 75% (see Experimental). The reaction mixture was treated with bacterial alkaline phosphatase and chromatographed on paper with water-saturated butanol (solvent *C*). Only those products with free phosphate hydroxyls underwent enzymic dephosphorylation (see below); furthermore, with aqueous butanol as solvent, the methyl esters remained at the start (see Table 1). This procedure made it possible to establish the overall yield of methyl esters and to obtain a clearer picture of the nature of the other products.

Chromatography as above showed the following distribution of products: methyl esters, 22%; cytidine, 26%; 2′(3′)-*O*-methylcytidine, 33%; 2′,3′-di-*O*-methylcytidine, 12%. An additional spot (R_F=0.18, 7%) exhibited a spectrum characteristic for 1,3-disubstituted cytosine. The latter was also found as a contaminant of the methyl ester fraction. It is therefore clear that alkylation of the more concentrated solution, under conditions where the extent of transformation of 5′-CMP to products was the same as for the dilute solution (75%), led to a marked increase in phosphate esterification and N_3 alkylation.

The foregoing suggested the following mechanism for methylation (or ethylation) of 5′-CMP: Three types of nucleophilic centres are involved, the ionized ribose hydroxyls (RO^-), the ionized phosphate group ($ROPO_3^{2-}$) and the cytosine ring N_3 all react variously with the electrophilic centre of dimethylsulphate in the transition state. The participation of the nucleophile in formation of the transition state increases directly in the order RO^-, $ROPO_3^{2-}$ and N_3. The reaction takes on, to an increasing extent, the character S_N2, and to a lesser extent the character S_N1. This is supported by the fact that increase in 5′-CMP concentration, i.e. an increase in the probability of intermolecular reactivity, leads to an increase in the yield of methyl esters and ring N_3 alkylated products.

The reactivity of a nucleophile, according to reaction schemes S_N1 and S_N2, is dependent on its electronic structure. Strongly basic and weakly polarized nucleophiles, the nucleophilic centres of which are highly electronegative atoms with a large negative charge (i.e. "hard" nucleophiles) are known to react preferentially according to the scheme S_N1. By contrast, nucleophiles with contrasting properties ("soft" nucleophiles) react preferentially according to S_N2. More precisely, the character

of the transition state is dependent on the electronic structures of both the nucleophilic and electrophilic reagents. In the present instance we are dealing with the reactivities of three nucleophilic centres with respect to the same electrophilic reagent, dimethylsulphate (or diethylsulphate) (Edwards & Pearson, 1962; Pearson & Songstad, 1967; Klopman, 1968).

A qualitative evaluation of the "hardness" of the nucleophilic centres of 5′-CMP on the basis of the foregoing criteria provides results in agreement with experimental data: the sequence in the "hardness" of the centres involved ($RO^- > ROPO_3^{2-} > N_3$) is in the direction expected for the relative tendency of these centres to react according to S_N1. The influence of the foregoing factors on the reactivities of the nucleophilic centres of 5′-CMP are undoubtedly more significant than simple competition between these centres for the alkylating reagent. More extensive studies on the nature of the foregoing reaction mechanism are under way.

Enzymic hydrolysis and dephosphorylation of alkylated products. The alkylated products of 5′-CMP (B_m, C_m, D_m, E_m, F_m and B_e) were subjected to enzymic dephosphorylation in order to establish their structures, using non-specific bacterial alkaline phosphatase, and *Vipera ammodytes* venom as a source of 5′-nucleotidase and phosphodiesterase. This venom is contaminated with only traces of non-specific phosphatase.

The bacterial phosphatase readily dephosphorylated B_m and C_m to give products identified chromatographically (on paper) as 2′-*O*-methylcytidine and 2′,3′-di-*O*--methylcytidine, respectively. The product of dephosphorylation of B_m was further shown, by chromatography on Dowex OH^- (see Fig. 1) to consist of a mixture

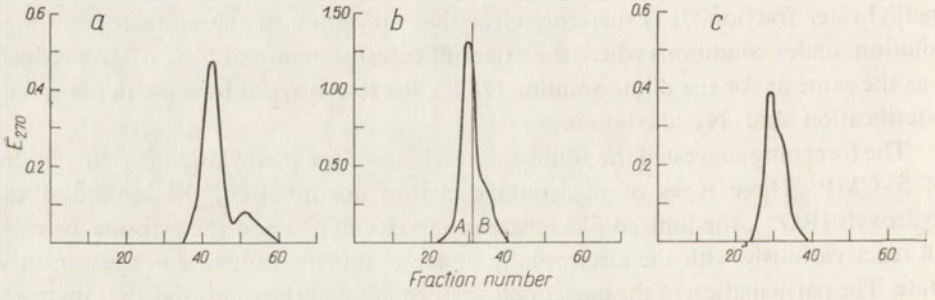

Fig. 1. Chromatography of 2′(3′)-*O*-alkylcytidines on Dowex OH^-, 1X2, 200/400. Column dimensions 22×1.25 cm. Elution with 30% aqueous methanol, with collection of 2.5 ml fractions at intervals of 3 min: (*a*) Fractionation of products of dephosphorylation of 2′(3′)-*O*-methyl-5′-CMP; (*b*) Fractionation of products of dephosphorylation of 2′(3′)-ethyl-5′-CMP; (*c*) Rechromatography of pooled fractions *B* from (*b*).

of 2′-*O*-methylcytidine and 3′-*O*-methylcytidine in the ratio 88:12. D_m, E_m and F_m were resistant to the bacterial enzyme and were consequently considered to be methyl phosphate esters, and are discussed separately, below.

The viper venom readily dephosphorylated B_m to give 2′(3′)-*O*-methylcytidine; that dephosphorylation in this instance was due to 5′-nucleotidase is testified to by

the fact that, during the 3 h required for 90% dephosphorylation, 2′(3′)-CMP was unaffected and 24 h incubation were required to reveal trace dephosphorylation of the latter compound. It had been previously noted by Janion *et al.* (1970) that *Vipera ammodytes* venom dephosphorylated 2′-*O*-methyl-CMP, although at a slower rate than for 5′-CMP. In the present instance the complete dephosphorylation of B_m shows that 3′-*O*-methyl-5′-CMP is also a substrate for 5′-nucleotidase. By contrast C_m, which was shown to be 2′,3′-di-*O*-methyl-5′-CMP, since it was converted by alkaline phosphatase to 2′,3′-di-*O*-methylcytidine, proved virtually resistant to the venom 5′-nucleotidase.

Dephosphorylation of the products D_m, E_m and F_m, which are resistant to alkaline phosphatase (which indicates that they are methyl phosphate esters), must involve the combined action of the venom phosphodiesterase and 5′-nucleotidase. The hydrolysis of nucleotide 5′-methyl esters by venom phosphodiesterase has been previously reported (Szer & Shugar, 1961). Hydrolysis and dephosphorylation of D_m gave only one product, cytidine, whereas E_m was transformed under similar conditions to a product chromatographically identical with 2′-*O*-methylcytidine. However, enzymic conversion of E_m was slower than for D_m and was incomplete after 24 h incubation. The product F_m was also hydrolysed slowly by the snake venom phosphodiesterase to 2′,3′-di-*O*-Me-5′-CMP (identified by chromatography with product C_m), but this, in turn, proved relatively resistant to the venom 5′-nucleotidase, since 24 h incubation led to the appearance of only traces of 2′,3′-di-*O*-methylcyti-

Table 2

Final products of hydrolysis by Vipera ammodytes *venom of the various products of alkylation of 5′-CMP*

Results are expressed in terms of % dephosphorylation (which, in the case of the methyl phosphate esters D_m, E_m and F_m must be preceded by phosphodiesterase hydrolysis of the esters) and chromatographic identification of the final product. For further details, see Results and Discussion.

Substrate	Approximate % dephosphorylation after incubation for			Final product of dephosphorylation
	1 h	3 h	24 h	
5′-CMP	100	100	100	cytidine
5′-CMP*	100	100	100	cytidine
2′(3′)-CMP	0	0	traces	cytidine
B_m	70	90	100	2′(3′)-*O*-methylcytidine
B_m*	0	traces	80	2′(3′)-*O*-methylcytidine
B_e	10	—	100**	2′(3′)-*O*-ethylcytidine
C_m	—	0	traces	2′,3′-di-*O*-methylcytidine
D_m	—	—	100	cytidine
E_m	—	—	90	2′(3′)-*O*-methylcytidine
F_m	—	—	traces	2′,3′-di-*O*-methylcytidine

* No $MgCl_2$ in incubation medium.
** 30 h incubation.

dine. It is even likely that 2′,3′-di-*O*-Me-5′-CMP is fully resistant to the 5′-nucleotidase, the trace dephosphorylation observed being then due to the traces of non-specific phosphatase present in the venom (see Table 2).

Summing up, the enzymic tests show that the methylation products of 5′-CMP, i.e. B_m, C_m, D_m, E_m, F_m are, respectively, the 5′-phosphates of 2′(3′)-*O*-methylcytidine and 2′,3′-di-*O*-methylcytidine, and the 5′-methylphosphates of cytidine, 2′(3′)-*O*-methylcytidine and 2′,3′-di-*O*-methylcytidine.

For the ethylated products of 5′-CMP, only B_e was subjected to analysis, since the remaining products appeared to be analogous to those formed by methylation, and were not required for further study. The product B_e was converted by the bacterial phosphatase to a mixture of 2′ and 3′-*O*-ethylcytidine (see below). B_e was also dephosphorylated by *Vipera ammodytes* venom, but at a considerably lower rate than B_m.

5′-Nucleotidase levels in different snake venoms. The availability of six snake venoms from various sources suggested the utility of comparing the levels of 5′-nucleotidase and non-specific phosphatase levels in these venoms. Such data should prove useful in assessing the value of a particular venom either as a source of 5′-nucleotidase activity in enzymic studies, or as starting material for isolation and purification of this enzyme. A study of the levels of 5′-nucleotidase, non-specific phosphatase, phosphodiesterase and endonuclease levels in several venoms has been reported by Richards *et al.* (1965).

5′-Nucleotidase activity was measured against 5′-CMP and 2′(3′)-*O*-Me-5′-CMP as substrates, and non-specific phosphatase activity with the use of 2′(3′)-CMP. Because of the low level of the latter enzyme in all the venoms herein examined, a 100-fold higher concentration of venom was employed with 2′(3′)-CMP as substrate, as compared with 5′-CMP. Furthermore, because of the lower susceptibility to 5′-nucleotidase of 2′(3′)-*O*-Me-5′-CMP relative to 5′-CMP, the venom concentration employed was also 100-fold greater. The results are shown in Table 3. It will be noted from the table that the levels of activity against 5′-CMP are approximately in the same order as for 2′(3′)-*O*-Me-5′-CMP. The one exception is *Vipera ammodytes*, which is apparently more active against 2′(3′)-*O*-Me-5′-CMP than might be expected from its activity against 5′-CMP. Particularly noteworthy is the high level of 5′-nucleotidase activity of *Vipera russelii* as compared to the other venoms. It is, of course, possible that these differences are due, in part, to the methods employed for isolation and drying of the various venoms, and to the method of storage. For several snake venoms examined by Richards *et al.* (1965), the range in levels of 5′-nucleotidase activity was somewhat less than that reported here. The very low non-specific phosphatase levels for all the venoms (bearing in mind the use of a 100-fold higher concentration of venom in this case) points to the value of these venoms as good sources of 5′-nucleotidase. An apparent exception to this is *Naja nigricollis* for which Richards *et al.* (1965) found a level of non-specific phosphatase activity of the order of 20% that for 5′-nucleotidase.

The present results are in agreement with the previous report of Janion *et al.* (1970) on the dephosphorylation of 2′-*O*-Me-5′-CMP by snake venom 5′-nucleo-

Table 3

Relative levels of 5′-nucleotidase activity of various dried snake venoms, measured against 5′-CMP and 2′(3′)-O-methyl-5′-CMP as substrates; and of non-specific monophosphoesterase activity, measured against 2′(3′)-CMP as substrate

Venom	% dephosphorylation of		
	5′-CMP	2′(3′)-*O*-Me-5′-CMP	2′(3′)-CMP
Vipera russelii	100	100	traces
Vipera ammodytes	34*	88**	traces
Crotalus terrificus	57	77	0
Agkistrodon piscivorus	57	47	0
Crotalus adamanteus	47	41	0
Bothrops atrox	16	17	0

* In the absence of Mg^{2+} in the incubation medium, there was no detectable dephosphorylation after 3 h incubation, and only about 20% after 24 h.

** In the absence of Mg^{2+}, trace dephosphorylation after 3 h and about 80% after 24 h.

tidase. It has however, been reported by Honjo *et al.* (1964) that 2′-*O*-methylnucleoside-5′-phosphates, isolated from yeast RNA, are completely resistant to snake venom 5′-nucleotidase. It is rather difficult to comment on these findings, since the authors give no details regarding conditions employed. Hudson *et al.* (1965) also reported resistance of 2′-*O*-methylnucleoside-5′-phosphates to *Vipera russelii* venom, during hydrolysis of RNA containing 2′-*O*-methyl residues, in apparent disagreement with our results (Tables 2 and 3). However, an examination of the conditions used by these authors shows that the incubation medium did not contain Mg^{2+} ions, which are necessary for 5′-nucleotidase activity.

With a view to testing the validity of our findings relative to those of Hudson *et al.* (1965), a qualitative examination was made of the relative rates of dephosphorylation by snake venom 5′-nucleotidase of 5′-CMP and 2′(3′)-*O*-methyl-5′-CMP, in the presence and absence of Mg^{2+} ions. The results, exhibited in Tables 2 and 3, demonstrate clearly a very marked decrease in the rates of dephosphorylation in the absence of Mg^{2+}.

2′(3′)-O-Alkyl ribonucleoside 5′-pyrophosphates as substrates for polynucleotide phosphorylase. Considerable attention has been devoted, during the past 3 years, to the synthesis of polynucleotides in which the ribose 2′-OH ha s been modified in some way. Interest in such polynucleotides stems in large part from the desire to elucidate the role of the 2′-hydroxyl in the differing conformations of RNA and DNA. Such model polymers are also of considerable value for biological investigations, e.g. in studies of *in vitro* translation, interferon induction, etc.

The procedure most readily applicable to the preparation of such model polynucleotides is polymerization of the 5′-pyrophosphates with the aid of polynucleotide phosphorylase, which has been shown to exhibit an extraordinary lack of substrate specificity, thus allowing for a wide range of modified substrates.

2′-*O*-Methylcytidine-5′-pyrophosphate and 2′-*O*-methyluridine-5′-pyrophosphate have already been shown to be suitable substrates for polynucleotide phosphorylase,

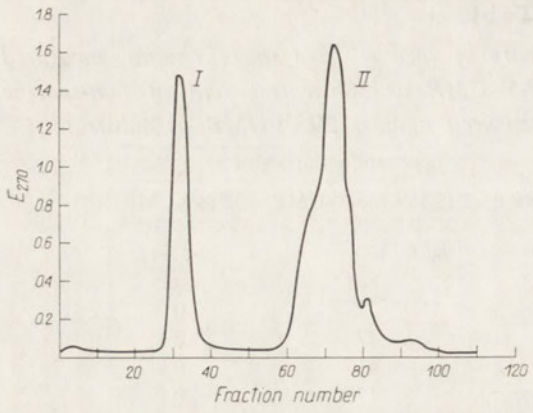

Fig. 2. Typical elution profile of incubation mixture of 2′(3′)-*O*-methyl-5′-CDP with polynucleotide phosphorylase, on a 65×2 cm column of Sephadex G-50 (fine), with 0.1 M-triethylammonium bicarbonate as eluent, and collection of 3-ml fractions at 5-minute intervals: (*I*) polymer; (*II*) unreacted monomer.

the resultant products being poly(2′-OMeC) and poly(2′-OMeU) (Janion *et al.*, 1970; Żmudzka & Shugar, 1971). In these instances synthesis of the substrates was achieved by phosphorylation of 2′-*O*-methylcytidine and conversion of the 5′-phosphate, *via* the morpholidate, to the 5′-pyrophosphate; the corresponding 2′-*O*-methyluridine-5′-pyrophosphate was obtained by deamination of 2′-*O*-cytidine-5′-phosphate to 2′-*O*-methyluridine-5′-phosphate and conversion of the latter to the 5′-pyrophosphate (Janion *et al.*, 1970; Żmudzka & Shugar, 1971).

The demonstration in the present study that 2′-*O*-methylcytidine-5′-phosphate may be obtained directly from inexpensive commercially available 5′-CMP offers an alternative and simpler pathway to substrate synthesis. The same procedure is also applicable to the 2′-*O*-ethyl derivative of 5′-CMP, as well as to a wide variety of cytosine nucleosides with substituents on the 5-position of the cytosine ring (such as 5-methyl, etc.) or on the exocyclic amino group.

One possible disadvantage to the foregoing procedure is that alkylation under our conditions of 5′-CMP gives a mixture of the 2′-*O*-alkyl and 3′-*O*-alkyl isomers which has hitherto defied attempts at separation by chromatographic methods. It was consequently necessary to establish whether the 3′-*O*-methyl isomer (present in the mixture to the extent of about 10%) interfered in the polymerization reaction or was able to act as a chain terminator.

Polymerization trials were therefore run with 2′(3′)-*O*-methylcytidine-5′-pyrophosphate as substrate and the results compared with those previously reported for 2′-*O*-methylcytidine-5′-pyrophosphate (Janion *et al.*, 1970). It was, in fact, found that 2′(3′)-*O*-Me-5′-CDP gave a polymer which was reasonably homogeneous and with a sedimentation coefficient as high as that obtained by polymerization of 2′-*O*-Me-5′-CDP. However, the overall yield of polymer product was only about 36% as compared to a maximum of about 60% for the polymer from 2′-*O*-Me-5′-CDP (Janion *et al.*, 1970). It is consequently possible that the 12% 3′-*O*-Me-5′-CDP present in 2′(3′)-*O*-Me-5′-CDP inhibits the polymerization reaction to some extent.

Another possibility which must be envisaged is that the 3′-isomer may undergo incorporation, in which case it would act as a chain terminator. This is ruled out

by the high sedimentation constant of the polymer product, and its relative homogeneity in the ultracentrifuge, as well as by an analysis of the incubation mixture during polymerization, which demonstrated preferential incorporation of the 2′-isomer. These findings are in agreement with those of Maeckey & Gilham (1971), who found that the 2′-isomer in a mixture of 2′(3′)-*O*-(α-methoxyethyl)-adenosine-5′-pyrophosphate was the preferred substrate in the addition, by means of polynucleotide phosphorylase, of an adenylate residue to a trinucleotide.

The foregoing results do not, of course, fully exclude the possibility that the 3′-isomer acts to a small extent as a chain terminator. Complete elimination of such an eventuality would require the use of a mixture of labelled 3′-*O*-Me-5′-CDP and unlabelled 2′-*O*-Me-5′-CDP as substrate.

Initial attempts to polymerize 2′(3′)-*O*-Et-5′-CDP under conditions analogous to those for polymerization of 2′(3′)-*O*-Me-5′-CDP were unsuccessful. Changes in pH of incubation, substrate concentration, ratio of substrate to Mn^{2+} cations, as well as the use of Mg^{2+} and Ca^{2+} cations, were without effect. Attention was then directed to 2′(3′)-*O*-Et-5′-UDP, bearing in mind the occasional specificity of polynucleotide phosphorylase with respect to the base residue (Beers, 1957). Qualitative trials with fraction III of the *M. luteus* enzyme (Matthaei *et al.*, 1967), as well as with the enzyme from *E. coli* (Williams & Grunberg-Manago, 1964), were positive, the *E. coli* enzyme being considerably more effective, and polymerization rates were also increased by reducing the ratio of Mn^{2+} to substrate to 1:2.2 from the 1:0.8 ratio used with 2′(3′)-*O*-Me-5′-CDP.

The resulting poly(2′-*O*-EtU), the preparation and properties of which are elsewhere described (Kuśmierek *et al.*, 1973b), readily formed a helical structure with much more pronounced stability than poly(2′-OMeU) (Żmudzka & Shugar, 1971), in accord with the enhanced stability of poly(2′-OEtA) relative to poly(2′-OMeA) (Khurshid *et al.*, 1972).

On the basis of the foregoing, further attempts to polymerize 2′(3′)-*O*-Et-5′-CDP, with the conditions employed for polymerization of 2′(3′)-*O*-Et-5′-UDP (see Experimental) were undertaken and demonstrated reasonably good polymerization yields. The preparation and a study of the properties of poly(2′-OEtC) are therefore under way and will be reported elsewhere.

It is of interest that a 2′-*O*-ethyl (or 2′-*O*-methyl) substituent only partially sterically hinders the activity of polynucleotide phosphorylase, as is also evident from reports on polymerization of 2′-*O*-Et-5′-ADP (Khurshid *et al.*, 1972; Tazawa *et al.*, 1972). Bulkier 2′(3′)-*O* substituents, such as α-methoxyethyl (Maeckey & Gilham, 1971) or acyl (Kaufman *et al.*, 1971), have a much more profound effect, viz. enzymic addition of only one residue of the 5′-pyrophosphate to the 3′-OH of an oligonucleotide primer, subsequent addition of a second residue being virtually non-existent. Such "substrates" have been employed by the foregoing authors for the enzymic stepwise synthesis of oligonucleotides of defined sequence by the hydrolytic removal of the 2′(3′)-*O* substituent following each addition of a residue to the oligonucleotide chain.

EXPERIMENTAL

5′-CMP and 2′(3′)-CMP were products of Waldhof (Mannheim, G.F.R.). *E. coli* alkaline phosphatase BAPSF 9IC, 200 units/ml, was obtained from Worthington (Freehold, N. J., U.S.A.). *E. coli* polynucleotide phosphorylase (Williams & Grunberg-Manago, 1964) was a gift from Dr. Marianne Grunberg-Manago, and the *M. luteus* (Matthaei *et al.*, 1967) enzyme was kindly provided by Dr. H. Matthaei.

Vipera russelii venom was a gift from Dr. P. M. Bhargava of the Regional Research Laboratory, Hyderabad (India). The *Vipera ammodytes* venom was provided through the courtesy of the Institute Ruder Boskovic, Zagreb (Yugoslavia). The remaining four venoms (see Table 3) were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Ascending chromatography made use of Whatman papers nos. 1 and 3, and Eastman (Rochester, N. Y., U.S.A.) thin-layer 6065 cellulose sheets.

U.v. absorption spectra were run on a Zeiss (Jena, D.D.R.) VSU-2 spectrophotometer. Sedimentation constants of polymers were obtained with a MOM (Budapest, Hungary) ultracentrifuge fitted with u.v. optics.

Methylation of 0.015 M-*5′-CMP and isolation of 2′(3′)-*O*-methyl-5′-CMP*: To a vigorously stirred solution of 1.1 g (3 mmol) of Na_2-5′-CMP in 200 ml of 0.5 N-NaOH at room temperature was added, over a period of three hours, five 3-ml portions of dimethylsulphate (total 150 mmol), each of which was followed by 6 ml 10 N-NaOH. After several hours at room temperature, the reaction mixture was neutralized with 5 N-H_2SO_4. Paper chromatography with solvent *A* exhibited 6 products, the relative proportions of which were estimated by the $E_{270}^{pH\,12}$ values: A_m - 25%, B_m - 35%, C_m - 31%, D_m - 3%, E_m - 3%, F_m - 3%. Each exhibited u.v. spectra at various pH values similar to 5′-CMP. Samples were subjected to enzymic dephosphorylation, as described below.

The entire reaction mixture was deposited on Whatman paper no. 3 (0.7 mg/cm) and developed twice in the same direction with the solvent *A*. Band B_m was eluted with water. Dowex H^+ was added to the eluate to acid reaction, and the resin filtered off and washed with water. The combined filtrates contained 0.91 mmol (7 700 $E_{270}^{pH\,12}$ units) of 2′(3′)-*O*-methyl-5′-CMP (30% yield). The solution was brought to dryness under reduced pressure, dried under vacuum over KOH, and employed in this form for subsequent reactions.

Ethylation of 0.015 M-*5′-CMP and isolation of 2′(3′)-*O*-ethyl-5′-CMP*: The procedure was analogous to that for methylation. To 3 mmol of Na_2-5′-CMP in 200 ml 0.5 N-NaOH was added, over a period of 15 h, 5 × 4 ml (total 160 mmol) of diethylsulphate, each portion being followed by 6 ml 10 N-NaOH. Yields of the various products were: A_e (35%), B_e (29%), C_e (19%), D_e (6%), E_e (6%), F_e (5%). The overall yield of isolated 2′(3′)-*O*-ethyl-5′-CMP was 0.7 mmol (23%).

Methylation of 0.15 M-*5′-CMP*: To a constantly stirred solution of 55 mg (0.15 mmol) Na_2-5′-CMP in 1 ml 0.5 N-NaOH was added, over a period of 2 h, 4 × 10 µl (0.4 mmol) dimethylsulphate. The reaction mixture was left for several hours at room temperature and then neutralized with H_2SO_4. A 40 µl aliquot was mixed

with an equal volume of 0.5 M-tris-HCl buffer, pH 8, and to this was added 2 µl of a solution of bacterial phosphatase, followed by incubation for 20 h at 37°C. Chromatography with solvent *C* showed 5 u.v. absorbing spots with R_F values (and relative intensities based on $E_{280}^{pH\,2}$) as follows: $R_F = 0.05$ (22%, mixture of phosphate esters); $R_F = 0.10$ (26%, cytidine); $R_F = 0.18$ (7%, N_3-methylated product(s)); $R_F = 0.27$ (33%, 2′(3′)-*O*-methylcytidine); $R_F = 0.48$ (12%, 2′,3′-di-*O*-methylcytidine). The spot with $R_F = 0.05$ was contaminated with N_3-methylated products.

Methylation of 2′,3′-di-O-*methyl-5′-CMP*: To 130 $E_{270}^{pH\,12}$ units (0.015 mmol) of 2′,3′-di-*O*-Me-5′-CMP in 1 ml 0.5 N-NaOH was added, with stirring and over a period of 2 h, 4 × 20 µl (0.8 mmol) dimethylsulphate. Each portion of dimethylsulphate was followed immediately by addition of 40 µl 10 N-NaOH. Chromatography then showed the presence of one product (13%) identical in solvent systems *A* and *B* with the methyl ester of 2′,3′-di-*O*-Me-5′-CMP (product F_m in methylation of 5′-CMP). Prolongation of the reaction time to 24 h at 37°C was without further effect.

Deamination of 2′(3′)-O-*Et-5′-CMP*: 2700 $E_{270}^{pH\,12}$ units (0.32 mmol) of 2′(3′)-*O*-Et-5′-CMP and 700 mg $NaNO_2$ were dissolved in 2 ml of 25% acetic acid and themixture heated for 2 h at 55°C, following which starting product had disappeared. The mixture was taken up in 500 ml water and the solution brought to pH ~ 8.5 with conc. NH_4OH. The solution was then deposited on a 26 × 1.8 cm column of Dowex 1X2 (HCO_3^- form) 200/400, which was eluted with 1000 ml portions, successively, of water, then 0.05 M, 0.10 M, 0.15 M and 0.2 M-triethylammonium bicarbonate. The 0.10 M and 0.15 M eluates contained monovalent anions (absence of u.v. absorption at 260 nm, presence of u.v. absorption at 230 nm), while the 0.20 M eluate contained all the 2′(3′)-*O*-Et-5′-UMP in the form of the triethylammonium salt. The latter eluate was brought to dryness under vacuum, the residue evaporated several times from methanol and finally dissolved in a small volume of water. Some Dowex(H^+) resin was added to the aqueous solution to give a strongly acid reaction and the resin then removed by filtration and washed with water. The combined filtrates were brought to dryness to yield 0.25 mmol (based on $E_{260}^{pH\,2}$ units (80% yield)) of the free acid of 2′(3′)-*O*-Et-5′-UMP, chromatographically homogeneous and with spectral properties similar to those for UMP.

Enzymic dephosphorylation of 5′-CMP alkylation products. The alkylation products B_m,C_m,D_m,E_m,F_m and B_e, following isolation on Whatman paper no. 3 with solvent *A*, were rechromatographed on the same paper with solvent *B*. The appropriate spots were eluted and, following concentration, employed directly for enzymic studies.

a. Alkaline phosphatase. To a sample of 5 $E_{270}^{pH\,12}$ units of product in 100 µl of 0.2 M-tris-HCl buffer, pH 8.0, was added 1 µl of enzyme solution and incubation conducted at 37°C. Simultaneous controls were run with 5′-CMP, as well as with 2′(3′)-CMP. The course of dephosphorylation was followed by chromatography on Eastman (Rochester, N. Y.) 6065 cellulose plates with solvent *A*. Two hours incubation led to complete dephosphorylation of 5′-CMP, 2′(3′)-CMP, and products B_m, B_e and C_m. Products D_m, E_m and F_m were unaffected even after 24 h incubation. Chromatography with appropriate controls, using solvents *A*, *B* and *C* (Table 1),

showed that the products of dephosphorylation of 5′-CMP and 2′(3′)-CMP were cytidine, that of B_m 2′(3′)-*O*-methylcytidine, of B_e 2′(3′)-*O*-ethylcytidine and of C_m 2′,3′-di-*O*-methylcytidine.

b. Vipera ammodytes venom. To a sample of 5 $E_{270}^{pH\,12}$ units of alkylation product in 100 µl of 0.2 M-tris-HCl buffer, pH 9.0, was added 1 µmol $MgCl_2$ and 0.25 mg crude snake venom. Controls consisted of 5′-CMP and, to correct for possible traces of non-specific phosphatases, 2′(3′)-CMP. Incubations were at 37°C and aliquots for chromatography were removed at intervals of 1, 3 and 24 h. Chromatographic procedures were as in the preceding section (Table 1). The results of enzymic dephosphorylation are presented in Table 2.

5′-Nucleotidase activity of various snake venoms. Activities of several available venoms were compared, using the following incubation conditions: About 5 $E_{270}^{pH\,12}$ units of substrate and 1 µmol $MgCl_2$ in 100 µl of 0.2 M-tris-HCl buffer, pH 9.0. With 5′-CMP as substrate, the amount of venom added to the incubation mixture was 0.0025 mg and the incubation time was 3 h. With 2′(3′)-*O*-methyl-5′-CMP, the amount of venom employed was 0.25 mg and incubation time 3 h. With 2′(3′)--CMP, 0.25 mg venom was added and incubation prolonged to 24 h. Incubation temperature was 37°C in all cases. Following incubation, the mixtures were chromatographed with solvent system *A* and the extent of dephosphorylation estimated from the optical densities of the eluates of the nucleosides and nucleotides. The results are shown in Table 3.

Synthesis of 5′-pyrophosphates of 2′(3′)-O-*alkylnucleosides*: These were prepared from the 5′-monophosphates *via* the morpholidates according to standard procedures as described by Moffatt & Khorana (1961). The procedure involved some modifications and is described elsewhere (Janion *et al.*, 1970).

Ratio of 2′,3′ isomers in 2′(3′)-O-*alkyl-5′-CMP and -CDP*: To a solution of several mg nucleotide in 0.5 - 2.0 ml 0.2 M-tris-HCl buffer, pH 8.0, was added 1 - 2 µl of alkaline phosphatase. Samples were incubated at 37°C for 2 - 3 h (for monophosphates) or overnight (for pyrophosphates), and then subjected to preparative chromatography on Whatman paper no. 3, using the solvent *B*. The band corresponding to 2′(3′)-*O*-alkylcytidine (100% dephosphorylation) was eluted with water, the solution brought to dryness, and the residue taken up in about 1 ml of 30% aqueous methanol. This was deposited on a 22 × 1.25 cm column of Dowex 1X2 200/400 (OH^-) which had been previously equilibrated with 30% methanol. The nucleoside was then eluted with 30% methanol, the eluate being controlled spectrally by measurements at 270 nm. A typical elution pattern is shown in Fig. 1.

In the case of 2′(3′)-*O*-methyl-CMP and -CDP, the ratio of the 2′- to the 3′-isomer was 88:12 (Fig. 1a). For the *O*′-ethylated nucleosides, the column separation of isomers was markedly inferior (Fig. 1b). However, the asymmetry of the elution pattern suggests that the ratio of isomers is similar to that obtained with the *O*′-methylcytidines.

Polymerization of 2′(3′)-O-*methyl-5′-CDP*: The reaction mixture contained, in a volume of 1 ml, 5 mg (100 $E_{270}^{pH\,12}$ units, 12 µmol) of the sodium salt of 2′(3′)-*O*--methyl-5′-CDP; 200 µl of 0.5 M-tris-HCl buffer, pH 8.5; 100 µl of 0.01 M-NaN_3;

100 µl 0.005 M-Na-EDTA; 300 µl 0.05 M-$MnSO_4$; and 100 µl polynucleotide phosphorylase (*Micrococcus luteus*, fraction III of Matthaei *et al.*, 1967). The reaction was conducted for 3 days, its course being followed by chromatography on Eastman 6065 cellulose plates with the solvent system isopropanol - 1% acetic acid - 2.5% ammonium oxalate (3:2:1, by vol). With this system, the polymer formed was retained at the start, compounds of manganese were located at R_F 0.45, pyrophosphates at about 0.5, monophosphates at about 0.6. The reaction was terminated by immersion of the reaction tube for 2 - 3 min in boiling water. The reaction mixture was then brought to room temperature and centrifuged to remove oxides of manganese and denatured protein. The supernatant was examined by passage through a Sephadex G-50 column (see below), or following supplementary deproteinization by the standard phenol procedure and dialysis, once against 0.01 N-NaCl with 0.001 M-Na-EDTA, then twice against water.

In 0.1 M-NaCl with 0.05 M-tris-HCl buffer, pH 8, the neutral form of poly(2′-*O*-MeC) exhibited an s_{20} of 6.9 to 9.3 for various samples. For a preparation with $s_{20} = 6.9$, the acid form, in 0.1 M-NaCl with 0.05 M-acetate buffer, pH 4.4, showed an s_{20} of 10.2.

Analysis of polymerization mixture: The reaction mixture was centrifuged to remove manganous oxides and denatured protein (see above), the supernatant deposited on a column of Sephadex G-50 (fine) and the column eluted with 0.1 M-triethylammonium bicarbonate, with collection of 3-ml fractions at intervals of 5 - 6 min. A typical elution pattern, based on measurement of absorption at 270 nm, is shown in Fig. 2.

Calculations of polymer yield in this manner, on the assumption of previous determinations of 37% hyperchromicity (Janion *et al.*, 1970), gave values ranging from 34 to 37%. In one experiment involving the use of a twofold lower enzyme concentration, the overall polymer yield was only 12%.

The isolated monomer peak eluate was brought to dryness, the residue evaporated several times from methanol, dephosphorylated with alkaline phosphatase, and the ratio of the 2′ to the 3′ isomers determined by chromatography on Dowex (OH^-), as above. The results showed that, as the polymerization reaction proceeded, the ratio of the 3′ isomer to the 2′ isomer in the incubation medium increased from an initial value of 12% to about 20%, as would be anticipated if no incorporation of the 3′ isomer occurred.

Deamination of poly(2′-OMeC): 12.9 $E_{270}^{pH\,7}$ units (2 µmol) of poly(2′-OMeC), $s_{20} = 6.9$, was dissolved in 150 µl water, followed by addition of 50 µl glacial acetic acid and 40 mg $NaNO_2$. The mixture was allowed to react for 13 min at 80°C, brought to room temperature, and then dialysed successively against 0.1 M-NaCl with 0.01 M-EDTA, 0.01 M-NaCl with 0.001 M-EDTA, and finally twice against water. The final yield of deaminated polymer was 16.5 $E_{270}^{pH\,7}$ units, corresponding to a 97% yield of poly(2′-OMeU). Spectral data: λ_{max} 260 nm, λ_{min} 232 nm, $\varepsilon_{max}/\varepsilon_{min} = 3.23$. The s_{20} value for the polymer in 0.02 M-cacodylate buffer, pH 7, was 9.7.

Polymerization of 2′(3′)-O-ethyl-5′-UDP: Following a series of trial runs, polymerization conditions employed (not necessarily optimal) were as follows: Na salt

of 2′(3′)-*O*-ethyl-UDP, 11 μmol; tris buffer, pH 8.5, 150 μmol; $MnSO_4$, 5 μmol; NaN_3, 1 μmol; Na-EDTA, 0.5 μmol; *E. coli* polynucleotide phosphorylase (Williams & Grunberg-Manago, 1964), 25 μl; total volume of incubation mixture, 0.75 ml. Incubation was at 37°C and the polymerization reaction was followed by t.l.c. estimation of appearance of polymer, as described above for polymerization of 2′-*O*-Me-5′-CDP. Following 24 h incubation, about 50% substrate incorporation into polymer was noted. The reaction was terminated by heating to 100°C, the resulting precipitate of manganese and some protein centrifuged off, and the supernatant deproteinized by the phenol procedure. The polymer solution was then dialysed successively against 0.1 M-NaCl and 0.01 M-Na-EDTA, 0.01 M-NaCl and 0.001 M-Na-EDTA, and finally twice against water. The yield of isolated polymer was 21.5 E_{260} units (18°C) which, when corrected for hypochromicity, corresponded to about 30%. The s_{20} value for the polymer in 0.1 M-NaCl was 20.

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ALKILOWANIE CYTYDYNO-5′-FOSFORANU: MECHANIZM ALKILOWANIA, WPŁYW *O*′-ALKILOWANIA NA PODATNOŚĆ NUKLEOTYDÓW PIRYMIDYNOWYCH NA DZIAŁANIE NIEKTÓRYCH ENZYMÓW NUKLEOLITYCZNYCH ORAZ SYNTEZA 2′-*O*-ALKILOPOLINUKLEOTYDÓW

Streszczenie

1. Alkilowanie 5′-CMP siarczanami dwualkilowymi w środowisku alkalicznym daje 2′(3′)-*O*--alkilo-5′-CMP i 2′,3′-dwu-*O*-alkilo-5′-CMP jako główne produkty oraz trzy dodatkowe produkty zidentyfikowane jako estry alkilofosforanowe. W kontrolowanych warunkach nie zachodzi w zauważalnym stopniu alkilowanie atomu N_3 pierścienia cytozyny, jak również egzocyklicznej grupy aminowej.

2. Badano mechanizm reakcji alkilowania 5′-CMP. Różnice w reaktywności grup hydroksylowych rybozy, grupy fosforanowej i atomu N_3 pierścienia cytozyny przypisano różnicom w tendencji reagowania tych centrów nukleofilowych według mechanizmu S_N1 lub S_N2.

3. Wyodrębnione 2′(3′)-*O*-Me-5′-CMP i 2′(3′)-*O*-Et-5′-CMP zawierają 85 - 90% ważnego biologicznie izomeru. 2′(3′)-Et-5′-CMP przeprowadzono przez dezaminację azotynem w 2′(3′)-*O*--Et-5′-UMP. Wszystkie wymienione 5′-monofosforany przeprowadzono *via* pochodne morfolidowe w 5′-pirofosforany.

4. Stwierdzono, że otrzymane 2′(3′)-*O*-alkilo-5′-pirofosforany są substratami fosforylazy polinukleotydowej. 3′-Izomer obecny w mieszaninie w ilości 10 - 15% w niewielkim stopniu hamuje polimeryzację i nie działa jako terminator łańcucha.

5. 2′(3′)-*O*-Me-5′-CMP i 2′(3′)-*O*-Et-5′-CMP były defosforylowane przez 5′-nukleotydazę z jadu węża, jednakże o wiele wolniej niż 5′-CMP, natomiast 2′,3′-dwu-*O*-Me-5′-CMP był stosunkowo odporny. Estry metylowe *O*′-metylopochodnych 5′-CMP były również substratami fosfodwuesterazy zawartej w jadzie węża, jednakże gorszymi niż ester metylowy 5′-CMP.

6. Oszacowano względne aktywności 5′-nukleotydazy w sześciu różnych jadach węży wobec 5′-CMP oraz 2′(3′)-*O*-Me-5′-CMP jako substratów.

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CYTOPLASMIC FACTORS AFFECTING PROTEIN BIOSYNTHESIS IN HYPERTROPHIC RAT KIDNEY*

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1. Elongation factors were isolated from hypertrophic kidney 48 h after unilateral nephrectomy and from kidney of intact animals, and their activity in cell-free system was examined. 2. The incorporation of [^{14}C]Phe-tRNA into polyphenylalanine was more than doubled in the system containing elongation factors from hypertrophic kidney. 3. The experiments with the separated elongation factors showed that in hypertrophic kidney the activity of the aminoacyl-tRNA binding factor (EF_1) is enhanced, and that of the peptidyl-tRNA transfer factor (EF_2) remains unchanged.

In most mammals, unilateral nephrectomy leads to adaptive growth of the remaining kidney and restoration of lost renal function. The compensatory growth of the renoprival kidney starts within a few hours after the operation and continues until optimum size has been reached. It is still not clear, however, what are the mechanisms by which the renoprival kidney is capable of increased rate of protein biosynthesis, which is evidenced by the increasing protein content observed during compensatory renal hypertrophy (Halliburton, 1969). It should be noted that the enhanced synthesis of protein starts even before the rise in DNA synthesis (Goss & Dittmer, 1969).

The hypertrophic kidney is used in our work as a model system for studying factors involved in increased translation of genetic information. In our previous works (Sendecki *et al.*, 1972, 1973) we have found that unilateral nephrectomy causes an increase in the amount of heavier polyribosomes and in the activity of ribosomes in the process of translation.

The present experiments were undertaken to determine the activity of elongation factors (EF_1 and EF_2) in regulation of protein biosynthesis in normal and hypertrophic kidney.

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MATERIALS AND METHODS

Animals. Male rats (120 - 150 g) of the albino strain were maintained on standard diet and water *at libitum*. Nephrectomy was performed as described by Sendecki *et al.* (1972). At 48 h after the operation the animals were decapitated and kidney and liver were removed to ice-cold buffer. All further work was carried out in the cold (2 - 4°C). For each experiment, 10 operated and 5 control rats were used.

Chemicals and radioactive materials: L-[U-^{14}C]Phenylalanine (spec. act. 0.1 mCi/0.088 mg) was from the Institute of Radioisotopes (Prague, Czechoslovakia). [^{14}C]Phenylalanyl-tRNA was prepared from liver of intact animals by the method of Moldave (1963). Sephadex G-200 and Ficoll were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), *N*-ethylmaleimide from Calbiochem (San Diego, Calif., U.S.A.), cycloheximide (Actidione) from Carl Roth (Karlsruhe, G.F.R.). Other chemicals were obtained from the same sources as in the previous work (Sendecki *et al.*, 1973).

Analytical methods. Protein was determined according to Lowry *et al.* (1951) using bovine serum albumin as standard. In some experiments, protein concentration was also determined by the method of Warburg & Christian (1942). The two methods agreed within 5%. RNA, tRNA and poly U were determined spectrophotometrically as described previously (Sendecki *et al.*, 1973).

Kidney and liver ribosomes were obtained by the method of Moldave *et al.* (1971) and purified according to Moldave & Skogerson (1967) as described by Sendecki *et al.* (1973). Prior to the assays, the absorption of the ribosomal suspension was determined at 235, 260 and 280 nm. In the ribosome preparations used, the E_{260}/E_{280} ratio exceeded 1.7, and the E_{260}/E_{235} ratio exceeded 1.4 (Trachewsky *et al.*, 1972).

Preparation of elongation factors. Partially purified preparation of EF_1 and EF_2 (precipitate at 0.3 - 0.7 ammonium sulphate sat. of the pH 5 supernatant) was obtained from control and hypertrophic kidney according to Moldave *et al.* (1971). For resolution of the two activities the preparation was desalted on Sephadex G-25 column, applied to a column of Sephadex G-200 (Gąsior & Moldave, 1965; Girgis & Nicholls, 1971) and eluted with 0.05 M-tris-HCl buffer, pH 7.5, containing 0.08 M-KCl, 4 mM-$MgCl_2$ and 4 mM-dithiothreitol. For further purification, fractions containing EF_1 activity (usually no. 1 - 4) were pooled, concentrated with Ficoll and rechromatographed on a second column of Sephadex G-200; then glycerol was added to 20% final concentration and the mixture concentrated with Ficoll and stored in small portions at -20°C. To fractions 12 - 14, containing EF_2 activity, glycerol was added to 15% final concentration, and the mixture was stored in small portions at -20°C.

[^{14}C]*Phe-tRNA incorporating system.* The incorporation of [^{14}C]phenylalanine and radioactivity measurements were performed as described previously (Sendecki *et al.*, 1973). The amount of [^{14}C]Phe-tRNA, elongation factors and poly U applied in particular experiments, is indicated in legends to Tables and Figures.

The incorporation is expressed as pmol of [^{14}C]phenylalanine incorporated per 1 mg of ribosomal or cytoplasmic factor protein, 500 d.p.m. corresponding to 1 pmol of incorporated [^{14}C]phenylalanine.

RESULTS

At least two cytoplasmic factors are known to be necessary for the synthesis and elongation of polypeptide chain. Enzymic binding of aminoacyl-tRNA to ribosome-mRNA complex involves the aminoacyl-tRNA binding factor, EF_1, and the translocation reaction is catalysed by EF_2. The activities of these two enzymes are usually investigated in cell-free systems for amino acid incorporation. In our experiments, incorporation of [^{14}C]phenylalanine from [^{14}C]Phe-tRNA into poly--Phe was measured in cell-free systems which contained ribosomes and cytoplasmic factors from control or hypertrophic kidney. In some experiments, liver ribosomes were used.

Both kidney and liver ribosomes were depleted of cytoplasmic protein by washing with KCl solutions of high ionic strength, and then sedimented through 1 M-sucrose. In a system containing Mg^{2+}, GTP and poly U, the washed ribosomes were unable to incorporate amino acids without the addition of elongation factors. In the presence of elongation factors, the synthesis of poly-Phe (cf. Table 3) was inhibited in 88% by 2 mM-cycloheximide, an inhibitor of translocation (Obrig *et al.*, 1971) and in 80% by 2 μM-*N*-ethylmaleimide, the SH-group inhibitor (cf. McKeehan & Hardesty, 1969).

The results presented in Table 1 show that in systems containing ribosomes from control or hypertrophic kidney, the partially purified preparation of elongation factors from hypertrophic kidney supported incorporation of phenylalanine to a much higher extent than did the factors from control kidney. The incorporation in the presence of ribosomes from hypertrophic kidney was always higher than with ribosomes from control kidney. In the system in which both the ribosomes and the cytoplasmic factors were derived from hypertrophic kidney, the incorporation was 3 - 5 times as high as in the control system.

These results are in agreement with those of our previous experiments (Sendecki *et al.*, 1973) in which the ribosomes obtained from hypertrophic kidney showed much higher activity in cell-free systems than control ribosomes. Thus it seems possible to suppose that the increased translation of poly U in preparations from hypertrophic kidney is dependent upon the increase of activity of ribosomes as well as of elongation factors.

To eliminate the effect of changes occurring in kidney ribosomes during compensatory growth, in further experiments liver ribosomes were used. Their use presented the following advantages: first, the activity of liver ribosomes in poly-Phe synthesis was higher than that of normal kidney ribosomes, which could be related to lower nucleolytic activity of liver preparations (Sendecki *et al.*, 1973); secondly, liver ribosomes on storage at -20°C did not lose their activity for several weeks whereas preparations from control kidney became partly inactivated within three weeks (Sendecki *et al.*, 1973).

Table 1

Synthesis of [^{14}C]*polyphenylalanine from* [^{14}C]*Phe-tRNA with ribosomes and elongation factors from kidney of control and unilaterally nephrectomized rats*

The reaction mixture (0.25 ml) contained: 60 mM-tris-HCl buffer, pH 7.6, 80 mM-NH_4Cl, 2 mM-dithiothreitol, 0.2 mM-GTP, 6 mM-$MgCl_2$, poly U (100 μg), 80 μg of [^{14}C]Phe-tRNA (14 000 d.p.m.), kidney ribosomes (100 μg protein) and partially purified kidney elongation factors (300 μg protein). Incubation time was 20 min at 37°C.

Expt. no.	Kidney ribosomes	Kidney elongation factors	[^{14}C]Phenylalanine incorporated (pmol/mg ribosomal protein/min)	Increasing factor
1	Control	control	5.6	1.0
	Control	hypertrophic	17.6	3.1
	Hypertrophic	control	15.6	2.8
	Hypertrophic	hypertrophic	29.3	5.2
2	Control	control	7.2	1.0
	Control	hypertrophic	18.6	2.6
	Hypertrophic	control	16.6	2.3
	Hypertrophic	hypertrophic	24.0	3.2
3	Control	control	6.4	1.0
	Hypertrophic	hypertrophic	32.2	5.0

The kinetics of phenylalanine incorporation in the presence of elongation factors from normal or hypertrophic kidney was practically the same irrespective whether liver or control kidney ribosomes were used. At optimum Mg^{2+} ion concentration and saturating amount of poly U, in the presence of control elongation factors the incorporation was linear for the first 10 min of the reaction, and with elongation factors from hypertrophic kidney the synthesis of poly-Phe was much higher and practically linear for at least 20 min (Fig. 1).

In Table 2 are presented the results of experiments in which the activity of elongation factors from normal and hypertrophic kidney was studied with liver ribosomes. In the system in which incorporation of [^{14}C]phenylalanine was stimulated by cytoplasmic factors from hypertrophic kidney, synthesis of poly-Phe was 2.5-fold higher than in systems in which the factors were derived from control kidney.

The increased synthesis of poly-Phe from [^{14}C]Phe-tRNA observed in the presence of partially purified elongation factors could be attributed to increased binding of Phe-tRNA to poly U-ribosomes complex, regulated by EF_1, and/or to increased peptidyl-tRNA translocation dependent on EF_2.

To determine which of these reactions becomes enhanced in hypertrophic kidney, the EF_1 and EF_2 activities of control and hypertrophic kidney were separated by passing the partially purified preparation through a Sephadex G-200 column. Owing to a relatively high difference in the molecular weight of the two factors, very good

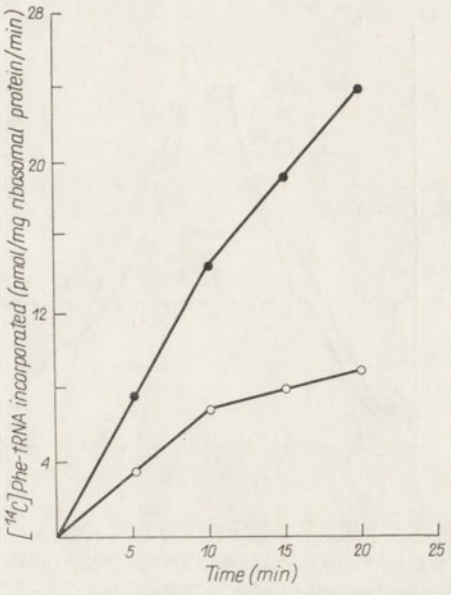

Fig. 1. Time-course of polyphenylalanine synthesis in cell-free systems from (○), control and (●), hypertrophic kidney. The incubation mixture (0.5 ml) was composed as described for Table 1 and contained poly U (200 μg), [^{14}C]Phe-tRNA (150 μg, 20 000 d.p.m.), ribosomes (250 μg protein) and partially purified elongation factors (1000 μg protein). Samples of 0.1 ml were withdrawn and radioactivity measured.

separation was achieved. In Fig. 2a is presented the elution pattern of the partially purified preparation from control and hypertrophic kidney, and in Fig. 2b the pattern of EF_1 and EF_2 activities, expressed as pmol of incorporated phenylalanine per 1 mg of protein. It was found that in the system containing EF_1 from hypertrophic kidney and EF_2 from control kidney, the synthesis of [^{14}C]polyphenylalanine was more than twice as great as when both factors were from the control kidney. Similar results were obtained when EF_1 from hypertrophic or control kidney were supplemented with EF_2 from liver.

Table 2

Incorporation of [^{14}C]*Phe-tRNA into polypeptide in ribosomes from liver, incubated with elongation factors from control and hypertrophic kidney*

The incubation mixture (0.25 ml) was composed as described in the legend to Table 1, and contained liver ribosomes (100 μg protein), partially purified kidney elongation factors (300 μg protein), poly U (100 μg), [^{14}C]Phe-tRNA (80 μg, 12 000 d.p.m.). The incubation time was 20 min at 37°C.

Expt. no.	Kidney elongation factors	[^{14}C]Phenylalanine incorporated (pmol/mg ribosomal protein/min)	Increasing factor
1	Control	19.0	1.0
	Hypertrophic	48.0	2.5
2	Control	21.6	1.0
	Hypertrophic	56.5	2.6
3	Control	17.1	1.0
	Hypertrophic	37.5	2.2

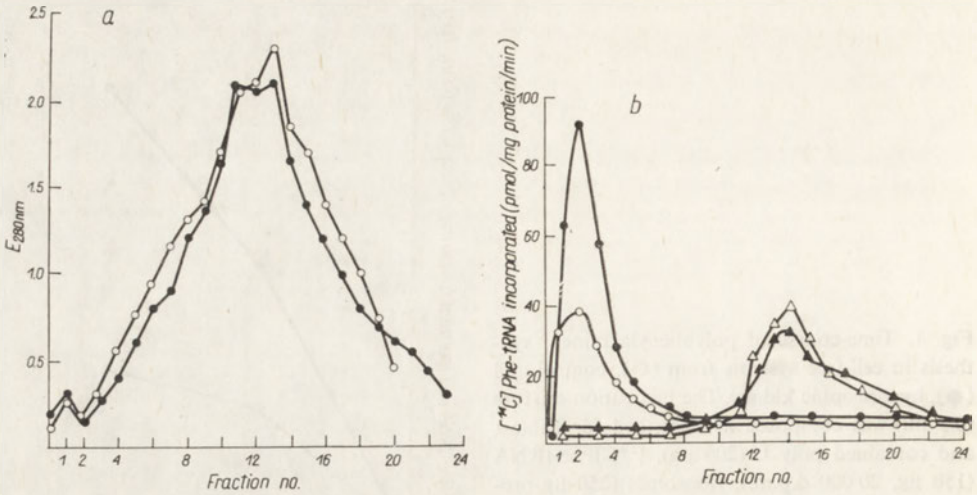

Fig. 2. Resolution of EF_1 and EF_2 activities on a Sephadex G-200 column, from (○), control and (●), hypertrophic kidney. The partially purified preparation of elongation factors was chromatographed on a Sephadex G-200 column (25×2.5 cm) and: (*a*), extinction at 280 nm was determined; (*b*), the activity of EF_1 and EF_2 was measured in the system described in the legend to Table 1, using liver ribosomes and the elongation factors from control and hypertrophic kidney, resolved by Sephadex G-200 chromatography. The incubation mixture (0.25 ml) contained liver ribosomes (50 μg protein), poly U (100 μg), [^{14}C]Phe-tRNA (80 μg, 14 000 d.p.m.) and: ○, ●, 0.05 ml of the eluate and 0.05 ml of control fraction no. 13; △, ▲, 0.05 ml of the eluate and 0.05 ml of the control fraction no. 3. Radioactivity was measured as described in Methods, and incorporation expressed as pmol of incorporated [^{14}C]phenylalanine per 1 mg protein of EF_1 or EF_2 per minute.

Table 3

Requirements for the synthesis of polyphenylalanine from [^{14}C]*Phe-tRNA, in cell-free system from hypertrophic kidney*

The incubation mixture (0.25 ml) was composed as described for Table 1 and contained poly U (100 μg), [^{14}C]Phe-tRNA (80 μg, 14 000 d.p.m.), kidney ribosomes (100 μg protein) and separated elongation factors from hypertrophic kidney (EF_1, 50 μg protein; EF_2, 50 μg protein). The incubation was for 20 min at 37°C.

Incubation system	[^{14}C]Phenylalanine incorporated (pmol/mg ribosomal protein/min)	% of control
Complete (control)	37.0	(100)
Mg^{2+} omitted	0.9	2.5
GTP omitted	0.3	0.9
Poly U omitted	3.2	8.6
EF_1 and EF_2 omitted	0.3	0.8
EF_1 omitted	1.2	3.2
EF_2 omitted	1.4	3.7
Cycloheximide, 0.5 mM, added	12.1	33.0
Cycloheximide, 2 - 4 mM, added	4.4	12.0
NEM, 2 μM, added	6.3	20

In Table 3 are presented the results of experiments in which both the ribosomes and the separated elongation factors were derived from hypertrophic kidney. When the system contained only one of the factors, irrespective whether EF_1 or EF_2, the incorporation of [^{14}C]phenylalanine was less than 4%. It should be noted that when neither factor was present, the incorporation was below 1%. Inhibition of polyphenylalanine synthesis by cycloheximide was observed both in the system containing elongation factors from hypertrophic and from control kidney, the percentage of inhibition being in both cases the same.

Table 4

Synthesis of [^{14}C]*phenylalanine from* [^{14}C]*Phe-tRNA by liver ribosomes and resolved* EF_1 *and* EF_2 *from control and hypertrophic kidney*

The incubation mixture (0.25 ml) was composed as described for Table 1 and contained liver ribosomes (50 μg protein), rechromatographed EF_1 (15 μg protein) and EF_2 (80 μg protein).

Expt. no.	Kidney elongation factors		[^{14}C]Phenylalanine incorporated (pmol/mg ribosomal protein/min)	Increasing factor
	EF_1	EF_2		
1	Control	control	15.8	1.0
	Control	hypertrophic	14.9	0.9
	Hypertrophic	hypertrophic	30.5	2.0
	Hypertrophic	control	36.0	2.3
2	Control	control	18.5	1.0
	Control	hypertrophic	17.3	0.9
	Hypertrophic	hypertrophic	35.7	2.0
	Hypertrophic	control	32.8	1.9

The effect of homologous or heterologous elongation factors on the rate of phenylalanine incorporation was studied in the system containing liver ribosomes. From the results presented in Table 4 it is evident that the ability to enhance poly--Phe synthesis is a property of EF_1 from hypertrophic kidney. In the system containing control EF_1 and control or hypertrophic EF_2, the incorporation was practically the same, whereas it was doubled in the presence of hypertrophic EF_1, irrespective whether EF_2 present in the system was from control or hypertrophic kidney.

DISCUSSION

Translation of cellular mRNA is known to be regulated by several factors. The role of cytoplasmic factors controlling the rate of protein synthesis in animal cells is, however, still poorly understood. Castañeda (1969) suggested that protein synthesis is regulated on the translation level by two cytoplasmic enzymes, so-called elongation factors, the aminoacyl-tRNA binding factor (EF_1) and the peptidyl-tRNA transfer factor (EF_2). Taking into account that the two factors are not specific

towards amino acids, Castañeda called this regulation "coarse or unspecific type of regulation".

Due to the lack of specificity of the elongation factors, it was possible to use the polypeptide-synthesizing system directed by poly U as a model system for studying the activities of these factors in protein biosynthesis.

Only a few investigations have been carried out on the cell-free system isolated from hypertrophic kidney. Brade *et al.* (1972) induced kidney hypertrophy by administration of folic acid, and observed that the high-speed supernatant stimulated incorporation of amino acids in the cell-free system to a much higher extent than did the supernatant from control kidney. On the other hand, Halliburton (1969) found no differences in the incorporation of radioactive amino acids between systems containing microsomes and cell sap from hypertrophic or control kidney.

From the presented here experiments it is evident that the cell-free system isolated from kidney hypertrophied as a result of unilateral nephrectomy, incorporated [^{14}C]phenylalanine from [^{14}C]Phe-tRNA more actively than the system from control kidney. The higher polypeptide synthesis was due both to enhanced activity of ribosomes and the factors present in the cytoplasm. The resolution of the elongation factors from the pH 5 supernatant showed that EF_1 present in hypertrophic kidney is twice as active as in control kidney, whereas the activity of EF_2 is unaltered. The enhanced activity of EF_1 could be demonstrated not only in the system containing control or hypertrophic kidney ribosomes, but also in the system containing liver ribosomes. In preliminary experiments (unpublished) it was observed that in the presence of GTP and 6 mM-Mg^{2+}, stimulation of binding of [^{14}C]Phe-tRNA to the ribosome-poly U complex by EF_1 from hypertrophic kidney was twice as large as with the factor from control kidney.

The increased activity of EF_1 in hypertrophic kidney is in agreement with the experiments of Girgis & Nicholls (1971, 1972) who demonstrated the limiting activity of EF_1 in normal kidney, liver and brain, and with the results of Willis & Starr (1971) who observed that the very low activity of EF_1 in rat spleen increased considerably following immunization of the animal.

The results of the present work are not incompatible with participation of factors other than EF_1 in stimulation and regulation of the rate of protein synthesis in hypertrophic kidney. In our experiments, however, the enhanced incorporation could not be dependent on the increased amount of (1), mRNA, as the incorporation was directed by poly U; and the incorporation dependent on endogenous mRNA was of the same order of magnitude in the systems from control and hypertrophic kidney, ranging from 8 to 10% of the activity observed with poly U.

(2) The enhanced phenylalanine incorporation could not be due to increased activity of aminoacyl-tRNA synthetases as precipitation at pH 5 removed from the high-speed supernatant aminoacyl-tRNA synthetases together with charged and free tRNA's; moreover, in the systems studied only Phe-tRNA complex was used.

(3) The increased synthesis could not be due, either, to the activity of soluble initiation factors which are present in supernatants from liver and other tissues

(Zasloff & Ochoa, 1971; Gąsior *et al.*, 1971, 1972; Leader & Wool, 1972), as they are active only in the presence of 40 s subunits (Gąsior *et al.*, 1971, 1972). In our experiments (Sendecki *et al.*, 1973) the optimum magnesium ion concentration was 6 - 8 mM, and its lowering led to a decrease in the incorporation; moreover, the 40 s subunits were most probably removed from the ribosomal preparations during centrifugation of the salt-washed ribosomes through discontinuous sucrose gradient.

Participation in the increased polyphenylalanine synthesis of the initiation factors described by Prichard *et al.* (1970), and Schafritz & Anderson (1970) seems also unlikely, as these factors were isolated by washing the ribosomes or microsomes with 0.5 - 1.0 M-KCl or NH_4Cl solution. Moreover, the lack of a shift in the optimum concentration of Mg^{2+} indicates that these factors are not responsible for increased incorporation of phenylalanine in our systems. Gee-Clough & Arnstein (1971) isolated from the 0.4 - 0.6 $(NH_4)_2SO_4$ sat. fraction of the high-speed supernatant of reticulocytes, a factor which stimulated protein biosynthesis. The Sephadex G-200 elution pattern of this factor suggests that it might be present in our EF_1 preparation; however, it could not have affected the results of our experiments, as the kidney preparations of EF_1 were several times frozen and thawed, and the factor of Gee-Clough & Arnstein is known to be rapidly destroyed under these conditions.

(4) The increased activity of ribosomes in hypertrophic kidney could be thought to be related to the absence of inhibitors of protein synthesis described by Nolan & Hoagland (1971) and Metafora *et al.* (1971, 1972), which are present in ribosomes and microsomes and absent from the supernatant. If this were the case, the presence of relatively high concentrations of GTP and dithiothreitol in our experiments would also have resulted in an increase of the activity of control ribosomes similar to that observed for ribosomes from hypertrophic kidney.

(5) Changes in ribonuclease activity could also be considered, as in ribosomes and cytoplasm of regenerating liver Shortman (1962) and Arora & de Lamirande (1967) found decreased activity of this enzyme. However, in the cytoplasm of control and hypertrophic kidney the RNase activity was found to be the same (Sendecki *et al.*, 1973).

The above-presented results indicate that during compensatory hypertrophy of the kidney, similarly as in other tissues in which protein biosynthesis is increased (Girgis & Nicholls, 1971; Willis & Starr, 1971; Myron *et al.*, 1972), the cytoplasmic factors participate actively in the unspecific regulation of the rate of translation.

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CZYNNIKI CYTOPLAZMATYCZNE BIORĄCE UDZIAŁ W BIOSYNTEZIE BIAŁKA W PRZERASTAJĄCEJ NERCE SZCZURA

Streszczenie

1. Porównywano aktywność czynników cytoplazmatycznych, biorących udział w biosyntezie łańcucha polipeptydowego, izolowanych z nerek kontrolnych i z nerek przerastających w 48 godzin po jednostronnej nefrektomii.

2. W bezkomórkowych układach syntetyzujących polifenyloalaninę w obecności czynników cytoplazmatycznych z nerek przerastających włączanie [^{14}C]fenyloalaniny było ponad dwukrotnie wyższe w porównaniu z układami izolowanymi z nerek kontrolnych.

3. W badaniach z rozdzielonymi czynnikami cytoplazmatycznymi stwierdzono, że aktywność czynnika wiążącego aminoacylo-tRNA (EF_1) jest znacznie wyższa w nerce przerastającej. Jednocześnie nie stwierdzono różnic w aktywności czynnika przenoszącego peptydylo-tRNA (EF_2) w badanych układach.

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PREPARATIVE PHOTOCHEMICAL SYNTHESIS OF ISOGUANOSINE RIBO- AND DEOXYRIBONUCLEOSIDES AND NUCLEOTIDES, AND ISOGUANOSINE-3′,5′-CYCLIC PHOSPHATE, A NEW cAMP ANALOGUE*

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The previously reported photochemical conversion of the N_1-oxides of adenine and adenosine to isoguanine and isoguanosine has been extended to the synthesis, on a preparative scale in 20 - 30% overall yield, of various nucleotides of isoguanine, including isoguanosine-2′(3′)-phosphate, isoguanosine-5′-phosphate, deoxyisoguanosine-5′-phosphate, and isoguanosine-3′,5′-cyclic phosphate, a new analogue of cAMP. The procedure is equally applicable to other adenine nucleosides and nucleotides. The nucleosides isoguanosine and deoxyisoguanosine may be prepared in the same way, or by enzymic dephosphorylation of the nucleotides. The 5′-phosphates of isoguanosine and deoxyguanosine are substrates of snake venom 5′-nucleotidase. Isoguanosine-3′,5′-cyclic phosphate was a substrate for 3′,5′-cyclic phosphodiesterase, the hydrolysis rate being about one-half that for cAMP.

Cherbuliez & Bernhard (1932) originally isolated from croton beans (*Croton tiglium* L.), a purine derivative which, on acid hydrolysis, yielded isoguanine and D-ribose. Crotonoside was subsequently shown to be identical with 9-β-D-ribofuranosylisoguanine (iso-G)[1] by Davoll (1951), who prepared the nucleoside by deamination of 2,6-diamino-9-β-D-ribofuranosylpurine. Iso-G has since also been obtained by Yamazaki *et al.* (1968) *via* the methylation and amination of 6-thioxanthosine.

Practically nothing has hitherto been reported regarding the possible biological role of iso-G, nor of its possible function in croton beans. It has, however, been found that, in addition to 6-mercaptopurine and 2-amino-6-mercaptopurine, isoguanine

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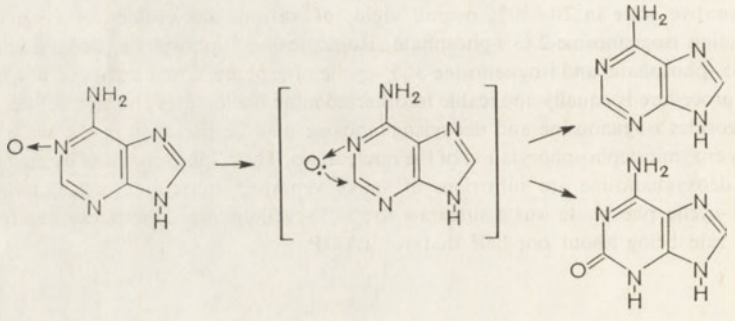
[1] In addition to the usual abbreviations recommended by the International Commission on Biochemical Nomenclature of IUPAC, the following are employed in this text: iso-G, isoguanosine; iso-dG, deoxyisoguanosine; 5′-iso-GMP, isoguanosine-5′-phosphate; 2′(3′)-iso-GMP, isoguanosine-2′(3′)-phosphate; 5′-iso-dGMP, deoxyisoguanosine-5′-phosphate; iso-cGMP, isoguanosine-3′,5′-cyclic phosphate.

is an inhibitor of IMP pyrophosphorylase (Hagen, 1973). Isoguanine has also been found to be a powerful competitive, and in some instances non-competitive, inhibitor of xanthine oxidase (Leonard *et al.*, 1962). Some of the properties of iso-G have been reported by Ravindranathan & Miles (1965).

Our interest in iso-G stemmed from a desire to prepare homopolymers of this nucleoside with a view to studying their structure, messenger and template properties, etc., since iso-G may be considered as a base analogue of adenosine. Furthermore, during the course of synthesis of a variety of alkylated derivatives of isoguanine destined for studies on the tautomerism of the heterocyclic base and its base pairing properties, it was noted that isoguanine exhibits appreciable emission in aqueous medium at room temperature, so that it could conceivably be used as a fluorescent probe.

In the search for a suitable procedure for the synthesis of isoguanine nucleosides and nucleotides, it occurred to us that a relatively simple pathway to such syntheses might be provided by the photochemical transformation of the N_1-oxide of adenine originally reported by Brown *et al.* (1964), and leading to formation of adenine and isoguanine as shown in Scheme 1. The mechanism of this reaction has been discussed elsewhere (Brown *et al.*, 1964; Cramer & Schlingloff, 1964; Kazimierczuk



Scheme 1.

et al., 1973) and will not be further elaborated upon here. The possibility of extension of this reaction to nucleosides of adenine is indicated by the observations of Cramer & Schlingloff (1964), who showed that u.v. irradiation of the N_1-oxide of adenosine leads to a mixture of adenosine and iso-G. They also demonstrated formation of a third, ring-opened, product which, on exposure to dilute alkali (pH ~ 10), cyclized to iso-G, thus increasing the yield of the latter.

MATERIALS AND METHODS

Adenosine-5′-phosphate, adenosine-2′(3′)-phosphate, 2′-deoxyadenosine-5′-phosphate and adenosine-3′,5′-cyclic phosphate (cAMP) were all products of Waldhoff Papierfabrik (Mannheim, G.F.R.).

The N_1-oxides of the mononucleotides were prepared as described by Stevens *et al.* (1959) and McCormick (1966) for adenine derivatives. The N_1-oxide of cAMP was synthesized according to Jastorff & Freist (1972).

Escherichia coli alkaline phosphatase was obtained from Worthington (Freehold, N.J., U.S.A.). *Vipera ammodytes* snake venom (kindly supplied by Dr. V. Skaric of Zagreb, Yugoslavia) was used as a source of 5′-nucleotidase. 3′,5′-Cyclic phosphodiesterase was prepared from rabbit brain according to the procedure of Drummond & Perrott-Yee (1961).

Rates of photochemical transformation were obtained by irradiation of approximately 10^{-4} M aqueous solutions suitably buffered in 10-mm path-length spectrophotometer cuvettes. The irradiation source was a British Thermal Syndicate mercury resonance lamp (95% of emitted radiation at 254 nm) and the radiation was first passed through a 5-mm layer of 30% acetic acid to remove traces of radiation below 230 nm. For measurements of quantum yields, light intensities were measured by actinometry in the same cuvette, using 5′-UMP for which the quantum yield for hydration of the 5,6 bond is 0.021 (Shugar, 1960).

For preparative purposes, larger volumes of about 10^{-3} M solutions of the nucleotides were irradiated in a photochemical reactor constructed from a Phillips (Eindhoven, Holland) 40-watt germicidal lamp elsewhere described (McLaren & Shugar, 1964). In this reactor provision is made for filtering the radiation through a layer of sodium acetate to remove traces of radiation below 220 nm.

U.V. absorption spectra were obtained with a Zeiss (Jena, D.D.R.) VSU-2 instrument, using 10-mm cuvettes. Measurements of pH, using 0.01 M buffers, were carried out with a Radiometer PHM 22 meter. Extremes of acid or alkaline pH were calculated from the concentrations of acid or alkali.

Paper chromatography was ascending, using the solvent systems indicated in Table 2. Whatman no. 1 paper was used for analytical purposes.

RESULTS AND DISCUSSION

Adenosine-1-oxide itself was found to undergo the same photochemical transformation to isoguanosine as adenine-1-oxide to isoguanine, the quantum yields for both reactions being very similar (Table 1). The percentage transformation of adenosine-1-oxide to iso-G varies with the pH of the medium, being maximal at alkaline pH, where it attains a value as high as 60% (Cramer & Schlingloff, 1964). However, when initial trials demonstrated that the N_1-oxides of adenosine nucleotides also underwent photochemical transformation similar to that for the nucleoside N_1-oxide, efforts were concentrated entirely in this direction for two reasons: (a) direct synthesis of the nucleotides obviates the necessity of phosphorylating the nucleoside, and obviously makes the nucleoside available by subsequent dephosphorylation with non-specific acid or alkaline phosphatases; (b) adenosine nucleotides are readily available commercially and are relatively inexpensive as starting materials.

The course of photochemical transformation of a 10^{-4} M aqueous solution of the N_1-oxide of 5′-AMP is illustrated in Fig. 1. The course of the reaction with time

Table 1

Quantum yields for photochemical transformation of the N_1-oxides of adenosine nucleotides, together with the yields for the N_1-oxides of adenine and adenosine

N_1-oxide of	Quantum yield (mol/einstein)
Adenine	0.107*
Adenosine	0.11
Adenosine-5′-phosphate	0.12
Adenosine-2′(3′)-phosphate	0.13
Deoxyadenosine-5′-phosphate	0.090
Adenosine-3′,5′-cyclic phosphate	0.090

* Data from Levin *et al.* (1964).

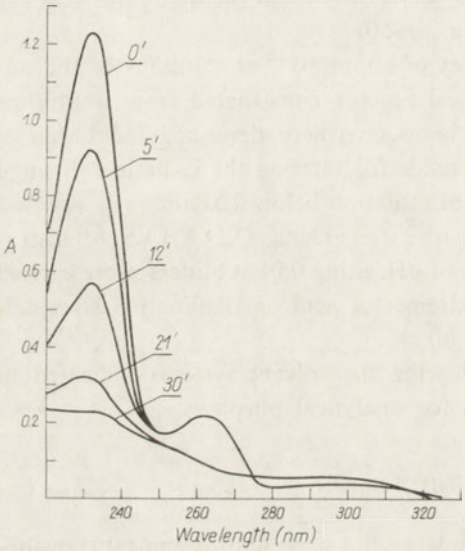


Fig. 1. Photochemical transformation of the N_1-oxide of 5′-AMP, 10^{-4} M in aqueous medium at pH 7. Disappearance of the N_1-oxide followed by decrease of characteristic absorption maximum at 232 nm. Figures beside each curve indicate irradiation times in minutes. Irradiation source was British Thermal Syndicate mercury resonance lamp as described in Materials and Methods.

of irradiation is readily followed by the disappearance of the characteristic absorption maximum of the N_1-oxide at 232 nm. The behaviour of the other adenosine nucleotides was fully analogous, except for small differences in rate. The quantum yields for these transformations are quite high (Table 1), in some instances exceeding that for adenine-1-oxide (Brown *et al.*, 1964).

Following the demonstration that the N_1-oxides of various adenosine nucleotides undergo similar photochemical transformations with approximately the same high quantum yields, each of these was irradiated in the photochemical reactor, and the resulting isoguanosine nucleotides isolated by column chromatography, as described under Experimental. The overall recovery of nucleotides in the case of the 5′-monophosphates of iso-G and iso-dG was usually in excess of 30%. For the 2′(3′)-phosphate and 3′,5′-cyclic phosphate the yields were somewhat lower, about 20%.

Table 2

Ascending paper chromatography of relevant adenine nucleosides and nucleotides, their N_1-oxides, and the corresponding isoguanine photoconversion products

R_F values of various compounds on Whatman paper no. 1 with the following solvent systems: (*A*) *n*-butanol - acetic acid - water, 2:1:1; (*B*) isopropanol - conc. NH_4OH - water, 6 :1:3; (*C*) dioxane - ethanol - water - conc. NH_4OH, 4:3:2:1. All ratios by vol.

Compound	R_F value with solvent		
	A	*B*	*C*
Adenine	0.55	0.55	0.62
Isoguanine	0.48	0.36	0.41
Adenosine	0.63	0.64	0.76
Iso-G	0.38	0.33	0.45
Iso-dG	0.40	0.43	0.52
5′-AMP	0.33	0.35	0.12
N_1-Oxide of 5′-AMP	0.25	0.31	0.09
5′-Iso-GMP	0.17	0.14	0.03
N_1-Oxide of 2′(3′)-AMP	0.31	0.37	0.12
2′(3′)-iso-GMP	0.25	0.25	0.05
N_1-Oxide of 5′-dAMP	0.22	0.32	0.06
5′-Iso-dGMP	0.17	0.23	0.05
cAMP	0.28	0.65	0.62
N_1-Oxide of cAMP	0.20	0.45	0.46
Iso-cGMP	0.17	0.39	0.25

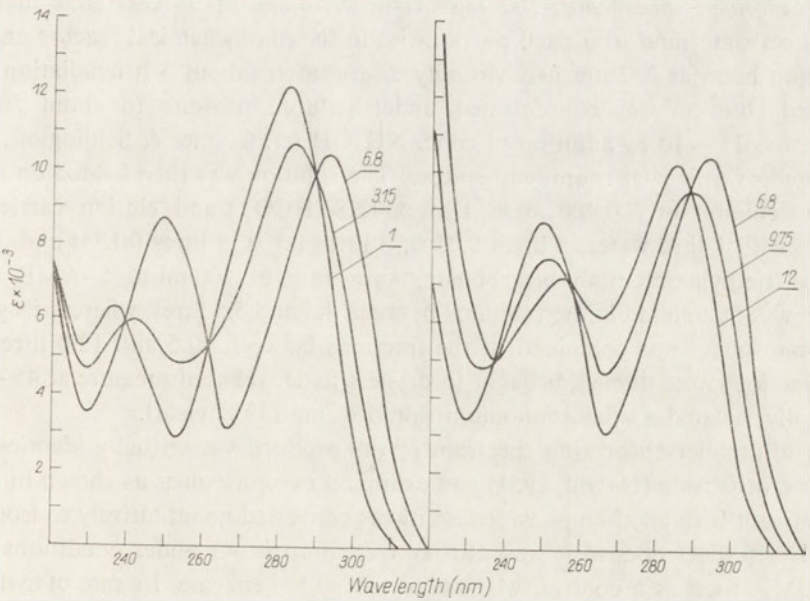


Fig. 2. Absorption spectra at indicated pH values of isoguanosine-5′-phosphate, showing two equilibria with (*a*) p*K* value of 3.4 (for protonation of N_1) and (*b*) p*K* 9.8 (for dissociation of the N_3 proton).

All of the products, the R_F values for which are shown in Table 2, were chromatographically homogeneous and exhibited pH-dependent absorption spectra in agreement with those presented by Davoll (1951) for iso-G. In Fig. 2 is presented the absorption spectrum of 5′-iso-GMP as a function of pH. As might have been anticipated, spectral titration demonstrates two pK values, pK_1 = 3.4 for protonation of a ring nitrogen (probably the N_1), and pK_2 = 9.8 for dissociation of the N_3 proton. The structures of the resulting ionic forms are under investigation and will be reported upon elsewhere (Kazimierczuk & Shugar, in preparation).

Identification of the isoguanosine nucleotides was further confirmed in the case of the 2′(3′)- and 5′-monophosphates by conversion to the corresponding nucleoside with bacterial phosphatase; and by susceptibility of the 5′-phosphates, but not the 2′(3′)-phosphate, to 5′-nucleotidase.

In the case of isoguanosine-3′,5′-cyclic phosphate, supplementary identification was provided by its susceptibility to hydrolysis by 3′,5′-cyclic phosphodiesterase to 5′-iso-GMP. The rate of enzymic hydrolysis was about 50% that for cAMP. Tests on the possible biological activity of the analogue are under way.

Finally, it should be emphasized that the foregoing findings point the way to reasonably simple syntheses of a variety of other isoguanine nucleoside and nucleotide derivatives from the corresponding adenine nucleosides and nucleotides e.g. ADP, nucleoside antibiotic analogues of adenosine such as puromycin, tubercidin, etc. Additional trials to this end are under way.

EXPERIMENTAL

Isoguanosine-5′-phosphate: 500 mg of the N_1-oxide of 5′-AMP was dissolved in 1.5 litres water and irradiated portionwise in the photochemical reactor until the absorption band at 232 nm had virtually disappeared (about 3 h irradiation). The irradiated solution was concentrated under reduced pressure to about 500 ml, brought to pH ~10 by addition of conc. NH_4OH (cf. Cramer & Schlingloff, 1964) and stored overnight at room temperature. The solution was then loaded on a 40 × × 2.5 cm column of 200/400 mesh Dowex 1X8 (HCO_3^-) and elution carried out stepwise with 1 litre water, 3 litres 0.05 M, 3 litres 0.1 M, 3 litres 0.15 M and 3 litres of 0.2 M-triethylammonium bicarbonate, at a rate of 200 ml/h. 5′-AMP, about 200 mg, was first eluted in the fractions between 4.5 and 5.5 litres, whereas isoguanosine-5′-phosphate was contained in the fractions between 10.5 and 12.0 litres. The latter fractions were pooled, brought to dryness under reduced pressure at 45 - 50°C, and finally isolated as the ammonium salt, 184 mg (33% yield).

The ultraviolet absorption spectrum of the product was virtually identical with that for crotonoside (Davoll, 1951) and exhibited two pK values as shown in Fig. 2. On treatment with alkaline phosphatase it was converted quantitatively to isoguanosine. With 5′-nucleotidase it was also converted to iso-G under conditions where 2′(3′)-CMP, used as a control, was unaffected by the enzyme. Its rate of hydrolysis by 5′-nucleotidase was similar to that for 5′-CMP.

Isoguanosine-2′(3′)-phosphate: 430 mg of the N_1-oxide of 2′(3′)-AMP in 1.3 litres water was irradiated portionwise in the reactor to disappearance of the 232 nm

absorption band. The irradiated solution was reduced in volume to 400 ml, brought to pH 10 with conc. NH_4OH and left overnight at room temperature. It was then deposited on a 40×2.5 cm column of Dowex 1X8 (HCO_3^-) and elution carried out stepwise with 1 litre water, and 3 litres each of 0.05 M, 0.10 M, 0.15 M and 0.20 M-triethylammonium bicarbonate. 2′(3′)-AMP came through in the fractions between 4.5 and 5.0 litres, and isoguanosine-2′(3′)-phosphate between 10 and 11.5 litres. The latter fractions were pooled, concentrated to dryness and the residue converted to the ammonium salt to give 105 mg (22%) of the desired product.

The product was resistant to 5′-nucleotidase, as expected but, on treatment with alkaline phosphatase, it was converted quantitatively to iso-G.

Deoxyisoguanosine-5′-phosphate: 300 mg of a crude preparation of the N_1-oxide of 5′-dAMP was dissolved in 1 litre water and irradiated in the photochemical reactor until the absorption maximum at 232 nm had disappeared (about 3 h irradiation). The irradiated solution was concentrated to 500 ml, brought to pH 10 with conc. NH_4OH and left overnight at room temperature. The solution was then deposited on a 35×2 cm column of Dowex 1X8 (HCO_3^-), which was washed with 1 litre water. The column was then eluted stepwise with 3 litres 0.1 M, 1 litre 0.15 M and 2 litres 0.2 M-triethylammonium bicarbonate. All the 5′-dAMP was contained in the fractions between 4.5 and 4.9 litres eluent, while the 5′-iso-dGMP was included in the eluents between 5.1 and 5.6 litres. The 5′-iso-dGMP fractions were pooled, brought to dryness and converted to the ammonium salt to yield 95 mg (30% overall yield).

The product was converted quantitatively to iso-dG on treatment with alkaline phosphatase or 5′-nucleotidase, the resulting u.v. spectrum remaining unchanged.

Isoguanosine-3′,5′-phosphate: 150 mg of crude cAMP-N_1-oxide (contaminated with about 10% cAMP) was dissolved in 150 ml water and irradiated in the reactor in the normal manner. The irradiated solution was brought to pH 10 and left overnight at room temperature. The solution was then loaded on a 30×1.5 cm column of Dowex 1X8 (HCO_3^-), which was washed with 1 litre water. The column was then eluted with triethylammonium bicarbonate in the form of a stepwise gradient, starting with 1 litre 0.05 M and increasing the concentration by 0.025 M for each successive litre. cAMP was eluted in the fractions between 4.0 and 5.5 litres, and the desired iso-cGMP in the fractions between 6.0 and 7.1 litres. The latter fractions were pooled and brought to dryness to give 32 mg of the triethylammonium salt of iso-cGMP (18% yield). On treatment with 3′,5′-cyclic phosphodiesterase as described by Drummond & Perrott-Yee (1961), it was converted to 5′-iso-GMP. The rate of opening of the cyclic phosphate ring was followed by chromatography, and was somewhat slower than the rate for cAMP.

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PREPARATYWNA SYNTEZA FOTOCHEMICZNA RYBO- I DEZOKSYRYBONUKLEOZYDÓW I NUKLEOTYDÓW IZOGUANINY ORAZ 3′,5′-CYKLICZNEGO FOSFORANU IZOGUANOZYNY, NOWEGO ANALOGU cAMP

Streszczenie

Opisaną w literaturze przemianę fotochemiczną N_1-tlenków adeniny i adenozyny prowadzącą do powstania izoguaniny i izoguanozyny zastosowano do syntezy na skalę preparatywną z 20 -30% wydajnością następujących nukleotydów izoguanozyny: 2′(3′)-fosforanu izoguanozyny, 5′-fosforanu izoguanozyny, 5′-fosforanu dezoksyizoguanozyny i 3′,5′-cyklicznego fosforanu izoguanozyny, nowego analogu cAMP. Procedurę tę można zastosować do innych nukleozydów i nukleotydów adeniny. Izoguanozynę i dezoksyizoguanozynę można otrzymać w taki sam sposób, albo też przez enzymatyczną defosforylację nukleotydów. 5′-Fosforany izoguanozyny i dezoksyizoguanozyny są substratami dla 5′-nukleotydazy z jadu węża. 3′,5′-Cykliczny fosforan izoguanozyny jest substratem dla 3′,5′-cyklicznej fosfodwuesterazy; hydroliza tego nukleotydu zachodzi około dwu razy wolniej niż hydroliza cAMP.

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RECENZJE KSIĄŻEK

ERYTHROCYTES, THROMBOCYTES, LEUKOCYTES. Recent Advances in Membrane and Metabolic Research (E. Gerlach, K. Moser, E. Deutsch & W. Willmanns, eds). Georg Thieme Verlag, Stuttgart 1973; str. 523, cena DM 110.

Książka zawiera referaty i doniesienia wygłoszone an II Międzynarodowym Sympozjum nt. "Metabolizm i Przepuszczalność Błon Erytrocytów i Trombocytów", które odbyło się we Wiedniu w lipcu 1972 r. Porównanie tego sympozjum z poprzednim wykazuje znaczny wzrost zainteresowań w zakresie struktury i funkcji błon składników morfotycznych krwi. Książka składa się z trzech następujących rozdziałów: erytrocyty, trombocyty i leukocyty.

Rodział 1 - Erytrocyty obejmuje pięć podrozdziałów: Błony, struktura i funkcja; Błony, przepuszczalność i transport; Metabolizm — aspekty ogólne; Metabolizm 2,3-DPG i transport tlenu; Metabolizm i enzymy. Na łączną ilość 53 referatów, 35 dotyczy struktury i funkcji błony erytrocyta. Zwrócono szczególną uwagę na model błony erytrocytów oraz stosowane metody badań, podkreślając ograniczoność interpretacji danych z doświadczeń wykonywanych na cieniach erytrocytów. Położono szczególny akcent na znaczenie oddziaływania hemoglobina - błona w biologicznym transporcie anionów przez błonę. Obok prac omawiających badania nad chemiczną strukturą błony krwinki czerwonej przedstawiono wyniki doświadczeń nad rekonstrukcją lipoprotein błon. W grupie prac na temat metabolizmu krwinek czerwonych dominowało zagadnienie roli i mechanizmu działania 2,3-DPG. Omówiono zarówno aspekty teoretyczne, jak i praktyczne tego niezwykle ciekawego problemu.

Rozdział 2 - Trombocyty obejmuje trzy podrozdziały: Błona i funkcja; Agregacja; Metabolizm. W sumie zaprezentowano 40 referatów, z tego aż 34 prace dotyczą dwóch pierwszych zagadnień. Szczególnym przedmiotem zainteresowania były glukoproteidy, ich struktura i rola jako elementów błony i frakcji subkomórkowych płytek. Wiele miejsca poświęcono tzw. czynnikowi 4, który w określonych warunkach fizjologicznych uwalniany jest z wnętrza płytki. Znalazły się tam także artykuły na temat wpływu cyklicznego AMP i prostaglandyn na metabolizm i agregację płytek.

Rozdział 3 - Leukocyty liczy 170 stron, obejmuje trzy podrozdziały: Normalna i leukemiczna granulopoeza; Proliferacja limfocytów; Wpływ leków na metabolizm leukocytów normalnych i białaczkowych.

W tym rozdziale dominują prace poświęcone metabolizmowi kwasów nukleinowych, biosyntezie RNA, a także immunologicznym aspektom fagocytozy. Spośród prac omawiających mechanizm działania leków na metabolizm leukocytów białaczkowych na uwagę zasługują prace Huennekensa i wsp. dotyczące działania pochodnych kwasu folowego.

Książka wydana jest starannie, zawiera indeks rzeczowy; po każdym referacie podana jest literatura i bardzo ciekawa dyskusja niekiedy przekraczająca objętość referatu. Książka stanowi cenną pozycję nie tylko dla pracowników interesujących się składnikami morfotycznymi krwi, lecz także i przede wszystkim jest ona wartościowym dziełem dla osób zajmujących się biologicznym transportem.

Stefan Angielski

PROTIDES OF THE BIOLOGICAL FLUIDS. Proceedings of the Twentieth Colloquium, Brugge 1972 (H. Peeters ed.) Pergamon Press, Oxford, New York, Toronto, Sydney, Braunschweig 1972; str. 611, cena 17Ł.

Kolejny tom z serii *Protides of the Biological Fluids* zawiera materiały z dwudziestej międzynarodowej konferencji odbytej w Brugge w 1972 r. Zagadnienia tego sympozjum koncentrowały się wokół trzech zasadniczych problemów z rozległej dziedziny dotyczącej chemii białek. Pierwszy z nich to immunoglobuliny i substancje skrobiowate, drugi to aktywne polipeptydy, pochodzące z białek osocza, trzeci problem dotyczy nowej o dużych perspektywach metody biochemicznej — techniki mikrokalorymetrii dostosowanej do systemów biologicznych.

Jednym z bardziej interesujących zagadnień omawianych w pierwszej części niniejszej publikacji jest zależność między budową immunoglobulin a zaburzeniami w metabolizmie białek osocza, jak hyperglobulinemia, występowanie białek Bence-Jonesa w moczu, oraz skrobiawica, przy czym mogą one występować oddzielnie lub równocześnie. Hyperglobulinemia spowodowana jest wybiórczą i wzmożoną syntezą immunoglobulin monoklonalnych przy równoczesnym zmniejszeniu syntezy innych przeciwciał, w wyniku czego obserwuje się zmniejszoną odporność osobniczą.

Tematem innych, licznych prac było zbadanie substancji skrobiowatej, odkładającej się w tkankach w zaburzeniu zwanym skrobiawicą. Obecnie wiadomo, że substancja ta składa się głównie z białek charakteryzujących się nierozpuszczalnością w wodnych i organicznych rozpuszczalnikach, niewrażliwością na działanie enzymów oraz mających wiele wspólnych cech z lekkim łańcuchem immunoglobulin.

W części książki poświęconej aktywnym polipeptydom osocza krwi, omówione są między innymi angiotensyna i renina, peptydy uzyskane z fibrynogenu pod wpływem trombiny, system kinin i inhibitory proteaz.

Artykuły w dziale zajmującym się mikrokalorymetrią dotyczą dwóch zasadniczych aspektów: termodynamicznych badań struktury białek oraz zastosowania techniki mikrokalorymetrycznej do badań złożonych systemów biologicznych, takich jak komórki i tkanki.

W artykule wstępnym znajduje się wykład H. Peetersa poświęcony pamięci Arne Tiseliusa, jednocześnie zawierający przegląd metod rozdziału białek i ich zastosowania.

Książka ta, bardzo starannie wydana, może być wartościowym źródłem najnowszych informacji dla wszystkich zajmujących się chemią białek.

Marek Ombach

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