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MARTA STRYJECKA-ZIMMER and T. BORKOWSKI

INCORPORATION OF [^{32}P]ORTHOPHOSPHATE INTO HISTONES OF CHICK EMBRYO BRAIN AND LIVER

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[^{32}P]Orthophosphate was introduced intra-amnionally to 14-day-old chick embryos, and after 4, 10 and 20 h the nuclear histone fractions of brain and liver were isolated. The highest radioactivity was observed after 10 h. The kinetics of ^{32}P incorporation into particular histone fractions of both tissues were somewhat different.

The dependence of phosphorylation of histone fractions on the cell development stage and metabolic activity, has been investigated by numerous workers (Ord & Stocken, 1969; Sung *et al.*, 1971; Balhorn *et al.*, 1972a,b; Barraclough & Campbell, 1973; Gurley *et al.*, 1973; Louie & Dixon, 1973), but the biological role of phosphorylation of histones has not been so far fully elucidated.

In the growing embryo, differentiation of particular tissues and mitosis proceed at different rates. In a 14-day-old chick embryo the brain tissue has achieved a considerable degree of differentiation whereas in liver the processes of mitosis still predominate. A comparison of histone phosphorylation within the same organism in tissues differing in mitotic activity may help to elucidate the role of phosphorylation of particular histone fractions.

In the previous work (Stryjecka & Borkowski, 1971) it was demonstrated that histones are phosphorylated *in vivo* in brain tissue of adult mammals. In the present work, the incorporation of ^{32}P into particular histone fractions from brain and liver of chick embryo, was compared.

MATERIALS AND METHODS

Material. For each experiment, fifty 14-day-old chick embryos were used. Radioactive phosphate, 80 μCi of [^{32}P]Na_2HPO_4 (spec. act. 330 mCi/mmol) obtained from the Institute of Nuclear Research (Świerk, Poland), in 0.1 ml of 0.9% NaCl solution, was introduced intra-amnionally, and after 4, 10 or 20 h the embryos were decapitated and brains and livers isolated.

The nuclei were isolated by the method of Mardell *et al.* (1961) and histones as described by Stryjecka (1971), using CM-cellulose chromatography (Johns *et al.*, 1960) for histone fractionation. The main three fractions, *f1*, *f2* and *f3*, eluted with a step-wise pH gradient were collected, precipitated with trichloroacetic acid, and washed several times with ether. Fraction *f4* eluted with 0.5 M-HCl, was not investigated.

Analytical methods. Protein was determined according to Lowry *et al.* (1951) and total phosphorus by the method of Hurst (1964).

Phosphoserine in the protein was detected electrophoretically after hydrolysis with 2M-HCl (Sanecka-Obacz, in preparation).

Radioactivity was measured in a sample containing 50 - 200 μg of protein in 0.1 - 0.3 ml of water. The scintillation fluid contained: dioxane, 100 ml; PPO, 400 mg; POPOP, 20 mg; naphthalene, 20.8 g; methanol, 60 ml, and toluene, 100 ml.

RESULTS

From brain nuclei of 14-day-old chick embryo, 0.7 mg of histone per 1 g fresh tissue weight was obtained, this value being similar to that reported by Bondy (1971). From liver nuclei, 1.4 mg of histone per 1 g was obtained.

Table 1

The content of phosphate in histone preparations from chick embryo brain and liver

	Brain	Liver
	μg P/mg protein	
Total histone	1.8	2.2
Fraction *f1*	1.2	0.5
Fraction *f2*	0.6	0.7
Fraction *f3*	1.9	1.8

All the histone fractions from brain and liver separated by chromatography were found to contain phosphate (Table 1). The CM-cellulose chromatography followed by several-fold precipitation with trichloroacetic acid precluded contamination of histones by inorganic and nucleotide phosphates. Moreover, it was found that phosphate was bound to protein mainly in the form of phosphoserine. Radioautography on Kodak AR-50 plates of the electrophoretograms of hydrolysed histones revealed only one radioactive spot, the mobility of which corresponded to that of phosphoserine (Fig. 1).

The content of phosphate in total histone from brain was somewhat lower than in liver histone. The highest amount of phosphate was found in both tissues in fraction *f3*, whereas the lowest in fraction *f2* from brain and *f1* from liver.

Differences in phosphate content of histone were reported by several workers. Ord & Stocken (1969) and Shepherd *et al.* (1971) found a different content of phos-

phate in fraction *f1* from tissues differing in mitotic activity, and a varying content during the cell cycle.

All the investigated chick embryo histone fractions incorporated radioactive phosphate. The radioactivity of brain histone 4 h after ^{32}P administration corresponded to 3.8% of the total radioactivity present in nuclei; in the case of liver nuclei the corresponding value was 2.2%. After 20 h, the ratio of histone radioactivity to the total radioactivity of nuclei decreased to 1.1%.

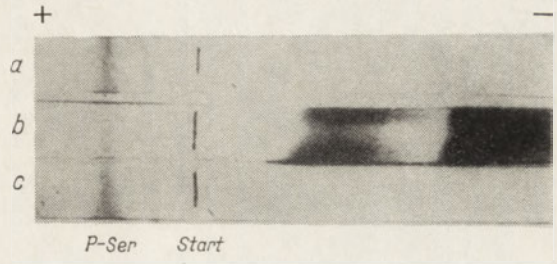

Fig. 1. Electrophoretic pattern of: *a*, standard phosphoserine stained with the molybdate reagent; and hydrolysate of total histone from chick embryo brain nuclei: *b*, stained with the ninhydrin reagent; *c*, stained with the molybdate reagent. The electrophoresis was carried out in formic-acetic acid buffer, pH 2.2, for 60 min at 800 V.

The maximum incorporation of radioactive phosphate into histones of brain and liver appeared after 10 h, the specific activity of liver histone being higher than in the preparation from brain (Fig. 2). The time-course of incorporation of the label into particular histone fractions from brain and liver was somewhat different (Fig. 3a,b). After 4 h, the brain fraction *f3* showed the highest specific activity, whereas in liver, fraction *f1*. After 10 h, both in liver and brain incorporation of ^{32}P into fractions *f3* largely predominated over incorporation into the two other fractions. After 20 h, the radioactivity decreased in all fractions but to the greatest extent in fraction *f3*.

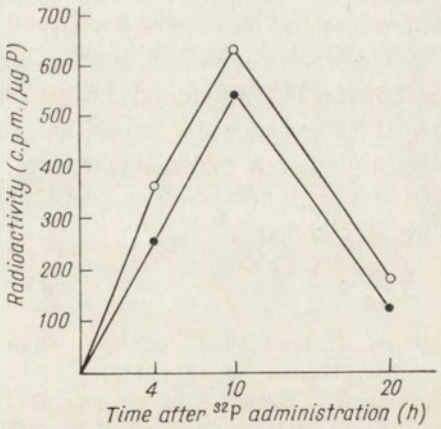

Fig. 2. Incorporation of ^{32}P into total histone from ●, brain and ○, liver nuclei of chick embryo.

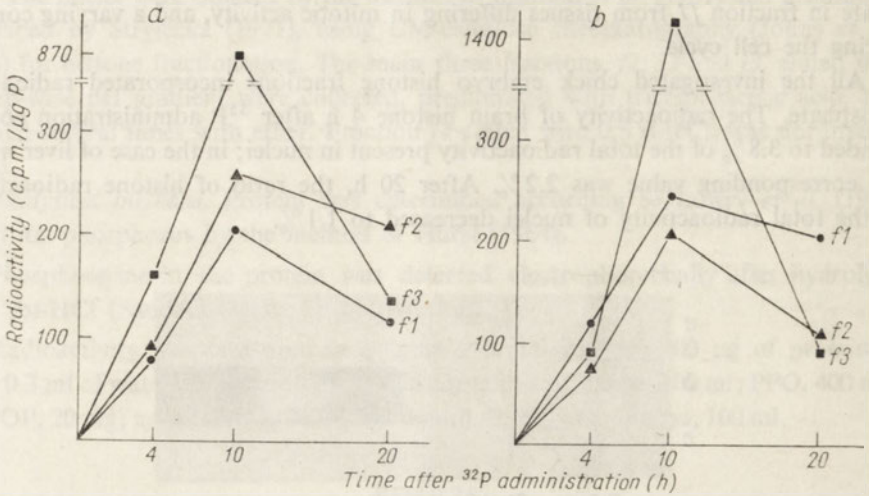

Fig. 3. Incorporation of ^{32}P into particular histone fractions prepared from: *a*, brain nuclei and *b*, liver nuclei of chick embryo.

The different turnover of phosphate in particular histone fractions of brain and liver nuclei, especially in fractions *f1* and *f2*, may be related to differences in the developmental rhythm of these two tissues. The dependence of phosphorylation of particular histone fractions on the phase of cell division, has been demonstrated by the workers cited in the introduction.

Although in our experiments *in vivo* the kinetics of ^{32}P incorporation into histone fractions cannot be correlated with the cell division phase, nevertheless the differences observed in embryonal tissues in the kinetics of histones phosphorylation may be explained by differences in the metabolism of DNA and RNA linked with mitosis. According to some authors, phosphorylation interferes with the interaction of histones with DNA (Kleinsmith *et al.*, 1966; Langan, 1968), or facilitates the transport of histones from cytoplasm to nuclei (Oliver *et al.*, 1972). Marks *et al.* (1973) demonstrated that in HeLa cells there was no correlation between phosphorylation of histones and increase of RNA synthesis and genome activity; they also suggested that the process of phosphorylation occurs in chromatin.

Louie *et al.* (1973) found in trout testis differences in the degree of phosphorylation of particular histone fractions, and suggested that the process of phosphorylation may also play a role different from the postulated derepression of the genome, and that phosphorylation and dephosphorylation of histones may regulate the structure of chromatin.

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INKORPORACJA [^{32}P]ORTOFOSFORANU DO HISTONÓW TKANKI MÓZGOWEJ I WĄTROBOWEJ EMBRIONÓW KURZYCH

Streszczenie

[^{32}P]Ortofosforan podawano doowodniowo 14-dniowym embrionom kurzym. Po 4, 10 i 20 godzinach od podania izotopu izolowano frakcje histonów z jąder komórek mózgowych i wątrobowych. Frakcje histonów obu badanych tkanek różniły się kinetyką inkorporacji ^{32}P, ale we wszystkich frakcjach najwyższą radioaktywność obserwowano po 10 godzinach.

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MARIA SANECKA-OBACZ and T. BORKOWSKI

PHOSPHORYLATION OF PROTEIN FROM RABBIT BRAIN RIBOSOMES

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1. Protein extracted with 0.25 M-HCl from rabbit brain ribosomes separated on CM-cellulose column chromatography into four phosphate-containing fractions, the phosphate being bound with serine and threonine residues. 2. After administration of $^{32}P_i$ *in vivo*, the rate and extent of radioactivity incorporation into particular fractions, were different.

Sanecka-Obacz & Borkowski (1970) reported that ^{32}P-labelled orthophosphate was incorporated *in vivo* into brain ribosomal proteins. Traugh & Traut (1972) demonstrated that the phosphorylation is catalysed by protein kinase present in cytoplasm, and Kabat (1971), Barden & Labrie (1973) and Walton & Gill (1973) found this enzyme also in ribosomes. Kabat (1970), Eil & Wool (1971) and Traugh *et al.* (1973) in experiments *in vitro* demonstrated that only certain ribosomal proteins underwent phosphorylation; the amount of detected phosphoprotein was dependent on the procedure used (Traugh & Traut, 1972; Eil & Wool, 1973).

In the previous work (Sanecka-Obacz & Borkowski, 1973) it was shown that fractions obtained by CM-cellulose chromatography of rabbit brain ribosomal protein extracted with 0.25 M-HCl, differed in the content of lysine and arginine, the ratio of basic to acidic amino acids, as well as in the rate and extent of incorporation of labelled lysine. The aim of the present work was to determine the content of phosphorus in particular fractions, and their phosphorylation *in vivo*.

MATERIALS AND METHODS

To adult rabbit of mixed breed, $Na_2H^{32}PO_4$ (Institute of Nuclear Research, Świerk, Poland) was injected into the cerebello-medullary cistern, under intravenous anaesthesia with Eunarcone, in a dose of 200 μCi/kg body wt. After 4, 16 and 46 h the animals were killed by air embolism and decapitated. From the brain, ribosomes were prepared, extracted with 0.25 M-HCl, and protein was precipitated with trichloroacetic acid as described by Sanecka-Obacz & Borkowski (1973). The protein was fractio-

nated on a column of CM-cellulose (Serva, Heidelberg, G. F. R.) by the method of Johns *et al.* (1960) elaborated for histone. The successive fractions eluted from the column were designated F_1, F_2, F_3 and F_4.

Phosphoserine and phosphothreonine in the protein were detected electrophoretically after mild acid hydrolysis (Sanecka-Obacz, in preparation) using as standards the preparations obtained as described by Zając (1962).

Gradient paper chromatography was performed after Bauer (1968).

Protein was determined according to Waddell & Hill (1956), and phosphorus by the method of Hurst (1964).

Radioactivity was measured in a Geiger-Müller counter.

RESULTS AND DISCUSSION

All the chromatographic protein fractions obtained from the 0.25 M-HCl extract of brain ribosomes, contained phosphorus but its content differed in the particular fractions (Table 1). It was the highest, 0.4%, in fraction F_3 eluted from the column with 0.02 M-HCl. Fractions F_4 and F_2, eluted with 0.5 and 0.01 M-HCl, respectively, had a smaller content, and in fraction F_1, eluted with acetate buffer at pH 4.2, the content of phosphorus was only 0.06%.

Table 1

Phosphorus content in the CM-cellulose chromatographic fractions of brain ribosomal protein extracted with 0.25 M-HCl

The results are mean values of 4 - 6 determinations, ±SD.

Fraction	Inorganic phosphorus (μg/mg protein)
Total protein	3.0±0.17
F_1 eluted at pH 4.2	0.6±0.05
F_2 eluted with 0.01 M-HCl	1.8±0.08
F_3 eluted with 0.02 M-HCl	4.0±0.5
F_4 eluted with 0.5 M-HCl	3.1±0.23

The fractions did not contain any inorganic phosphate which had been removed by dialysis, chromatography and several-fold precipitation with trichloroacetic acid. Neither did they contain RNA or DNA, as following alkaline hydrolysis according to Smidt & Tannhauser (1945) they gave a negative reaction both with orcine (Mejbaum, 1939) and diphenylamine (Bürton, 1956).

The phosphorus found in the particular fractions was bound with protein, as demonstrated by radioautography. On gradient paper chromatography the particular protein fractions were inhomogeneous and separated into several bands (Fig. 1). Radioautography on Kodak AR-50 plates showed radioactivity located only in areas corresponding to protein bands.

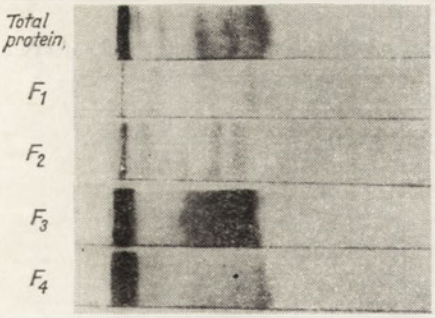

Fig. 1

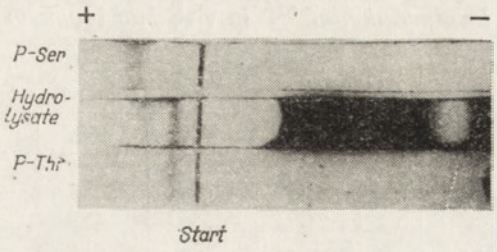

Fig. 2

Fig. 1. Paper gradient chromatograms of the brain ribosome protein and the particular fractions obtained by CM-cellulose column chromatography. The proteins were stained with Amido Black.

Fig. 2. Paper electrophoresis of brain ribosomal protein hydrolysate, stained with ninhydrin. The electrophoresis was carried out in formic-acetic acid buffer, pH 2.2, for 60 min at 800 V. Phosphoserine and phosphothreonine standards were stained with molybdate reagent after Saito & Akashi (1957).

The electrophoretic pattern of the products obtained by mild acid hydrolysis demonstrated the presence in the ribosomal proteins of phosphoserine and phosphothreonine (Fig. 2), in agreement with the findings of Eil & Wool (1973) and Traugh *et al.* (1973).

In experiments *in vitro* Kabat (1970) and Martini & Gould (1973) recovered in the ribosomal protein 50% of the radioactivity incorporated into ribosomes from [γ-^{32}P]ATP. However, in our experiments *in vivo* at 4, 16 and 46 h after administration of ^{32}P, the label present in the protein amounted to, respectively, only 2.5, 3 and 3.6%. The very low radioactivity of the investigated protein could be due to the fact that the experiments were performed *in vivo*, and that the protein analysed did not represent all the proteins present in ribosomes. Studies performed *in vitro* (Traugh & Traut, 1972; Martini & Gould, 1973; Barden & Labrie, 1973) demonstrated that some proteins or hydroxyl groups remain inaccessible to phosphorylation. Moreover, it was reported that washing of ribosomes with solutions of high ionic strength removed proteins incorporating phosphate most actively (Kabat, 1970; Traugh & Traut, 1972), the amount of removed protein being much higher in the case of previously phosphorylated ribosomes (Walton & Gill, 1973).

The incorporation of phosphate increased with time, but the increase in the particular protein fractions was not uniform (Table 2). In unfractionated protein, the increase after 16 h was 3.5 times that after 4 h, and after 46 h, 4.3. A similar time-course of the incorporation was observed in fraction F_4, in which the specific radioactivity of protein was the highest, and in fraction F_2, in which it was the lowest, about 1/7 that in fraction F_4. In fraction F_3, the increase in radioactivity between the 4th and 16th hour was small whereas later it increased rapidly to reach after 46 h a value almost eightfold that at 4 h. Incorporation of radioactivity into fraction F_1 was practically completed after 4 h.

Table 2

Incorporation of ^{32}P in vivo *into brain ribosomal protein extracted with 0.25* M-HCl

CM-cellulose fraction	Radioactivity					
	counts/min/mg protein			counts/min/μg P		
	4 h	16 h	46 h	4 h	16 h	46 h
Total protein	636	2257	2738	212	752	915
F_1	366	452	357	610	753	595
F_2	136	477	586	75	265	359
F_3	476	607	3760	119	152	940
F_4	1014	3164	4522	327	1020	1459

From the above data it appears that the amount of the incorporated label was not dependent on the content of phosphate in the protein. It seems of interest that, irrespective of the time of incorporation, the greatest amount of phosphate was incorporated into fraction F_4 which, as demonstrated by Sanecka-Obacz & Borkowski (1973), is an acidic protein. This is in agreement with the observations of other workers (Traugh & Traut 1972; Martini & Gould, 1973).

Since the particular protein fractions differ in the content of phosphorus, the radioactivity was calculated also per 1 μg P; the highest phosphorus specific radioactivity was found after 4 h in fraction F_1, whereas after 16 and 46 h, in fraction F_4 (Table 2).

The differences in the time-course of phosphate incorporation point to distinct differences in the rate of phosphate turnover in particular protein fractions. The highest rate was found for fraction F_1, the lowest for fraction F_3. Fractions F_2 and F_4 showed a similar turnover, although they differed largely in the amount of incorporated label. It should be noted that in the *in vivo* experiments on the incorporation of radioactive lysine, the proteins of fraction F_1 also showed the highest turnover rate (Sanecka-Obacz & Borkowski, 1973).

The differences in the rate and extent of phosphorylation of the phosphoproteins present in particular fractions of brain ribosomal proteins extracted with 0.25 M-HCl, may suggest their different roles in the function of ribosomes. The different pattern of phosphorylation of the corresponding ribosomal protein fractions from Ehrlich ascites tumour cells reported by Szwaj & Borkowski (1973) could be related to different metabolic activity of brain and tumour cells.

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FOSFORYLACJA BIAŁEK RYBOSOMÓW MÓZGU KRÓLIKA

Streszczenie

1. Białko ekstrahowane do 0.25 M-HCl z rybosomów mózgu królika poddane chromatografii na kolumnie z CM-celulozy dzieliło się na 4 frakcje. Wszystkie otrzymane frakcje zawierały fosfór związany z seryną i treoniną.

2. Poszczególne frakcje różniły się szybkością inkorporacji i ilością wbudowanego *in vivo* ^{32}P.

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J. ROGULSKI, A. PACANIS, WALERIA ADAMOWICZ and S. ANGIELSKI

ON THE MECHANISM OF MALEATE ACTION ON RAT KIDNEY MITOCHONDRIA

EFFECT ON OXIDATIVE METABOLISM

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1. Maleate inhibits oxygen uptake and phosphorylation in rat kidney mitochondria oxidizing 2-oxoglutarate; this inhibition is not affected by the degree of mitochondrial coupling or integrity of mitochondrial membrane. 2. Maleate inhibits also the oxidation of other CoA-dependent substrates, i.e. pyruvate, palmitoyl-carnitine, acetoacetate. Inhibition of oxygen uptake by mitochondria metabolizing isocitrate or malate at high maleate concentration appears to be caused by blocking of oxidative metabolism of 2-oxoglutarate. 3. Maleate has no inhibitory effect on isolated oxoglutarate dehydrogenase or the electron transfer system. Arsenate and high concentration of phosphate decrease the inhibitory effect of maleate. It is concluded that maleate affects the substrate-level phosphorylation and/or other reaction(s) involving succinyl-CoA.

It was shown previously in our laboratory that maleate at concentrations below 1 mM inhibited oxidation of 2-oxoglutarate in isolated mitochondria of rat kidney (Angielski & Rogulski, 1962; Rogulski & Angielski, 1963). The same degree of inhibition of citrate and malate oxidation appeared at maleate concentration 10 times higher. Succinate oxidation was not sensitive to maleate. The inhibition of 2-oxoglutarate oxidation was also observed in brain and heart muscle homogenates but no inhibitory effect, even at high maleate concentrations, was observed in liver homogenates (Angielski & Rogulski, 1962). Since it was found that the purified preparation of oxoglutarate dehydrogenase was not inhibited by maleate at high concentrations (Angielski, 1963), more detailed studies were now undertaken in an attempt to elucidate the mechanism of the inhibitory effect of maleate on rat kidney mitochondria. The results seem to indicate that the inhibitory effect of maleate concerns the formation of the first product of 2-oxoglutarate oxidation, i.e. succinyl-CoA.

MATERIALS AND METHODS

Mitochondria were prepared according to Hogeboom (1955) from kidneys and livers of rats starved for 12 h. Disintegration of mitochondria was accomplished by repeated freezing (solid CO_2 - acetone mixture) and thawing or by sonication (20

Kcyc/sec) for 30 sec at 6 A with the aid of MSE sonicator. The electron transfer particles (ETP)[1] were obtained according to Crane *et al.* (1956). 2-Oxoglutarate dehydrogenase was prepared by the method of Sanadi *et al.* (1952); the specific activity of the preparation was 2.4 μmoles/min/mg protein.

Respiration was measured by the conventional Warburg technique in a medium containing in a final volume of 2 ml: 10 mM-Tris-HCl buffer (pH 7.4), 20 mM-potassium phosphate buffer (pH 7.4), 25 mM-KCl, 8 mM-$MgSO_4$, 50 mM-substrate, 30 mM-glucose, 1 mM-ADP, 0.5 mg of yeast hexokinase (Sigma, Type III), 0.1 mM--EDTA, and 2.0 - 2.5 mg of mitochondrial protein. The centre well contained 0.2 ml of 2.0 M-KOH and filter paper. Incubations were carried out for 30 min at 30°C; gas phase, air. The reactions were started by the addition of glucose and hexokinase following a 5 min thermoequilibration, and were stopped by the addition of trichloroacetic or perchloric acid. Polarographic measurement of oxygen uptake was performed using Clark type oxygen electrode at 25°C in a medium containing in a final volume of 2 ml: 40 mM-Tris-HCl buffer (pH 7.4), 10 mM-potassium phosphate buffer (pH 7.4), 10 mM-KCl, 90 mM-sucrose, 5 mM-$MgSO_4$, 2.5 mM-ADP and 2.0 - - 2.5 mg of mitochondrial protein. The substrate concentrations were 10 mM except that of palmitoyl-carnitine which was about 0.2 mM.

Reduction of exogenous ferricytochrome *c* by 2-oxoglutarate or NADH was followed spectrophotometrically at 550 nm in a medium containing in a final volume of 3 ml: 70 mM-Tris-HCl buffer (pH 7.4), 5 mM-$MgSO_4$, 2 mM-KCN and 1 mg of cytochrome *c*. The concentrations of NADH and 2-oxoglutarate were 0.1 and 8 mM, respectively. In these experiments mitochondria were pretreated in water for 5 min and, after increasing the osmolarity to 0.25 M with sucrose, were frozen and thawed three times. About 100 μg and 5 μg of mitochondrial protein was used to measure the rate of reduction of cytochrome *c* by 2-oxoglutarate and NADH, respectively. The mitochondrial preparation was preincubated with the appropriate amount of inhibitor (maleate or amytal) before use.

Reduction of NAD by 2-oxoglutarate dehydrogenase and oxidation of NADH by ETP were measured spectrophotometrically at 340 nm in a single cuvette in a medium containing in a final volume of 3 ml: 15 mM-potassium phosphate buffer (pH 7.3), 0.1 mM-CoA, 3.3 mM-cysteine-HCl, 2 mM-2-oxoglutarate, 0.1 mM-NAD. The reaction was started by the addition of 20 μg of 2-oxoglutarate dehydrogenase preparation, and the increase in extinction with time was monitored. After the reaction slowed down, 90 μg of ETP was added and the decrease in extinction was followed. Maleate, when used, was preincubated with 2-oxoglutarate dehydrogenase preparation for 5 min at 0°C.

Protein was determined by the biuret method (Gornall *et al.*, 1949) or according to Warburg & Christian (1941). 2-Oxoglutarate was measured by the procedure of Friedemann & Haugen (1943). Inorganic phosphate was determined by the method of Gomori (1953). Isocitrate was measured enzymically according to Stern (1957) and acetoacetate by the method of Walker (1954).

[1] Abbreviations used: DNP, 2,4-dinitrophenol; ETP, electron transfer particles.

Sodium acetoacetate was prepared after Seeley (1955), monosodium salt of 2-oxoglutarate according to Krebs *et al.* (1961) and DL-palmitoyl-carnitine by the method of Bremer (1962). ADP, ATP, hexokinase, antimycin, rotenone, L(-)malic acid and sodium pyruvate were from Sigma Chem. Co. (St. Louis, Mo., U. S. A.); maleic acid from v/o Sojuzchimexport (Moscow, U. S. S. R.); NAD, NADH and isocitrate dehydrogenase were from Boehringer (Mannheim, G. F. R.); succinic acid from Reanal (Budapest, Hungary) and Tris from British Drug Houses (Poole, Dorset, England). Other reagents were from Polskie Odczynniki Chemiczne (Gliwice, Poland.)

RESULTS

Effect of maleate on oxidation and phosphorylation in mitochondria. Oxidation of 2-oxoglutarate and glutamate by the coupled mitochondria of kidney cortex is decreased by half in the presence of 0.5 mM-maleate (Table 1). The decrease in oxygen uptake is accompanied by a lowered esterification of inorganic phosphate. The degree

Table 1

The effect of maleate on oxidation and phosphorylation in the coupled and uncoupled mitochondria of rat kidney cortex

Each Warburg vessel contained in a final volume of 2.0 ml: 10 mM-Tris-HCl buffer (pH 7.4), 20 mM--potassium phosphate buffer, 25 mM-KCl, 8 mM-$MgSO_4$, 50 mM-sucrose, 10 mM-substrate, 30 mM--glucose, 1 mM-ADP, 0.5 mg of yeast hexokinase (Sigma, type III), 0.1 mM-EDTA, and 2.0 - 2.5 mg of mitochondrial protein; time of incubation 30 min; gas-phase, air; temperature 30°C. Oxygen uptake is expressed in μatoms/30 min/5 mg of protein.

Substrate	Maleate (mM)	Coupled mitochondria		Mitochondria uncoupled with 0.01 mM-DNP	
		Oxygen uptake	P/O	Oxygen uptake	P/O
2-Oxoglutarate	—	6.80	2.92	6.89	2.08
	0.5	2.94	2.58	2.98	1.83
	1.0	1.88	1.87	2.02	1.36
	5.0	1.43	1.40	1.61	1.23
Glutamate	—	6.38	2.45	7.99	1.79
	0.5	2.98	2.18	3.33	1.50
	1.0	2.92	1.72	2.99	1.06
	5.0	1.96	1.17	2.32	0.43
Malate	—	7.99	2.07	9.05	1.33
	0.5	7.12	1.89	8.57	1.25
	1.0	6.86	1.96	8.20	1.10
	5.0	3.99	1.60	5.29	0.33
Succinate	—	20.6	1.73	18.5	1.56
	0.5	19.3	1.69	18.4	1.45
	1.0	20.3	1.61	19.1	1.44
	5.0	18.0	1.74	17.2	1.54

of inhibition of the latter process is greater than that of oxygen uptake, which results in the decreased efficiency of phosphorylation. With the increase in maleate concentration from 1 to 5 mM, the P/O ratio decreases by about a unity. Oxidation of malate and the related phosphorylation is practically unaffected by maleate over the concentration range from 0.5 to 1.0 mM; at maleate concentration of 5 mM, oxidation is inhibited in 50% and the phosphorylation efficiency is slightly decreased. DNP at concentration of 0.05 mM lowers the phosphorylation accompanying oxidation of 2-oxoglutarate, glutamate and malate by about unity but has no effect on the inhibitory action of maleate. Moreover, maleate and DNP act synergistically in decreasing phosphorylation.

Oxidation of succinate and the respective phosphorylation process are completely insensitive to maleate even at concentrations exceeding 5 mM, both in coupled and uncoupled kidney mitochondria (Table 1), in agreement with our previous data (Angielski & Rogulski, 1962; Rogulski & Angielski, 1963). In contrast to the inhibition observed in kidney, maleate at concentration up to 5 mM has no effect whatsoever on oxygen consumption, phosphorylation and 2-oxoglutarate metabolism, both in coupled and uncoupled liver mitochondria (Fig. 1).

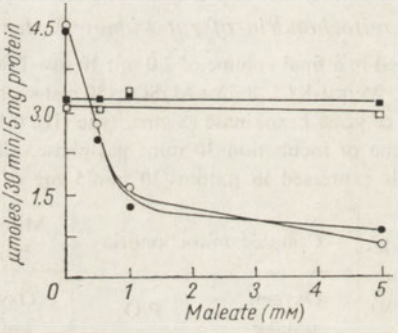

Fig. 1. The effect of maleate on the oxidation of 2-oxoglutarate by rat kidney (○) and liver (□) mitochondria. Experimental conditions as in Table 1. The results are expressed in µmoles/30 min/5 mg protein. Full symbols represent uncoupled mitochondria (0.01 mM-DNP).

Higher susceptibility of 2-oxoglutarate oxidation to maleate, as compared with that of malate and citrate (Angielski & Rogulski, 1962; Rogulski & Angielski, 1963) might be a secondary effect of inhibition of tricarboxylic acids cycle. Therefore, detailed investigations have been undertaken on the effect of maleate on isocitrate oxidation. As can be seen from the data given in Fig. 2, maleate has no effect on the consumption of isocitrate. However, the higher is maleate concentration, the greater the inhibition of oxygen uptake accompanied by a rising accumulation of 2-oxoglutarate. At maleate concentration of 5 mM, the relation of oxygen and isocitrate used and 2-oxoglutarate accumulated is stoichiometric which indicates that under these conditions oxidation of isocitrate takes place in one step and is not inhibited by maleate. The P/O ratio is practically unaffected which means that the effect on this ratio in mitochondria oxidizing 2-oxoglutarate should be referred to the substrate-level phosphorylation.

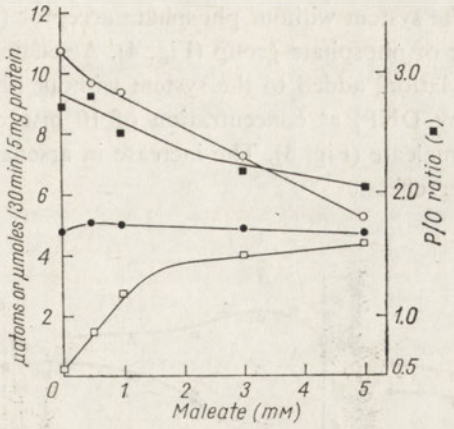

Fig. 2. The effect of maleate on isocitrate oxidation by rat kidney mitochondria. Experimental conditions as in Table 1. Oxygen uptake (○), isocitrate consumption (●), 2-oxoglutarate accumulation (□), and the P/O ratio (■).

In short-term polarographic experiments maleate shows only a slight inhibitory effect on 2-oxoglutarate oxidation by mitochondria in state 4. However, the same concentration of maleate inhibits very distinctly respiration in state 3, lowering the respiratory control ratio. Inhibition of mitochondrial respiration stimulated by DNP and by the phosphate acceptor system is similar. The results of manometric experiments confirmed the preliminary polarographic measurements and showed that respiration in state 4 is less inhibited than in state 3 (Table 2); the inhibition of oxygen uptake, phosphate esterification or substrate consumption does not exceed 65% in state 4, but in state 3 it reaches 90%.

Effect of phosphate and arsenate on the inhibition caused by maleate. It has been demonstrated that the higher is the phosphate concentration in the medium, the smaller is the inhibition by maleate both of oxygen and substrate consumption.

Table 2

Effect of maleate on the oxidation of 2-oxoglutarate in state 4 and state 3 in rat kidney mitochondria

The experimental conditions were as in Table 1 except that ADP was omitted in experiments on state 4 respiration. The concentration of maleate was 1 mM.

	State 4		State 3		Respiratory control ratio	P/O
	oxygen uptake	substrate consumption	oxygen uptake	substrate consumption		
	natoms or nmoles/min/mg protein					
Control	19.5	16.2	131.0	128.0	6.7	2.61
Maleate	7.0	5.5	15.4	16.0	2.2	1.66

This holds both for the system without phosphate acceptor (Fig. 3) and the system including the acceptor of phosphate group (Fig. 4). Arsenate, an uncoupler of substrate-level phosphorylation, added to the system without phosphate acceptor (respiration stimulated by DNP) at concentration of 10 mM diminishes by half the inhibition caused by maleate (Fig. 5). The increase in arsenate concentration up to 40 mM has no further effect.

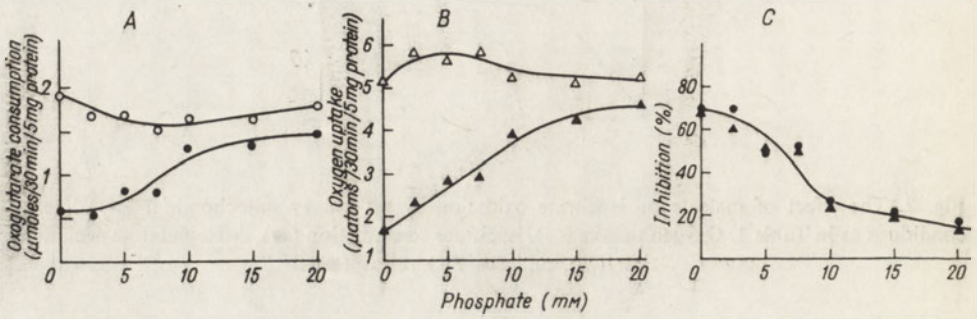

Fig. 3. The effect of phosphate on maleate-induced inhibition of 2-oxoglutarate oxidation in rat kidney mitochondria. The basic incubation medium was as in Table 1, except that the trapping system for phosphate was omitted. Since phosphate concentration varied, Tris-HCl buffer was added to maintain constant osmolarity of the medium. *A*, 2-oxoglutarate consumption without (○) and with (●) 1 mM-maleate; *B*, oxygen uptake without (△) and with (▲) 1 mM-maleate; *C*, inhibition of oxygen uptake (▲) and 2-oxoglutarate consumption (●).

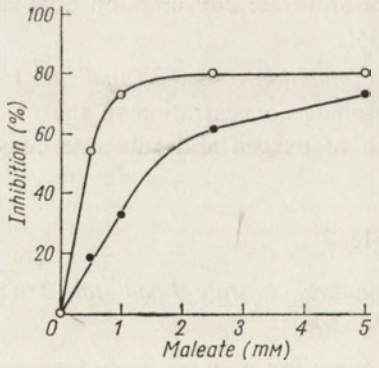

Fig. 4

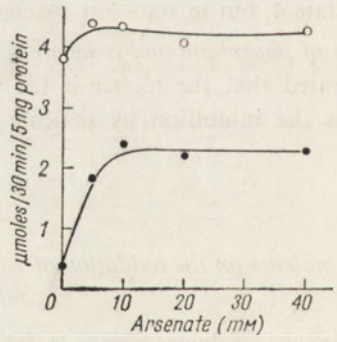

Fig. 5

Fig. 4. The effect of maleate on 2-oxoglutarate oxidation by kidney mitochondria at low and high concentration of phosphate. Experimental conditions as in Table 1; phosphate concentration: 20 mM (○) and 40 mM (●).

Fig. 5. The effect of arsenate on maleate-induced inhibition of 2-oxoglutarate oxidation in kidney mitochondria. Experimental conditions as in Table 1 except that phosphate concentration was 2.5 mM, the trapping system for phosphate was omitted and 0.01 mM-DNP was added. 2-Oxoglutarate consumption without (○) and with (●) 1 mM-maleate.

Influence of maleate on 2-oxoglutarate metabolism in the intact and disrupted kidney mitochondria. Anionic metabolites are known to affect the transport of similar metabolites through the mitochondrial membrane (Chappell & Haarhoff, 1967). One may assume, therefore, that inhibition by maleate of 2-oxoglutarate transport to kidney mitochondria may be the direct cause of the lowered metabolism of 2-oxoglutarate. It has been shown, however, that maleate inhibits 2-oxoglutarate oxidation by kidney mitochondria disrupted by repeated freezing and thawing or sonication, to the same or even higher extent as compared with the intact mitochondria (Table 3).

Table 3

Effect of maleate on the oxidation of 2-oxoglutarate by intact and disrupted rat kidney mitochondria

Experimental conditions as under Methods; the concentration of maleate was 1mM. The results are expressed in μatoms of O_2 and μmoles of 2-oxoglutarate utilized/30 min/5 mg protein.

Mitochondrial preparation	Oxygen uptake			2-Oxoglutarate consumption		
	control	maleate	inhibition (%)	control	maleate	inhibition (%)
Intact	7.05	1.91	73	4.38	1.05	76
Frozen-thawed	7.48	1.72	77	4.55	0.93	80
Sonicated	6.74	1.68	75	4.12	0.85	80

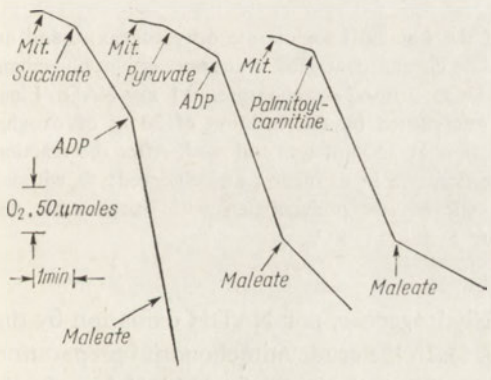

Fig. 6

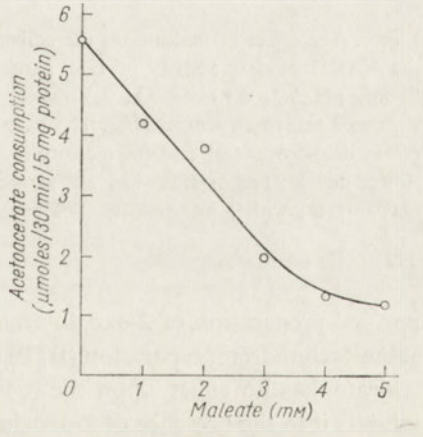

Fig. 7

Fig. 6. The effect of maleate on the oxidation of CoA-dependent substrates by kidney mitochondria. Oxygen uptake was recorded polarographically. Experimental conditions: 40 mM-Tris-HCl buffer, pH 7.4, 10 mM-potassium phosphate buffer, pH 7.4, 10 mM-KCl, 90 mM-sucrose, 5 mM-$MgSO_4$, and 2 mg of mitochondrial protein; concentration of substrates was 10 mM, except that of palmitoyl-carnitine which was about 0.2 mM; final volume 2 ml, temperature 25°C. Further additions of ADP and maleate to final concentrations of, respectively, 2.5 mM and 1 mM, are indicated in the Figure. For comparison, the tracing of oxygen uptake with succinate as substrate is included.

Fig. 7. The effect of maleate on acetoacetate metabolism in kidney mitochondria. Experimental conditions as in Fig. 5. The concentration of acetoacetate was 10 mM. Fumarate (0.5 mM) was included in the reaction mixture.

Effect of maleate on oxidation of other CoA-dependent substrates. It has been shown previously that maleate inhibits pyruvate and α-ketoisocapronate oxidation by kidney homogenate (Angielski & Rogulski, 1962; Angielski *et al.*, 1966). The metabolism of these substrates, like that of 2-oxoglutarate, is linked to CoA. Therefore, the effect of maleate on oxidation of some other CoA-dependent substrates was studied. As may be seen from Fig. 6, maleate distinctly inhibits oxidation of pyruvate and palmitoyl-carnitine; the oxidation of acetoacetate (Fig. 7) is inhibited, although to a smaller extent.

Effect of maleate on the activity of oxoglutarate dehydrogenase and the respiratory system. Maleate in concentrations up to 5 mM does not affect NAD reduction by the

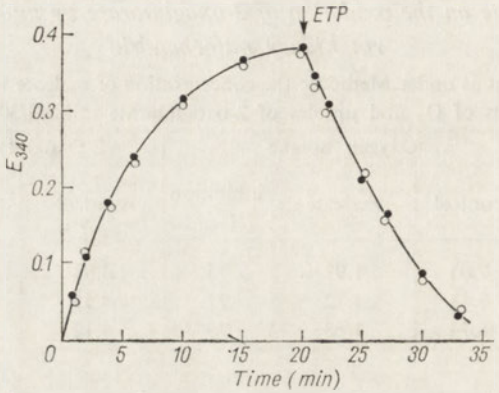

Fig. 8. The effect of maleate on the activity of the isolated oxoglutarate dehydrogenase, and on the NADH oxidase activity of ETP particles. The cuvette contained 15 mM-potassium phosphate buffer, pH 7.3, 0.1 mM-CoA, 3.3 mM-cysteine-HCl, 2 mM-2-oxoglutarate, 0.1 mM-NAD. Final volume 3 ml, room temperature. The reaction was started by the addition of 20 μg of oxoglutarate dehydrogenase, and the increase in extinction at 340 nm was followed. After the reaction slowed down, 90 μg of ETP was added and the decrease in extinction was observed: ○, without maleate; ●, with 5 mM-maleate. Before use, maleate was preincubated with oxoglutarate dehydrogenase for 5 min at 0°C.

purified preparation of 2-oxoglutarate dehydrogenase, nor NADH oxidation by the submitochondrial preparation (ETP) (Fig. 8). In the crude mitochondrial preparation maleate has no effect upon the reduction of cytochrome *c* by NADH but clearly inhibits it in the presence of 2-oxoglutarate (Fig. 9). This implies that maleate affects neither dehydrogenation of 2-oxoglutarate nor further steps of electron transfer. It seems that its action concerns the formation of the direct metabolite of 2-oxoglutarate, i.e. succinyl-CoA, or the substrate-level phosphorylation.

DISCUSSION

The results obtained substantiate our earlier data (Angielski & Rogulski, 1962; Rogulski & Angielski, 1963) pertaining to the effect of maleate on oxidative metabolism of 2-oxoglutarate in rat kidney mitochondria. Inhibition of oxygen uptake by mitochondria metabolizing other substrates of the Krebs cycle, i.e. citrate, isocitrate

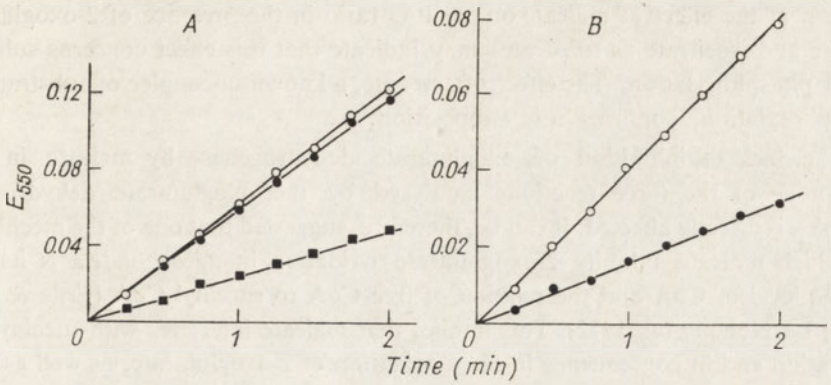

Fig. 9. The effect of maleate on the reduction of cytochrome *c* by NADH or 2-oxoglutarate. The cuvette contained 70 mM-Tris-HCl buffer, pH 7.4, 5 mM-$MgSO_4$, 2 mM-KCN, 1 mg of cytochrome *c*, and mitochondria disrupted by repeated freezing and thawing. Final volume 3.0 ml, room temperature. *A*, Reduction of cytochrome *c* by 0.1 mM-NADH; 5 μg of mitochondrial protein was used: ○, without inhibitor; ●, with 15 mM-maleate; ■, with 1.5 mM-amytal. *B*, Reduction of cytochrome *c* by 8 mM-2-oxoglutarate; 100 μg of mitochondrial protein was used: ○, without inhibitor; ●, with 1.5 mM-maleate.

and malate, appears to be directly caused by blocking oxidation at the 2-oxoglutarate step (Rogulski & Angielski, 1963). The inhibition of glutamate metabolism previously observed may be easily explained in the same terms since in kidney mitochondria oxidation of this substrate proceeds through the transamination step, and all inhibitors blocking 2-oxoglutarate metabolism inhibit simultaneously glutamate oxidation (Krebs & Bellamy, 1960). Maleate does not inhibit the conversion of citrate or isocitrate to 2-oxoglutarate but it lowers the concomitant oxygen uptake limiting the process to a one-step oxidation. The decrease in inhibition caused by high concentrations of inorganic phosphate, remains unexplained.

The data presented show that maleate has no inhibitory effect on electron transfer by the respiratory system, nor on the oxidative phosphorylation process. Since succinate oxidation is not susceptible to maleate action (Angielski & Rogulski, 1962), it seems possible that at partial inhibition of 2-oxoglutarate oxidation and complete lack of inhibition of succinate oxidation in the presence of maleate, the succinate resulting from 2-oxoglutarate oxidation is also oxidized which leads to a decrease in the overall P/O ratio. Therefore, it seems more reasonable to consider the ratio of phosphate esterified to 2-oxoglutarate metabolized. Although this ratio is also lowered by maleate, the dependence of the inhibition on maleate concentration is different than that of the P/O ratio.

It has been shown that inhibition of respiration by maleate in state 3 is higher than in state 4, and consequently we find a significant lowering of the respiratory control. The degree of mitochondrial coupling and integrity of mitochondrial membrane have no effect on the inhibition observed. The action of maleate is synergistic with that of dinitrophenol, which may suggest an uncoupling effect of maleate. A com-

parison of the effect of maleate on the P/O ratio in the presence of 2-oxoglutarate, malate and isocitrate as substrates may indicate that this effect concerns substrate--level phosphorylation. The effect of arsenate, a known uncoupler of substrate-level phosphorylation, confirms this supposition.

The lack of inhibition of oxoglutarate dehydrogenase by maleate indicates that none of the three reactions catalysed by the oxoglutarate dehydrogenase complex is directly affected. It can be, therefore, suggested that one of the mechanisms by which maleate inhibits 2-oxoglutarate oxidation in mitochondria is its effect on the level of CoA and the relation of free CoA to succinyl-CoA (Erfle & Sauer, 1969; La Noue *et al.*, 1972). This implies that maleate interferes with succinyl-CoA formation and in consequence inhibits oxidation of 2-oxoglutarate, as well as of the other CoA-dependent substrates. The inhibition of oxidative metabolism of pyruvate, palmitoyl-carnitine and acetoacetate by maleate, and the effect of arsenate observed, are in accordance with this interpretation.

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O MECHANIZMIE DZIAŁANIA MALEINIANU NA MITOCHONDRIA NERKI SZCZURA

WPŁYW NA PRZEMIANĘ OKSYDATYWNĄ

Streszczenie

1. Maleinian hamuje zużycie tlenu i fosforylację w mitochondriach nerki szczura utleniających ketoglutaran; na inhibicję nie mają wpływu stopień sprzężenia mitochondriów ani integralność błony mitochondrialnej.

2. Maleinian hamuje też utlenianie innych substratów, których metabolizm zależy od CoA, tj. pirogronianu, palmitylo-karnityny i acetooctanu. Zmniejszenie zużycia tlenu przez mitochondria metabolizujące izocytrynian lub jabłczan przy wysokim stężeniu maleinianu jest spowodowane zablokowaniem utleniania produktu reakcji, tj. ketoglutaranu.

3. Maleinian nie działa wprost na izolowaną dehydrogenazę ketoglutaranu ani na transport elektronów. Arsenian, podobnie jak i wysokie stężenie fosforanu, obniżają hamujące działanie maleinianu. Przypuszcza się, że miejscem działania maleinianu jest fosforylacja substratowa lub inne reakcje sukcynylo-CoA.

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TRANSPORT OF GLYCOSIDES OF OLEANOLIC ACID FROM SHOOT TO ROOT IN *CALENDULA OFFICINALIS**

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The results of ^{14}C-labelling of individual oleanolic acid glycosides from various parts of 60-, 90- and 140-day-old plants of *Calendula officinalis* confirmed our previous suggestion that oleanolic acid is transported from leaves to the root mainly in the form of pentaglycosides.

It has been shown previously (Kasprzyk *et al.*, 1973) that in the shoots of *Calendula officinalis* oleanolic acid glycosides are formed by successive addition of glucose in position 3 of oleanolic acid, with formation of monoglucoside I (3-glucoside) as the first member of one series of oleanolic acid glucosides.

Studies from our laboratory on the distribution of oleanolic acid glycosides in *C. officinalis* during vegetation have shown the presence of these compounds in the shoot of 30-day-old plants and in the root of 60-day-old ones (Miller, 1970). In roots, pentaglycosides are the first to appear, followed successively by glycosides with a lower number of sugar molecules: tetraglucoside, triglucoside, diglucoside and monoglucoside, and at last — in old plants — free oleanolic acid (Wasilewska, 1970).

These results suggested that pentaglycosides — the derivatives of 3-monoglucoside of oleanolic acid — are transported to roots from the shoots (Kasprzyk *et al.*, 1973). The present isotopic studies were designed to check this suggestion.

MATERIAL AND METHODS

Material. Plants of *Calendula officinalis* var. Radio were cultivated in a lumistate under conditions described previously (Kasprzyk *et al.*, 1968). Experiments were performed on the 60-, 90- and 140-day-old plants.

The root, the lower and upper parts of the stem, the leaf exposed to $^{14}CO_2$, and other leaves were separately fractionated by a previously described procedure (Kasprzyk *et al.*, 1973), and the isolated glycosides were hydrolysed under the previously employed conditions (Kasprzyk *et al.*, 1970).

* This study was carried out under project no. 09.1.7, coordinated by the Institute of Ecology of the Polish Academy of Sciences.

$^{14}CO_2$ *incorporation.* A single lower leaf of *C. officinalis*, non-detached from the whole plant, was exposed to $^{14}CO_2$ in a chamber with a tight-fitting cover. $^{14}CO_2$ was obtained by drop-wise addition of a 50% H_2SO_4 solution to a vessel situated inside the chamber and containing 100 μCi of $Na_2{}^{14}CO_2$ (specific radioactivity 16 mCi/mmol. The plant was irradiated for 3 h at 40 000 lux with luminescent lamps of Polish production; subsequently, the non-assimilated $^{14}CO_2$ was pumped out during 30 min; the air from the chamber was passed through a washer containing KOH solution. Under the conditions employed, about 97% of the administered $^{14}CO_2$ was assimilated. The plants were then transferred to a lumistate, and analysed after appropriate time intervals.

Radioactivity of the oleanolic acid glycosides was measured with a thin-window GM counter, with 3% yield.

RESULTS AND DISCUSSION

It has been shown (Kasprzyk *et al.*, 1968) that in *C. officinalis* oleanolic acid is transported both to the upper and lower parts of the plant in the form of glycosides. From the upper leaves it is transported mainly to the inflorescence, and from the lower ones to the roots. Since the transport in old plants takes place mainly in the downward direction, the use was made in the present study not only of the 60- and 90-day-old plants, but also of the 140-day-old ones. $^{14}CO_2$ was always administered to the lower leaves, which had completed growth. The results obtained at appropriate time intervals after precursor administration, were expressed in c.p.m. per given organ. This way of expressing the results permitted of better illustration of the transport of oleanolic acid glycosides, as compared with the results referred to fresh weight. The amount of metabolites, including oleanolic acid, which become labelled with $^{14}CO_2$ in one leaf during 3 h is limited, and the transport of this acid can be monitored more closely by determining the total radioactivity distribution among different organs.

Radioactivity measurements concerned both the glycosides derived from 3-glucoside of oleanolic acid (glucosides) and glycosides derived from 3-glucuronoside of oleanolic acid (i.e. glucuronosides). The latter compounds occur in all organs of *C. officinalis* during the whole vegetation period (Kasprzyk & Wojciechowski, 1967).

In the non-flowering 60-day-old plants analysed 5 days after $^{14}CO_2$ administration, the analysis of glucuronosides revealed that in the leaf treated with $^{14}CO_2$ and in other leaves, most of the radioactive label was incorporated into diglucuronoside D [3-(3′-galactosyl)-glucuronoside], and less, in decreasing order, into monoglucuronoside F [3-glucuronoside], triglucuronosides C [3-(3′-galactosyl)-glucuronoside,28-glucoside] and B [3-(3′-galactosyl,4′-glucosyl)-glucuronoside] and tetraglucuronoside A [3-(3′-galactosyl,4′-glucosyl)-glucuronoside,28-glucoside] (Fig. 1). Similar results were obtained with the plant analysed 10 days after ^{14}C labelling; in this case, in the $^{14}CO_2$-treated leaf most of the radioactivity was detected in glucuronoside C, i.e. in the glycoside with a higher number of sugars in the molecule. These results are consistent with the previously presented scheme of biosynthesis of

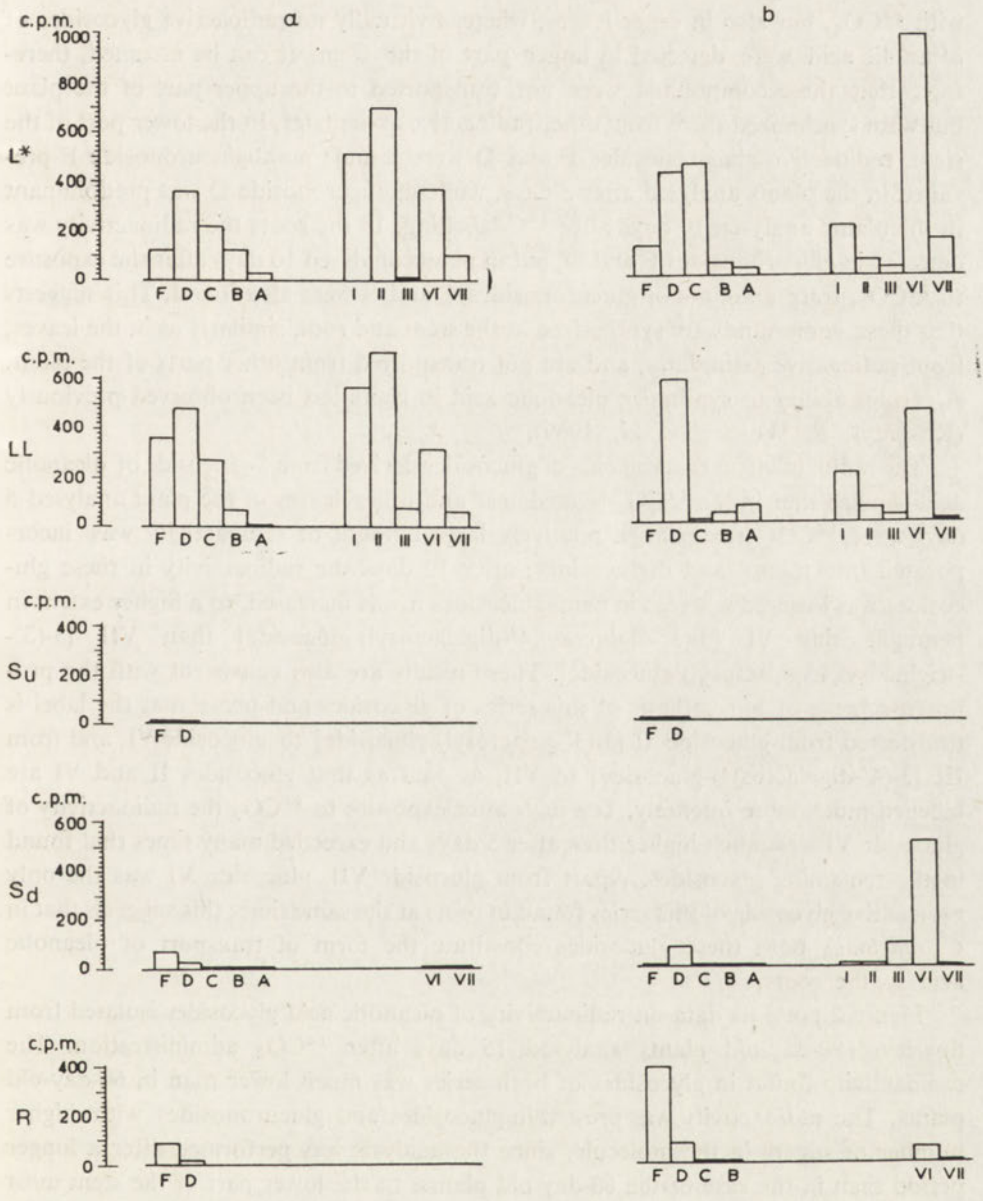

Fig. 1. Radioactivity of oleanolic acid glycosides from various parts of 60-day-old plants analysed on the 5th (*a*) and 10th (*b*) day after $^{14}CO_2$ administration. L*, the leaf fed with $^{14}CO_2$; LL, other leaves; S_u, part of the stem above L*; S_d, part of the stem below L*; R, root. The results are expressed in c.p.m./organ. F - A, glucuronosides; I - VII, glucosides. For further details see Text.

oleanolic acid glucuronosides (Kasprzyk *et al.*, 1970; Kintia *et al.*, 1974) and testify to their formation by way of successive addition of single sugars.

Radioactive glycosides of oleanolic acid were found not only in the leaf treated with $^{14}CO_2$, but also in other leaves, whereas virtually no radioactive glycosides of oleanolic acid were detected in upper part of the stem. It can be assumed, therefore, that these compounds were not transported to the upper part of the plant but were synthesized there from other radioactive assimilates. In the lower part of the stem, radioactive glucuronosides F and D were found; monoglucuronoside F prevailed in the plants analysed after 5 days, while diglucuronoside D was predominant in the plants analysed 10 days after ^{14}C-labelling. In the roots the radioactivity was detected in glucuronosides F and D, but in plants analysed 10 days after the exposure to $^{14}CO_2$, trace amounts of glucuronosides C and B were also found. This suggests that these compounds are synthesized in the stem and root, similarly as in the leaves, from radioactive assimilates, and are not transported from other parts of the plant. A certain ability to synthesize oleanolic acid in roots has been observed previously (Kasprzyk & Wojciechowski, 1969).

The radioactivity measurements of glucosides derived from 3-glucoside of oleanolic acid showed that in the $^{14}CO_2$-treated leaf and other leaves of the plant analysed 5 days after $^{14}CO_2$ treatment, a relatively large amount of radioactivity was incorporated into mono- and diglucosides; after 10 days the radioactivity in these glucosides was lowered whereas in pentaglucosides it was increased, to a higher extent in pentaglucoside VI [3-(3′-diglucosyl,4′-digalactosyl)-glucoside] than VII [3-(3′--triglucosyl,4′-galactosyl)-glucoside]. These results are also consistent with the proposed scheme of biosynthesis of this series of glycosides and prove that the label is transferred from glucoside II [3-(3′-galactosyl)-glucoside] to glucoside VI, and from III [3-(4′-digalactosyl)-glucoside] to VII, as well as that glucosides II and VI are labelled much more intensely. Ten days after exposure to $^{14}CO_2$ the radioactivity of glucoside VI was much higher than after 5 days and exceeded many times that found in the remaining glycosides. Apart from glucoside VII, glucoside VI was the only radioactive glycoside of this series found in roots at the same time; this suggests that in *C. officinalis* both these glucosides constitute the form of transport of oleanolic acid to the roots.

Figure 2 presents data on radioactivity of oleanolic acid glycosides isolated from flowering 90-day-old plants analysed 15 days after $^{14}CO_2$ administration. The radioactivity found in glycosides of both series was much lower than in 60-day-old plants. The radioactivity was present in glucosides and glucuronosides with higher number of sugars in the molecule, since the analysis was performed after a longer period than in the case of the 60-day-old plants. In the lower part of the stem most of the radioactivity was localized in glucoside VI but a small amount of labelled glucosides with a lower number of sugars in the molecule, i.e. glucosides III, II and I, was also detected. In the root — in addition to radioactive glucosides VI and VII — traces of radioactivity were found, as in the stem, in glucosides III, II and I. It seems that the appearance of these lower glucosides at this time of vegetation, is due to degradation of glucoside VI (Wasilewska, 1970).

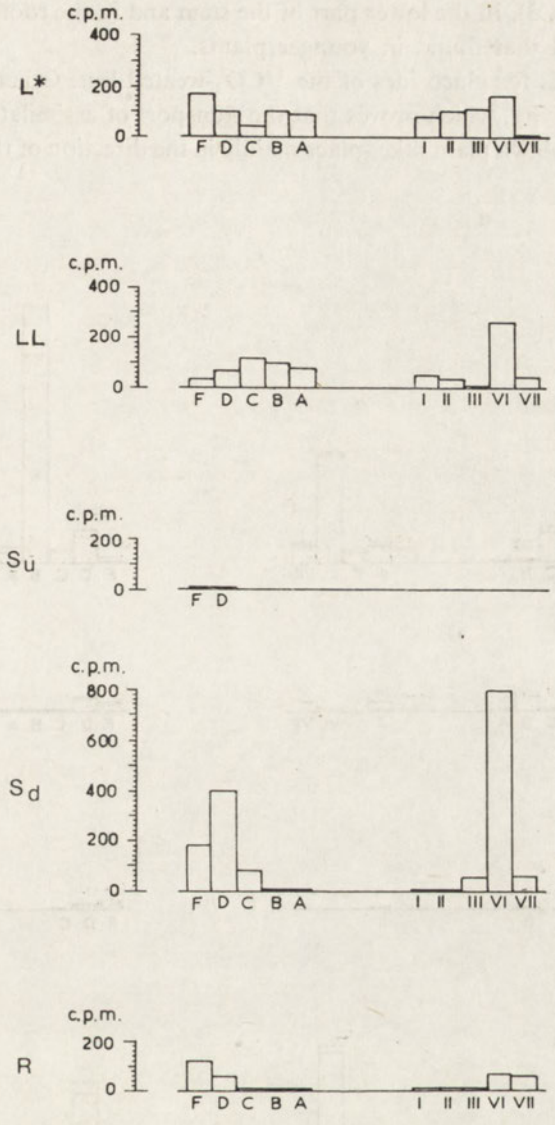

Fig. 2. Radioactivity of oleanolic acid glycosides from various parts of 90-day-old plants analysed on the 15th day after $^{14}CO_2$ administration. Abbreviations and symbols as in Fig. 1.

The amount and distribution of radioactivity in glucuronosides in the stem and root resembled those observed in 60-day-old plants analysed 10 days after $^{14}CO_2$ treatment.

Distribution of radioactivity in the oleanolic acid glucuronosides in the $^{14}CO_2$--treated leaf of the 140-day-old overblown plant analysed 10 days after exposure to the isotope, was similar to that observed for a 60-day-old plant. In the leaf treated with $^{14}CO_2$ and analysed after 20 days, radioactivity was greatly increased in glu-

curonoside B (Fig. 3). In the lower part of the stem and in the root, the radioactivity pattern resembled that found in younger plants.

The same holds for glucosides of the $^{14}CO_2$-treated leaf. Other leaves contained traces of radioactivity, which proves that the transport of assimilates from the lower leaves of the overblown plant takes place mainly in the direction of the root (Williams,

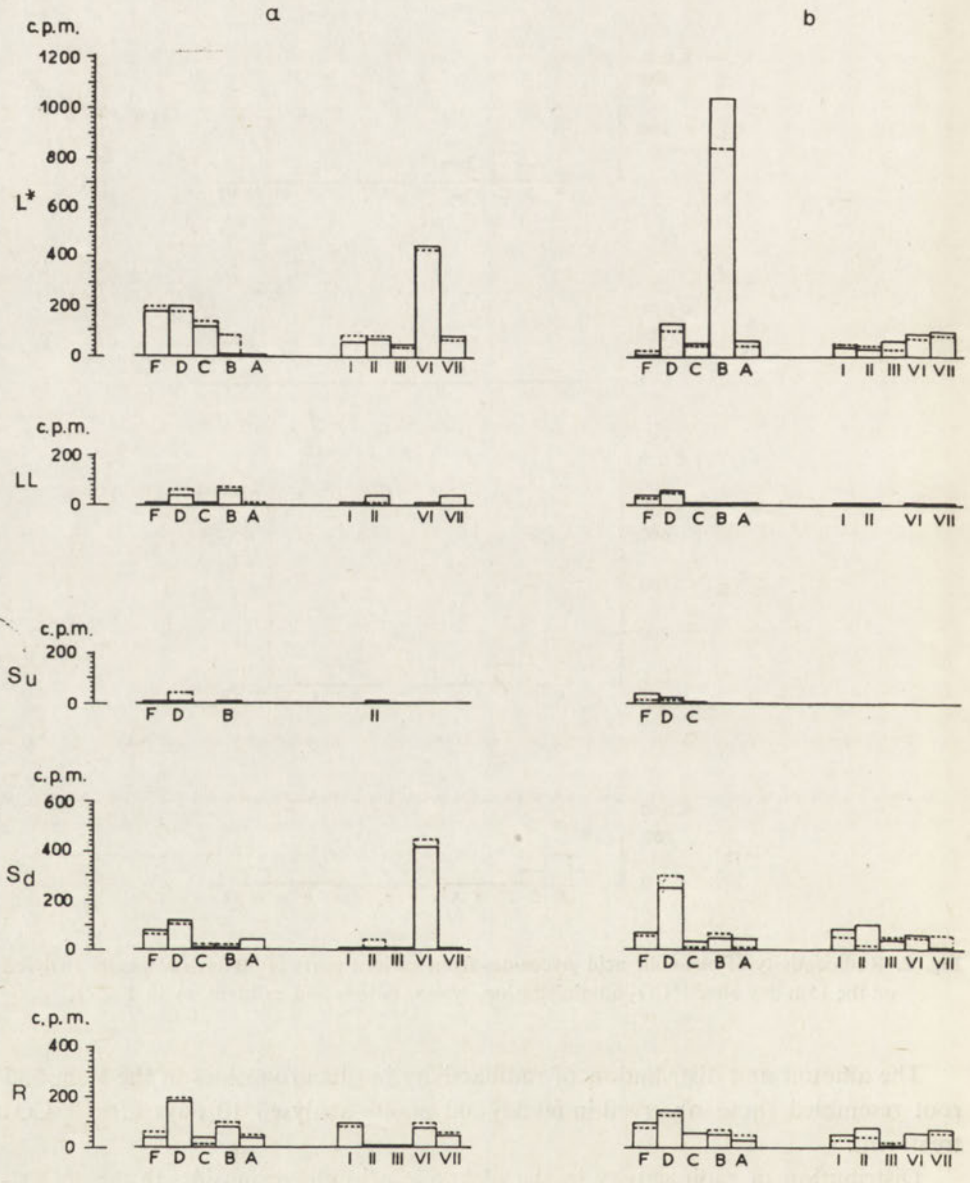

Fig. 3. Radioactivity of oleanolic acid glycosides from various parts of 140-day-old plants analysed on the 10th (*a*) and 20th (*b*) day after $^{14}CO_2$ administration. Abbreviations and symbols as in Fig. 1. Full and broken lines correspond, respectively, to experiment I and II.

1964). In the lower part of the stem of plants analysed 10 days after exposure, most of the radioactivity appeared in glucoside VI, and much less in the other glucosides. The same radioactivity patterns of glucosides were found in the lower part of the stem and in the roots of plants analysed 20 days after $^{14}CO_2$ administration. Total radioactivity in glycosides of both series was lower in the roots of plants analysed after 20 days, as compared with those analysed after 10 days, presumably due to degradation of glycosides to free oleanolic acid, which increasingly accumulated in older plants (Wasilewska, 1970).

The data presented indicate that in leaves the synthesis of glycosides proceeds the more intensely, the younger is the plant, and confirm also our supposition that in *C. officinalis* oleanolic acid is transported from the leaves to the root mainly in the form of glucoside VI and partially as glucoside VII, the biosynthesis of VI preceding that of VII. Finally, the detection of radioactive glucosides with lower number of sugar molecules in aging plants proved that these compounds are formed as a result of degradation of pentaglucosides.

The authors wish to thank Miss A. J. Buchowicz for skillful technical assistance.

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TRANSPORT GLIKOZYDÓW KWASU OLEANOLOWEGO Z PĘDÓW DO KORZENIA *CALENDULA OFFICINALIS* L

Streszczenie

Badano włączenie piętna z $^{14}CO_2$ w glikozydy kwasu oleanolowego w liściach, łodydze i korzeniu 60-, 90- i 140-dniowych roślin. Potwierdzono wcześniejsze przypuszczenie, że transport kwasu oleanolowego z liści do korzenia w nagietku odbywa się w postaci glikozydów VI i VII, przy czym główną rolę w tym procesie odgrywa glikozyd VI.

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THE LYSINE-RICH HISTONE *f1* FROM OX BRAIN

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It has been demonstrated that the lysine-rich histone *f1* can be prepared from ox brain by extraction with 0.15 M-sulphosalicylic acid, precipitation with tannin and liberation with caffeine in a strongly acidic medium. This indicates that the described procedure is suitable for preparation of histone *f1* not only from tissues rich in nuclei but also from those having a low content of nuclei.

In our laboratory, extraction of polypeptides and glycoproteins from various plant and animal tissues is performed using 0.15 M-sulphosalicylic acid (SSA). It has been found that SSA may also be used for extraction of the lysine-rich histone *f1*, which can be precipitated from the SSA-extract with tannin and liberated from the complex by caffeine. By this method, histone fraction *f1* was isolated from calf thymus (K. Maskos & Mejbaum-Katzenellenbogen, 1968), rabbit kidney (Wieczorek & Mejbaum-Katzenellenbogen, 1968), ox thymus, kidney, pancreas, thyroid gland and spleen, and from human leucocytes (Olichwier *et al.*, 1971), as well as from rabbit and rat skeletal muscle (Ch. Maskos & Mejbaum-Katzenellenbogen, 1971).

In the present work, an attempt was made to isolate histone *f1* by the above method from ox brain tissue, which has a low content of nuclei and differs largely in chemical composition from other tissues.

MATERIALS AND METHODS

Material. Ox brain was obtained immediately after death of the animal and transported from the slaughterhouse to the laboratory in ice. Duck blood was collected directly to a solution of sodium citrate of final concentration 1%. Except where indicated, all the procedures were carried out at a temperature not exceeding 5°C.

Isolation of nuclei. From ox brain, nuclei were obtained by the method of Bondy *et al.* (1970) which consists in homogenization of the tissue in 0.32 M-sucrose, centrifugation at 750 *g*, purification of nuclei by centrifugation in 2.4 M-sucrose at 34 000 *g* and washing with 0.14 M-NaCl.

To obtain nuclei from duck erythrocytes, blood was centrifuged for 45 min at 1000 *g*, and the cells were washed four times by stirring for 10 min with 0.14 M-NaCl - - 0.1 M-sodium citrate solution, then the erythrocytes were haemolysed by two cycles of freezing in solid CO_2 and thawing at room temperature. The nuclei were separated by sedimentation for 60 min at 1000 *g*, and washed twice with the above washing solution.

Sulphosalicylic-acid soluble proteins. The brain tissue, erythrocytes, or isolated nuclei were homogenized in a Unipan homogenizer with 4 vol. of 0.15 M-SSA for 2 min and centrifuged for 20 min at 1100 *g*. The supernatant was clarified by twofold filtration through a double layer of filter paper, and the extracted protein isolated by the tannin-caffeine procedure (Mejbaum-Katzenellenbogen, 1959a,b).

In this procedure, protein is precipitated with tannin and the tannin-protein complex washed three times with 0.14 M-NaCl. Then the protein is liberated from the complex into a small volume (0.2 - 0.5 ml) of 0.02 M-HCl or 0.14 M-NaCl by adding caffeine at a twofold excess in relation to protein. The tannin-caffeine sediment is centrifuged off, and the solution of liberated protein dialysed for 24 h against water and freeze-dried.

In the present work, the tannin-caffeine procedure was carried out under three sets of conditions: I, tannin was added directly to the SSA-extract (pH 1.5) to a final concentration of 1%, and protein was liberated by caffeine in 0.02 M-HCl medium; II, the SSA-extract was adjusted to pH 5.3 with NaOH, tannin was added at a threefold excess in relation to protein, and the protein was liberated in 0.02 M-HCl medium; III, tannin was added at a threefold excess at pH 5.3 (as under conditions II), and the protein liberated in 0.14 M-NaCl medium.

Analytical methods. Protein was determined by the turbidimetric tannin micromethod (Mejbaum-Katzenellenbogen, 1955).

CM-cellulose column chromatography was performed as in the previous work (Wieczorek & Mejbaum-Katzenellenbogen, 1968). About 100 mg of protein dissolved in 0.05 M-Na-acetate buffer, pH 4.4, was applied to the column (1 × 20 cm) and eluted with a potassium chloride concentration gradient (0 - 0.6 M-KCl) in the above buffer. Fractions of 3 ml were collected and protein was determined by measuring the extinction at 235 and 280 nm. Fractions corresponding to the histone *f1* peak were pooled, dialysed against water and freeze-dried.

Polyacrylamide-gel electrophoresis was carried out by the method described by Panyim & Chalkley (1969). Samples of about 20 μg of protein in 0.15 M-sucrose were applied to the gels and electrophoresis was run at room temperature for 2 h at 2.0 mA per tube. The electrode vessels contained 0.9 M-acetic acid. The gels were stained with 0.2% Amido Black 10B in 7% acetic acid for 12 h, and destained with 7% acetic acid.

Standard histone preparations were obtained from calf thymus. Total histone was prepared according to Davison *et al.* (1954) with some modifications. The nucleoprotein isolated by several-fold washing with acidified 0.14 M-NaCl (Phillips & Johns, 1959) was extracted three times with twice its volume of 0.25 M-HCl. Following

filtration, the histone solution was dialysed against 1 mM-HCl and freeze-dried (Johns *et al.*, 1961). The lysine-rich histone *f1* was prepared by method 1 of Johns (1964).

Reagents. Tannin was from U. S. S. R. (ser. no. 750666), Amido Black 10B from Grübler (Leipzig, G. D. R.), CM-cellulose Whatman CM-11 from Balston Ltd (Maidstone, Kent, England), acrylamide from B. D. H. Ltd (Poole, England), *N,N'*-methylene bisacrylamide from Koch-Light Lab. (Colnbrook, Bucks., England), *N,N,N',N'*-tetra-methylethylenediamine from Eastman Kodak (New York, U. S. A.). Other reagents were from P. O. Ch. (Gliwice, Poland).

RESULTS

The protein preparations from SSA-extracts of duck blood erythrocytes obtained by the tannin-caffeine procedure under three different sets of conditions, showed on polyacrylamide-gel electrophoresis the same patterns (Fig. 1a,b,c). In all cases,an intense band of lysine-rich histone *f1* was obtained, and two distinctly smaller fractions, the mobility of one of them being close to that of histone *f2a1*. Unlike the protein from duck erythrocytes, rich in nuclei, the SSA-soluble protein from brain tissue, in which nuclei form but a small fraction, showed electrophoretic patterns differing with the conditions of the tannin-caffeine procedure used. The protein precipitated with tannin at pH 1.5 or 5.3 and liberated from the complex by caffeine in acidic medium (0.02 M-HCl) gave practically the same patterns, with a faint band of histone *f1* and two bands with the highest cathodic mobility (Fig. 1d,e), the same as in the preparations from erythrocytes. On the other hand, when the brain protein was

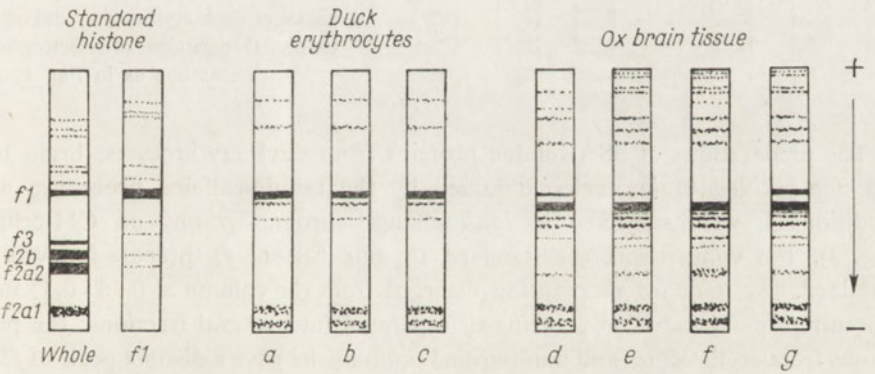

Fig. 1. Polyacrylamide-gel electrophoresis of sulphosalicylic acid-soluble protein preparations from duck erythrocytes and ox brain, obtained by the tannin-caffeine procedure under three sets of conditions. The amount of protein applied to the gel was 20 μg. Histone standards were obtained from calf thymus after Phillips & Johns (1959) and Johns (1964), and preparations from erythrocytes and brain as follows: *a* and *d*, protein was precipitated with 1% tannin directly from the SSA--extract and liberated with caffeine in 0.02 M-HCl medium (conditions I); *b* and *e*, the SSA-extract was adjusted to pH 5.3, the protein was precipitated with a threefold excess of tannin and solubilized with caffeine in 0.02 M-HCl medium (conditions II); *c* and *f*, the protein was precipitated as under conditions II and solubilized in 0.14 M-NaCl medium (conditions III); *g*, standard histone *f1* from thymus was added to the brain homogenate and proteins treated under conditions II.

liberated in neutral salt medium (0.14 M-NaCl), the electrophoretic fraction with the mobility of histone *f1* was absent (Fig. 1f); neither was it possible to recover the standard histone fraction *f1* added to the brain homogenate. None the less, the added standard histone *f1* was recovered under conditions I and II (Fig. 1g).

The electrophoretic patterns of brain proteins obtained by the three methods showed a very strong band somewhat faster than fraction *f1*, which did not correspond to any of the histone fractions, and was not identified.

The SSA-soluble proteins from duck erythrocyte nuclei, similarly as the proteins from whole erythrocytes, irrespective of the conditions of the tannin-caffeine procedure, gave on electrophoresis an intense band of the lysine-rich histone *f1*, and five faintly stained cathodic bands (Fig. 2a,b,c). The results for brain nuclei resembled those for proteins from whole brain tissue, except that the "non-histone" band was absent. The band of the lysine-rich histone *f1* appeared only in those preparations in which the protein was liberated by caffeine in 0.02 M-HCl medium (Fig. 2d,e), whereas this fraction was undetectable when the protein was liberated from the complex in 0.14 M-NaCl (Fig. 2f).

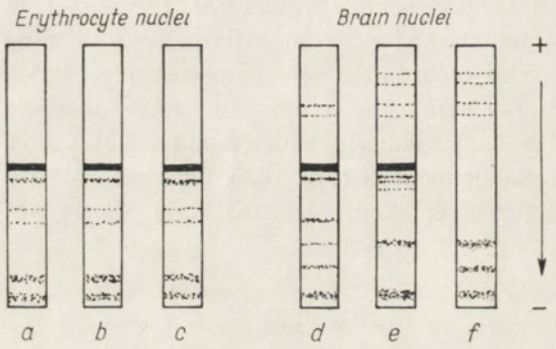

Fig. 2. Polyacrylamide-gel electrophoresis of SSA-soluble protein from isolated nuclei of duck erythrocytes and ox brain tissue. Designation of electrophoretograms *a*-*f* as in Fig. 1.

The preparations of SSA-soluble proteins from duck erythrocytes, brain tissue and non-purified brain nuclei, obtained by the tannin-caffeine procedure under conditions I, were subjected to ion-exchange chromatography on CM-cellulose (Fig. 3). For comparison, the standard thymus histone *f1* preparation was also examined. The standard preparation emerged from the column at 0.35 - 0.37 M-KCl concentration. The studied proteins all separated into several fractions; the preparations from erythrocytes and non-purified brain nuclei gave a distinct peak of *f1*, but this fraction was not detected in the preparation from whole brain tissue.

The fractions *f1* separated by chromatography were subjected to gel electrophoresis, and the obtained patterns compared with those for the corresponding preparations prior to chromatography (Fig. 4). Purification by CM-cellulose chromatography did not remove all the ballast proteins. The electrophoretograms of the studied chromatographic fractions *f1* showed fewer bands but one of the two distinct most cathodic fractions was still present. This fraction, probably of nuclear origin (see Fig. 2), was absent from the electrophoretogram of standard histone *f1* prepared according to Johns (1964).

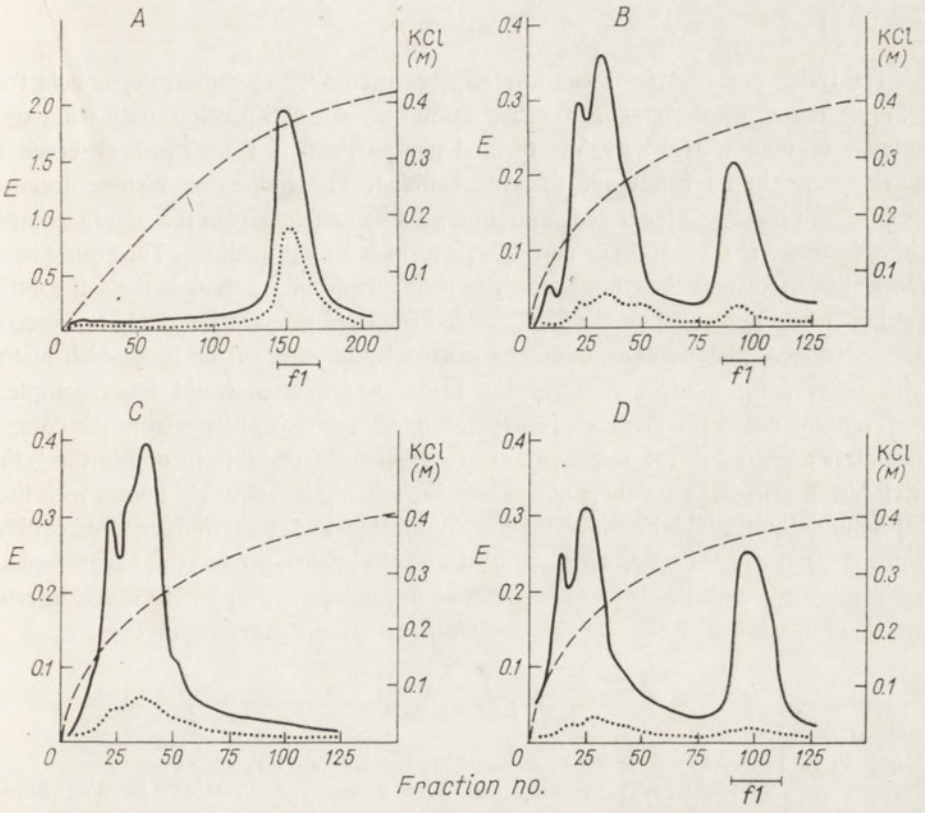

Fig. 3. Ion-exchange chromatography on CM-cellulose of proteins obtained from SSA-extracts by the tannin-caffeine procedure I. Protein, 100 μg, was applied and eluted with a KCl concentration gradient (— — —); *A*, standard lysine-rich histone *f1* (Johns, 1964); *B*, duck erythrocytes; *C*, ox brain; *D*, non-purified brain nuclei (precipitate at 750 *g* of the sucrose homogenate). ——, $E_{235\ nm}$; • • •, $E_{280\ nm}$.

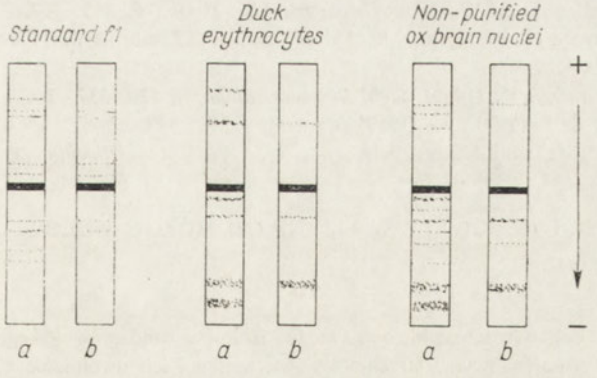

Fig. 4. Electrophoretic patterns of standard histone *f1* and the SSA-soluble proteins isolated from duck erythrocytes and non-purified ox brain nuclei. The amount of protein applied to the gel was 20 μg. *a*, preparations not subjected to chromatography; *b*, chromatographic fractions *f1* (see Fig. 3).

DISCUSSION

The lysine-rich histone *f1* can be readily extracted with sulphosalicylic acid from ox brain tissue, similarly as from other animal tissues, and precipitated with a small amount of tannin. However, the formed complex differs from those observed for other tissues in its behaviour towards caffeine. The lysine-rich histone fractions from all the tissues so far tested, were liberated by caffeine from the tannin complex either in acidic (0.02 M-HCl) or neutral salt (0.14 M-NaCl) medium. The brain histone *f1* was liberated from the complex only in acidic medium, whereas in the salt solution neither endogenous histone *f1* nor the added standard preparation could be detected. The described phenomenon could be caused by binding of the lysine-rich histone with other compounds present in the brain, which also would form complexes with tannin. When the medium, in which the liberation of protein from the complex occurs, is adjusted to an acidic pH value, it becomes possible to obtain the lysine-rich histone which under these conditions is probably less strongly bound with those compounds and could be more readily solubilized under the influence of caffeine. Since this phenomenon was also observed with the protein preparations from isolated brain nuclei, it might point to the presence in nuclei of compounds which interfere with liberation of the lysine-rich histone from the protein-tannin complex.

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HISTON BOGATY W LIZYNĘ (*f1*) MÓZGU WOŁOWEGO

Streszczenie

Wykazano, że bogaty w lizynę histon *f1* można izolować z mózgu wołowego stosując ekstrakcję 0.15 M-kwasem sulfosalicylowym, strącanie białek taniną i ich uwolnienie z kompleksu kofeiną w środowisku silnie kwaśnym. Opisaną metodę można stosować do preparacji histonu *f1* nie tylko z materiałów bogatych w jądra komórkowe, lecz także z materiałów o niskiej ich zawartości.

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THE EFFECT OF AZETIDINE-2-CARBOXYLIC ACID ON THE BIOSYNTHESIS OF COLLAGEN IN RAT GRANULOMA**

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L-Azetidine-2-carboxylic acid (Az) is incorporated *in vitro* into granuloma collagen, and simultaneously inhibits to a similar extent the incorporation of L-[U-^{14}C]proline and [2-^{3}H]glycine. Paper chromatography of alkaline hydrolysates of the Az-containing collagen showed that the incorporated Az was unaltered. The Az-containing collagen was less readily hydrolysed by bacterial collagenase, and more readily by trypsin; after the trypsin treatment, it was more susceptible to collagenase action.

The four-membered heterocyclic imino acid azetidine-2-carboxylic acid (Az) is a lower analogue of proline. Az occurs in plants in free form (Virtanen, 1955; Fowden, 1956; Fowden & Bryant, 1958) and was found to be incorporated into the actinomycins of *Streptomyces antibioticus* (Katz, 1960). The structural resemblance between Az and proline probably explains, in part, the antimetabolite action of Az toward proline (Fowden & Richmond, 1963; Puck & Kao, 1968); this action becomes manifest in the inhibition of protein synthesis (Fowden & Richmond, 1963) and the growth of microorganisms (Tristram & Thurston, 1966; Turnock & Wild, 1966; Baich & Smith, 1968), plants (Fowden & Bryant, 1959) and mammalian cells in tissue culture (Puck & Kao, 1968). It has also been reported that Az inhibits collagen synthesis *in vitro* in preparations from chick embryos (Zamaraeva *et al.*, 1969; Lane *et al.*, 1971a) and that [4-^{14}C]Az is indeed incorporated into collagan (Takeuchi *et al.*, 1969), and alters its morphological properties (Lane *et al.*, 1971b). These authors also demonstrated that fewer prolyl residues in protocollagen were hydroxylated when Az residues were present in the polypeptide chain. It has been

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postulated that this effect is probably due to the alteration of the polypeptide substrate interacting with protocollagen-prolyl-hydroxylase (Takeuchi & Prockop, 1969). Interference by Az residues with hydroxylation reaction was not limited to prolyl residues, but also was noted in the case of lysyl residues of protocollagen. Consequently, it is likely that the normal number of *O*-glycosylated hydroxylysyl residues are not formed. The latter deficiency, as well as other as yet unknown factors, may be responsible for the markedly reduced transport of low-molecular-weight collagen molecules from intra- to the extra-cellular space (Takeuchi & Prockop, 1969) which, in turn, may affect the extent of fiber formation in connective tissue.

The inhibitory action of Az on protein synthesis could be due to its incorporation into the peptide chain in place of prolyl residues (Fowden, 1964; Dancewicz & Altman, 1968; Takeuchi & Prockop, 1969; Takeuchi *et al.*, 1969; Lane *et al.*, 1971b); the incorporation would be facilitated by the presence of Az-tRNA synthetase demonstrated by Atherby & Bell (1964), Peterson & Fowden (1965) and Bublitz (1966). Moreover, Az may interfere with the availability of adequate supplies of proline precursors, e.g. glutamic acid, and thus limit protein synthesis (Tristram & Thurston, 1966; Baich & Smith, 1968; Ishibashi *et al.*, 1968). Az could also inhibit proline transport across the cell membrane and thus interfere with the maintenance of adequate concentrations of intracellular proline.

In the present work, the effect of Az on the biosynthesis of granuloma collagen, a protein exceptionally rich in pyrrolidine-containing imino acids, was investigated. It was thought that this study could help in finding ways leading to pharmacological reduction of fibrotic responses to various noxious agents *in vivo*. In a preliminary report, evidence for the incorporation of ^{14}C-labelled Az into granuloma collagen, has been presented (Dancewicz & Altman, 1967).

MATERIALS AND METHODS

Material. Granuloma was induced in rats by implanting polyvinyl sponges bilaterally under the dorsal skin according to the procedure described by Bouček & Noble (1955); the granulomas were harvested after 7 to 9 days of growth. The minced granuloma is capable of sustaining incorporation of proline or glycine into collagen for several hours at a reasonably rapid rate (Ernst *et al.*, 1966; Traelnes *et al.*, 1971).

Chemicals. L-[U-^{14}C]Proline was purchased from the Nuclear Corporation of New England (Boston, Mass., U. S. A.), [2-^{3}H]glycine from The Radiochemical Centre (Amersham, Bucks., England) and DL-[4-^{14}C]azetidine-2-carboxylic acid, as well as L-azetidine-2-carboxylic acid from Calbiochem (Los Angeles, Calif., U. S. A.). Bacterial collagenase was supplied by the Worthington Biochemical Co. (Freehold, N. J., U. S. A.) and was free of protease activity; trypsin, twice recrystallized, was purchased from the Sigma Chemical Co. (St. Louis, Mo., U. S. A.); gelatin, commercial, was from Knox Co., U. S. A.

Incubation procedure. The incubation medium used in the experiments was as described by Ernst *et al.* (1966) and Traelnes *et al.* (1971). The incubation was carried

out in Warburg vessels in an atmosphere of 95% O_2/5% CO_2 at 37°C for 3 h. At the end of the incubation period the minced granuloma tissue was separated from the medium, washed with an excess of ice-cold water and placed in cold acetone for 3 days for removal of lipids. Collagen was extracted and gelatinized by refluxing with boiling water overnight. Contaminating proteins were removed by treating the preparation with 3% trichloroacetic acid. The final product was precipitated by adding acetone to 90% (v/v), followed by three reprecipitations from aqueous solution with acetone. The product obtained has the physical and chemical properties of collagen, and will be referred to as collagen.

Analytical methods. Protein was determined by the method of Lowry *et al.* (1951), proline in the incubation medium by the method of Troll & Lindsley (1955), and α-amino nitrogen by the ninhydrin reaction with leucine as standard (Moore & Stein, 1954). Samples for amino acid analysis were prepared by hydrolysis in either 6 N-HCl or 2.5 N-$Ba(OH)_2$ for 18 h at 110°C, followed by neutralization. Paper chromatography of amino acids was carried out as described by Solberg (1965). DNA was determined by the method of Burton (1956).

Radioactivity was measured in a gas-flow counter after plating samples on rippled, stainless-steel planchets as infinitely thin layers when only ^{14}C was involved, and by means of liquid scintillation counting when both ^{14}C and ^{3}H were used. The counting errors did not exceed 3%.

RESULTS AND DISCUSSION

The data presented in Table 1 indicate that [^{14}C]Az was incorporated into granuloma collagen, and that the extent of incorporation was dependent on the Az concentration in the medium. The incorporation of [^{14}C]Az was time- and temperature-

Table 1

Incorporation of [4-^{14}C]*azetidine-2-carboxylic acid into granuloma collagen*

To 5 ml of Puck's N-16 medium fortified with L-glutamine and 5% calf serum, 100 mg of minced granuloma and Az as indicated, were added. The incubation was carried out for 3 h at 37°C. Mean values of duplicate experiments are given.

[4-^{14}C]Az in incubation medium		Molar ratio of Az to proline in incubation medium	^{14}C activity in collagen (c.p.m./mg protein)	Az content in collagen (μg/mg protein)
concentration (mM)	spec. act. (c.p.m./mmol)			
0.09	32.7×10^6	0.4	1788	5.4
1.09	2.7×10^6	4.2	617	22.8
2.09	1.4×10^6	8.1	339	24.2
5.09	0.6×10^6	19.7	256	42.6

-dependent (Table 2); at 37°C the incorporation increased linearly with time up to 4.5 h of incubation, and at 4°C the incorporation was very low. All attempts to remove the radioactivity from collagen after incubation with [^{14}C]Az, by dialysis and other purification procedures, were ineffective, thus providing further evidence for the incorporation of Az into collagen. The structure of the incorporated Az remained intact. Paper chromatographic analysis of alkaline hydrolysates of the isolated collagen showed a ninhydrin-positive spot identical with respect to R_F value and specific radioactivity with that obtained for [^{14}C]Az.

Table 2

Time-course of incorporation of [4-^{14}C]*azetidine-2-carboxylic acid into granuloma collagen*

Conditions as in Table 1; Az concentration in the medium was 34 μM (1.7 μCi of [^{14}C]Az/sample). Mean values of duplicate experiments are given.

Incubation time (h)	Temperature (°C)	^{14}C activity of collagen	
		c.p.m./mg protein	c.p.m./mg protein/μatom DNA-P
0*	4	3	5
1	4	16	13
2	4	21	30
1	37	63	111
2	37	110	175
4.5	37	204	319

* 2 - 3 min at 4°C.

Amino acid analysis of acid hydrolysates of the Az-containing collagen, gelatin or a mixture of standard amino acids with Az added, showed that Az was eluted at the position of threonine, increasing the values for threonine. This increase corresponded to the amount of Az present in the respective samples. Az was also recovered from acid hydrolysates of collagen by gas-chromatographic technique as a *tert.*-butyl ester with the R_T between that for glycine and proline.

The inhibitory effect of Az on the *in vitro* incorporation of [^{14}C]proline into granuloma collagen increased with increasing Az concentration (Table 3). Az inhibited also the incorporation of [^{3}H]glycine (Table 4). Thus, it appears that the effect of Az on amino acid incorporation into collagen was not specific for proline. In the granuloma collagen, the ^{3}H/^{14}C ratio remained virtually constant at different Az concentrations, and for both amino acids the inhibition of incorporation was of a similar order. The effect of Az on glycine incorporation may be due to conformational changes in the peptide chain containing Az residues; in that case inhibition of glycine incorporation would represent a secondary effect.

The occurrence of structural changes in granuloma collagen upon incorporation of Az was confirmed by its altered susceptibility to bacterial collagenase and trypsin.

Table 3

Effect of azetidine-2-carboxylic acid on incorporation of L-[U-^{14}C]*proline into granuloma collagen*

The incubation mixture was as in Table 1; each sample contained 3.09 μCi of [^{14}C]proline. Mean values from triplicate experiments at each Az concentration are given; the results did not differ by more than 5%.

Az concn. in incubation medium (mM)	Molar ratio of Az to proline in incubation medium	Spec. act. of collagen (c.p.m./mg protein)	Inhibition of proline incorporation (%)
0.0	0.0	20 837	0
0.4	1.9	16 616	20
1.1	6.0	13 221	37
2.2	12.0	11 428	45

Table 4

Effect of azetidine-2-carboxylic acid on incorporation of L-[U-^{14}C]*proline and* [2-^{3}H]*glycine into granuloma collagen*

The conditions of incubation were as in Table 1. Each sample contained 4.0 μCi of [^{14}C]proline and 50.0 μCi of [^{3}H]glycine per 1 ml of the incubation mixture. Mean values of duplicate experiments are given.

Az concn. in incubation medium (mM)	Molar ratio of Az to proline in incubation medium	Ratio of ^{3}H/^{14}C found in collagen	Inhibition of incorporation (%)	
			[^{14}C]proline	[^{3}H]glycine
0.00	0.0	34	0	0
0.35	0.3	33	1	3
0.71	0.6	32	12	16
1.78	1.7	32	20	25
3.53	3.3	32	43	45
6.90	6.1	34	54	54

The collagen used for the experiments was isolated from minced granuloma incubated alternatively with [^{14}C]proline without Az, with [^{14}C]proline and non-radioactive Az, or with [^{14}C]Az (Table 5). The granuloma collagen containing Az was less readily digested by collagenase than normal collagen, the action of the enzyme being to some extent dependent upon Az content in collagen. After 12 h of treatment with collagenase about 41% of the initial radioactivity and 0.37 μmol of α-amino nitrogen per mg of protein were recovered from normal collagen labelled with [^{14}C]proline, whereas only 7% of the initial radioactivity and 0.09 μmol of amino nitrogen were recovered from Az-containing collagen (0.15 μg Az/mg of protein, Exp. 2). One percent of the radioactivity was released from collagen labelled only with [^{14}C]Az

Table 5

Action of bacterial collagenase on granuloma collagen

Collagen (or gelatin), 5 mg, was dissolved in 5 ml of 0.005 M-$CaCl_2$ - 0.05 M-Tris-HCl buffer, pH 7.0, supplemented with 200 μg of collagenase, and incubated for 12 h at 37°C. Then the sample was dialysed for 12 h in a cold-room against 2 changes of water (100 ml each). The diffusate was concentrated in a flash evaporator to dryness, dissolved in a small volume of water, and analysed for α-amino N and radioactivity. For analysis of total liberated α-amino N, 50 μl portions were withdrawn before and during incubation, and after dialysis.

Granuloma collagen	Content of Az in collagen (μg/mg protein)	α-Amino nitrogen		Diffusible radioactivity (% of initial)
		liberated	diffusible	
1. Labelled with [^{14}C]proline	—	0.52	0.37	40.7
2. Labelled with [^{14}C]proline	0.15	0.14	0.09	7.3
3. Labelled with [^{14}C]Az	0.1	0.18	0.16	0.7
Gelatin, commercial	—	1.67	0.92	—

(0.1 μg Az/mg of protein), but the amount of dialysable α-amino nitrogen was twice as high as that in Exp. 2.

The decreased collagenase action and very small liberation of radioactivity from collagen containing [^{14}C]Az indicates that the number of Az residues released by the action of collagenase is small, and that the bacterial enzyme does not act on glycyl (or alanyl) peptide linkages in the immediate vicinity of Az residues. Since bacterial collagenase requires for its hydrolytic activity the presence of specific amino acid sequences composed of at least six residues, it is conceivable that alterations of the

Table 6

Sequential action of trypsin and bacterial collagenase on granuloma collagen

Substrate (collagen or gelatin), 2 mg, and trypsin, 200 μg, were incubated in 3 ml of 0.01 M-$CaCl_2$ - - 0.1 M-Tris-HCl buffer, pH 8.2, at 37°C for 2 h. Then the system was brought to pH 7.0 by adding 1.0 M-HCl, 200 μg of collagenase were added, and the incubation continued for another 2 h. In 50 μl portions, α-amino N and protein were determined. All values for α-amino N are expressed as μmol of leucine equivalents per mg protein.

Granuloma collagen	Content of Az in collagen (μg/mg protein)	α-Amino N liberated by trypsin	α-Amino N liberated by collagenase from material remaining after trypsin treatment
Labelled *in vivo* with [^{14}C]proline	0	0.22	1.42
Labelled *in vitro* with [^{14}C]proline in the presence of Az	0.1	0.56	1.01
Labelled *in vitro* with [^{14}C]Az	0.01	0.43	1.22
Gelatin, commercial	0	0.35	1.20

required sequences, e.g. by incorporation of Az, would reduce the number of sites available for attack by the collagenase and explain the small numbers of Az residues released by the enzyme.

Unlike bacterial collagenase which was less active toward granuloma collagen than toward standard gelatin (cf. Table 5), the activity of trypsin toward collagen was nearly the same as toward gelatin and toward Az-containing collagen even somewhat higher (Table 6). Following trypsin digestion, the Az-containing collagen subjected to collagenase action was hydrolysed to a similar extent as the preparation of collagen containing no Az. It appears plausible that tryptic digestion cleaves a sufficient number of Az-peptide bonds, so as to expose glycyl residues otherwise inaccessible to collagenase action, thereby relieving the inhibition of collagenase activity.

Table 7

Effect of free azetidine-2-carboxylic acid on the activity of bacterial collagenase toward gelatin

Commercial gelatin (dialysed), 2 mg, collagenase, 200 µg, and Az as indicated, were mixed in 5.0 ml of 0.005 M-$CaCl_2$ - 0.1 M-Tris-HCl buffer, pH 7.5, and incubated for up to 2 h at 37°C. Samples of 50 µl were withdrawn for determination of α-amino N. The results are expressed as µmol of leucine equivalents/mg of protein, and are mean values of duplicate experiments.

Az concn. (mM)	Ratio of Az to gelatin (µg Az/mg protein)	α-Amino N liberated	
		0.5 h	2 h
0	0.0	1.10	2.88
3.3×10^{-3}	0.3	1.12	2.97
3.3×10^{-2}	3.0	1.35	3.09
3.3×10^{-1}	30.0	1.30	3.65

That the low collagenase activity on the Az-containing substrates was not due to the presence of free Az contaminating the collagen preparations, was demonstrated in the experiment on the effect of the collagenase on gelatin in the presence of Az (Table 7). Az at concentrations ranging from 3.3×10^{-3} to 3.3×10^{-1} mM, did not affect the enzyme activity.

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WPŁYW KWASU AZETYDYNO-2-KARBOKSYLOWEGO NA BIOSYNTEZĘ KOLAGENU W ZIARNINIE SZCZURZEJ

Streszczenie

Kwas L-azetydyno-2-karboksylowy (Az) włącza się *in vitro* do kolagenu ziarniny i równocześnie hamuje w równym stopniu inkorporację L-[U-^{14}C]proliny, jak i [2-^{3}H]glicyny. Chromatografia bibułowa hydrolizatów alkalicznych kolagenu zawierającego Az wykazała, że inkorporowany Az nie uległ zmianie. Kolagen zawierający Az był mniej podatny na hydrolizę kolagenazą bakteryjną a bardziej podatny na działanie trypsyny. Po trypsynolizie kolagen zawierający Az był bardziej podatny na działanie kolagenazy.

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Added in proof: After the present manuscript had been submitted for publication, a paper on a related aspect of the topic "proline analogues" appeared (J. Uitto and D. J. Prockop, 1974, *Biochim. Biophys. Acta*, **336**, 234 - 251).

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EFFECT OF GIBBERELLIC ACID AND ABSCISIC ACID ON RNA SYNTHESIS IN MAIZE SEED SCUTELLUM*

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1. Incubation for 12 h of the isolated scutellum of maize seeds in the presence of gibberellic acid (GA_3) enhances several-fold RNA synthesis. Sedimentation analysis of total RNA in sucrose gradient revealed that rRNA synthesis was stimulated about sixfold, whereas that of the sRNA fraction only twofold. Radioactivity distribution after polyacrylamide-gel electrophoresis showed an additional slowly migrating peak, whose synthesis was stimulated about threefold. 2. Abscisic acid (ABA) at concentrations of 10^{-3} - 10^{-6} M did not cause meaningful inhibition of RNA synthesis, but markedly reduced the stimulatory effect of GA_3 on RNA synthesis. 3. The possible sequence of phytohormone action on particular parts of maize seeds during germination is also discussed.

It is generally accepted that plant growth substances and natural growth inhibitors are responsible for the induction and cessation of dormancy in higher plants. Khan *et al.* (1971) suggested that dormancy might be due not only to the presence of an inhibitor, but could also result from the lack of gibberellin or cytokinins. According to this hypothesis gibberellin is the primary stimulus for germination. Some evidence has been provided that gibberellic acid (GA_3) may affect the transcriptional process (Johri & Varner, 1968; Jarvis *et al.*, 1968; Zwar & Jacobsen, 1972; Wielgat *et al.*, 1974). It has also been reported that abscisic acid (ABA) is an inhibitor of RNA (Villiers, 1968; Bex, 1972) or DNA synthesis (Van Overbeek *et al.*, 1967; Stewart & Smith, 1972).

Effect of plant growth substances on RNA synthesis during germination of intact seeds can be demonstrated only at a very early stage of germination because the embryo itself produces hormones which participate in breaking dormancy and induce germination. For this reason aleurone layer of endosperm as well as scutellum from which the embryo axis has been removed, are much more convenient systems

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for the study of the effect of exogenous plant growth substances (Khan *et al.*, 1973; Wielgat *et al.*, 1974).

This paper deals with the effect of GA_3 and ABA on RNA synthesis in the isolated scutellum of maize seeds.

MATERIAL AND METHODS

Reagents. All reagents were of analytical grade. Phenol and *m*-cresol were used after distillation. Sucrose, RNase-free, was purchased from Schwarz-Mann (Orangeburg, N. Y., U. S. A.) and [^{32}P]orthophosphate (carrier-free, sodium salt) from the Institute for Nuclear Research (Świerk, Poland).

Plant material. Corn seeds (*Zea mays* c. v. Kb-260) were of Polish origin. The embryos were isolated from dry seeds and scutellum were obtained by removing axis from the embryo. For each experiment, 2-g samples (about 100 scutellum) were imbibed with phytohormones for 15 h at 2°C and then incubated for the time indicated at 22°C. All operations were performed under aseptic conditions as described previously (Wielgat *et al.*, 1974).

Isolation and fractionation of RNA. Isolation of total RNA and sucrose-gradient centrifugation were as described previously (Wielgat *et al.*, 1974). Polyacrylamide-gel electrophoresis of total RNA and the RNA fractions after sucrose-gradient centrifugation were performed according to Loening (1967). ^{32}P incorporation into the particular nucleic acid species was measured directly in the fractions collected in scintillation vials, using Tricarb Packard Scintillation Counter. After polyacrylamide-gel electrophoresis, gels were frozen in solid CO_2 with 96% ethanol, sliced into 2 mm slices, placed in the scintillation vials, solubilized with hydrogen peroxide and the radioactivity due to ^{32}P incorporation was measured in the scintillation counter.

RESULTS

The isolated maize scutellum were incubated for 12 h at 22°C with GA_3 and ABA. The total RNA was extracted therefrom and fractionated in the linear 5 - 20% sucrose gradient (Fig. 1). As can be seen from the presented profiles, GA_3 at concentration of 10^{-7} M enhanced several-fold incorporation of ^{32}P into 25s and 18s rRNA (Fig. 1a and 1b). Stimulation of ^{32}P incorporation by GA_3 at concentrations of 10^{-9}, 10^{-5} and 10^{-4} M was 20 - 30% lower. The most pronounced stimulation by GA_3 was found on 12 h incubation. Shortening of the incubation period to 4 or 8 h, or prolongation to 24 h resulted in a decrease of the stimulatory effect of GA_3 on RNA synthesis.

ABA at concentration of 10^{-3} M inhibited only very slightly RNA synthesis as compared with control (Fig. 1a and 1c) and at lower concentration (10^{-4} - 10^{-6} M) was ineffective. When the combined effect of GA_3 and ABA was studied, a significant depression of stimulatory effect of GA_3 was observed (Fig. 1b and 1d). These results imply that ABA does not affect RNA synthesis when applied alone, and depresses almost completely the stimulatory effect of GA_3.

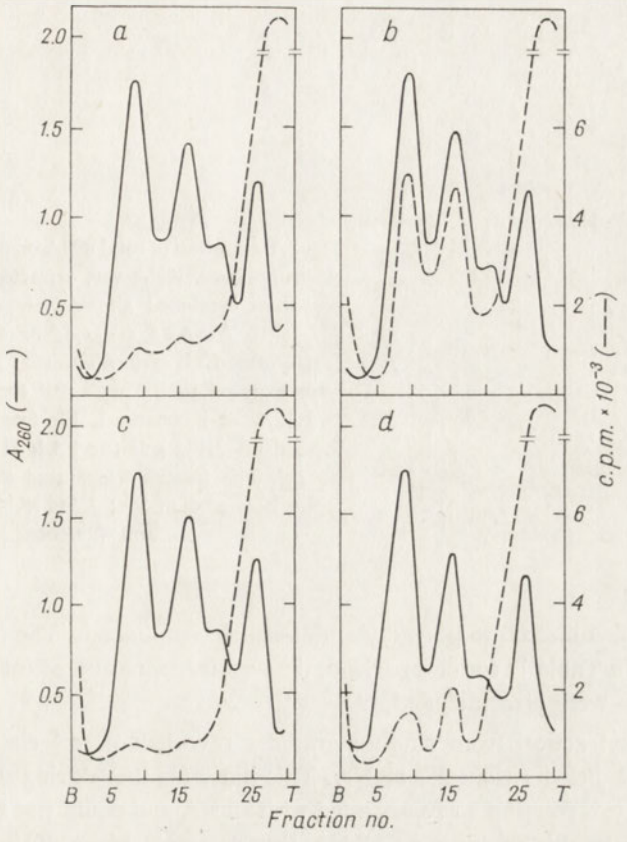

Fig. 1. Sucrose-density-gradient sedimentation patterns of total RNA extracted from control and hormone-treated maize scutellum. The tissue was incubated for 12 h at 22°C alternatively: *a*, without hormones; and with: *b*, GA_3 (10^{-7} M); *c*, ABA (10^{-3} M); *d*, GA_3 (10^{-7} M)+ABA (10^{-3} M). All samples were exposed to 0.2 mCi ^{32}P for 5 h (between 7 and 12 h of incubation). *B*, bottom; *T*, top of the gradient.

Distribution of radioactivity in the RNA species in the hormone-treated and untreated maize scutellum was recorded also in RNA subjected to polyacrylamide-gel electrophoresis (Fig. 2). As compared with the sucrose-gradient centrifugation, polyacrylamide-gel electrophoresis removed most of the non-defined ^{32}P impurities located in the sucrose gradient at sRNA zone (Loening, 1967) and enabled also resolution of the fraction located before the heavy rRNA peak, which could not be observed in the sucrose-gradient profiles. Significant stimulation of the synthesis of light and heavy rRNA by GA_3 and ineffectiveness of ABA were confirmed.

To get more information on the nature of the new fraction heavier than 25s rRNA, the samples were centrifuged in sucrose gradient, the first four tubes containing DNA were discarded (Fig. 1) and the remaining gradient was combined into two fractions corresponding to rRNA (tubes no. 5 - 20) and sRNA (tubes no. 21 - - 30). The material from the collected fractions was precipitated with 2 vol. of ethanol,

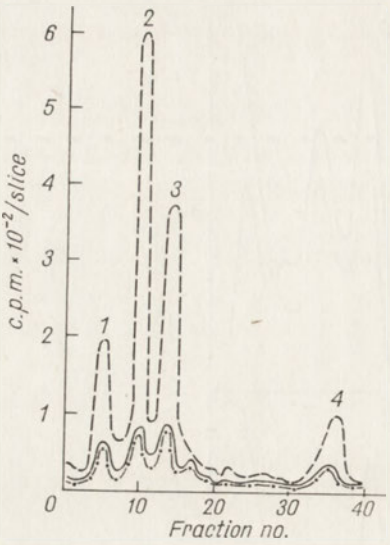

Fig. 2. Separation of total RNA on polyacrylamide-gel electrophoresis. RNA was extracted from the maize scutellum incubated alternatively with 10^{-7} M-GA_3 (- - -); 10^{-3} M-ABA (-· -· -); or without hormones (——) after 12 h of incubation at 22°C. All samples were exposed to 0.2 mCi ^{32}P for 5 h (between 7 and 12 h of incubation). Electrophoresis was performed on 2.4% gels for 1.5 h at 100 V and 5 mA per gel. The fractions separated were as follows: *1*, RNA heavier than 25s; *2*, 25s rRNA; *3*, 18s rRNA; and *4*, tRNA.

centrifuged and subjected to polyacrylamide-gel electrophoresis. The results obtained are presented in Table 1; for comparison, data on fractionation of total RNA by gel electrophoresis were also included.

The rRNA fraction from sucrose gradient revealed on gel electrophoresis an additional peak heavier than 25s rRNA. This indicates that during sucrose-gradient centrifugation this fraction was associated with rRNA and could not be separated as a single peak. In this fraction no sRNA was detected. Since during collection of both RNA fractions separated by the sucrose-gradient centrifugation the first four tubes (from the bottom of gradient) were discarded, the presence of DNA in rRNA should be excluded. The fraction representing sRNA, subjected to gel electrophoresis showed radioactivity only in the region of tRNA.

Table 1

^{32}P *incorporation into RNA fractions of maize scutellum*

The RNA fractions collected from the sucrose-gradient centrifugation and corresponding to rRNA (II) and sRNA (III) were subjected to gel electrophoresis as described in the text and under Methods. Radioactivity of unfractionated total RNA (I) and fractions II and III was recorded.

	c.p.m./fraction											
	heavier than 25s RNA			25s rRNA			18s rRNA			tRNA		
	I	II	III	I	II	III	I	II	III	I	II	III
Control (H_2O)	1050	350	0	1550	1850	0	1300	1600	0	1200	0	3500
GA_3 (10^{-7} M)	4500	950	0	8500	8500	0	7000	7000	0	3100	0	8100
ABA (10^{-3} M)	900	300	0	1300	1800	0	1200	1500	0	1000	0	3300

DISCUSSION

Treatment of the isolated scutellum from dry maize seeds with GA_3 results in significant stimulation of 25s and 18s rRNA synthesis. These results are consistent with our previous data (Wielgat *et al.*, 1974) based on the radioactivity distribution patterns obtained by the sucrose-gradient centrifugation of RNA from the intact maize seeds and embryo-less part of these seeds treated with phytohormones. In this paper, the results concerning the stimulatory effect of GA_3 on rRNA synthesis in maize scutellum were also confirmed by polyacrylamide-gel electrophoresis. By the applied technique an additional RNA fraction heavier than 25s rRNA was found.

The nature of this new RNA species, the synthesis of which was also stimulated by GA_3, is not yet clear. Localization of this fraction on the gel could imply that the new fraction may consist of DNA. However, this suggestion should be excluded since the same peak was found also when the RNA obtained after sucrose-gradient centrifugation and thus devoid of all DNA, was subjected to gel electrophoresis. It may be assumed that the additional radioactivity represents pre-ribosomal RNA, which was found by Bex (1972) in maize coleoptiles and by Richter (1973) in the cells of *Petroselium sativum* after two hours of labelling with [^{3}H]uridine and subsequent gel electrophoresis of total RNA. The new peak of radioactivity found after gel electrophoresis may also represent DNA-like RNA. Further study on the nature of this high mol. wt. RNA is necessary.

ABA alone at high concentration (10^{-3} M) apparently did not affect RNA synthesis after the 12 h contact with scutellum. Similar results were obtained by Chrispeels & Varner (1966) with the barley aleurone layer. In contrast to our observation, ABA at even lower concentrations (10^{-6} - 10^{-5} M) inhibited synthesis of various RNA fractions in pear embryos (Khan & Heit, 1969), in the excised bean axes (Walton *et al.*, 1970) and in maize coleoptile (Bex, 1972). In our experiments when isolated scutellum were incubated for 12 h with both GA_3 and ABA the stimulatory effect of GA_3 was almost completely abolished. This may imply that ABA antagonizes GA_3 in regulation of the transcriptional processes (Chrispeels & Varner, 1966, 1967).

Scutellum isolated from dry maize seeds was found to be very sensitive to GA_3 and may be a very convenient model for the study of hormonal effect on the metabolic events in this tissue during breaking of dormancy. This system is very similar with respect to RNA synthesis to the barley aleurone layer (Chrispeels & Varner, 1967). However, isolation of aleurone layer from dry maize seed is hardly possible. Scutellum in our opinion has an additional advantage for our studies, as compared with other parts of maize seeds, because in the seed the embryo axis is located in this tissue and thus all the processes concerned with breaking of dormancy and germination could be more directly recorded in scutellum (Dure, 1960).

We have found previously (Wielgat *et al.*, 1974) that RNA synthesis was significantly stimulated by exogenous GA_3 in intact maize seeds after 24 h of incubation. In the isolated embryo this process could be observed as early as within 3 - 5 h of incubation. In the isolated embryo-less part of seeds (endosperm with aleurone layer)

GA_3 started to stimulate RNA synthesis in the 4-h incubation period but the stimulatory effect was increasing slowly up to 24 h of incubation. In the isolated scutellum a rapid increase in the GA_3-stimulated RNA synthesis was observed between the 4th and 12th hour of incubation. On longer incubation this stimulatory effect was decreased and the extent of stimulation after 24 h was similar to that observed in the embryo-less part of seeds (Fig. 3).

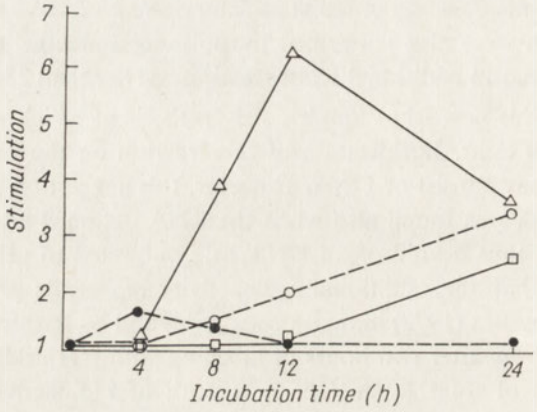

Fig. 3. Effect of growth substances on RNA synthesis in intact maize seed (□),scutellum (△), embryo (●), and embryo-less part of the seed (○). Stimulation of RNA synthesis by GA_3 is referred to the corresponding untreated tissue incubated for the time indicated, taken as 1. The results are mean values from 3 - 4 experiments. Data on the GA_3 effect on the intact seed, and the embryo and embryo-less parts, are from Wielgat *et al.* (1974)

The data on the successive changes in the sensitivity of RNA synthesis to GA_3 treatment in particular parts of maize seeds may indicate that in the intact seed phytohormones synthesized in embryo already after 4 - 5 h of germination (Fig. 3) are immediately transported into scutellum, where they stimulate synthesis of RNA and proteins (especially hydrolytic enzymes). It may be also assumed that after 24 h of germination the greatest stimulation of RNA synthesis by growth substances occurred in embryo and in the aleurone layer. Scutellum at that time is rather a "transit" area for the compounds necessary for embryo development and transferred from endosperm to the embryo.

A different lag in response of RNA synthesis in scutellum and aleurone layer of maize seed to GA_3 treatment may be also due to the different rate of synthesis of protein receptor (Matthysse & Philips, 1969; Matthysse & Abrams, 1970).

We thank Miss Krystyna Nowosielska for her excellent technical assistance.

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WPŁYW KWASU GIBERELINOWEGO I ABSCYZYNOWEGO NA SYNTEZĘ RNA W TARCZKACH KUKURYDZY

Streszczenie

1. Stwierdzono kilkakrotny wzrost syntezy RNA w izolowanych tarczkach kukurydzy po 12 godz. inkubacji w obecności kwasu giberelinowego (GA_3). Wirowanie ogólnego RNA w gradiencie sacharozy wykazało, że synteza rRNA była stymulowana około 6-krotnie, podczas gdy sRNA tylko 2-krotnie. Frakcjonowanie RNA na żelu poliakrylamidowym uwidoczniło dodatkową wolno wędrującą radioaktywną frakcję, której synteza była stymulowana około 3-krotnie.

2. Kwas abscyzynowy (ABA) w różnych stężeniach (10^{-3} - 10^{-6} M) nie powodował wyraźnego hamowania syntezy RNA, natomiast znosił stymulujący wpływ GA_3 na syntezę RNA.

3. Przedyskutowano kolejne etapy działania fitohormonów w poszczególnych częściach nasion kukurydzy podczas kiełkowania.

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COMPARISON OF SOME DIAMINOPYRIMIDINES AND AMETHOPTERIN AS INHIBITORS OF INSECT CELL PROLIFERATION AND OF INSECT FOLATE-METABOLIZING ENZYMES

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1. Two new antifolates 2,4-diamino-5-(1-adamantyl)-6-methyl (DAMP) and 6-ethyl (DAEP) pyrimidines were compared with amethopterin as inhibitors of cell proliferation from the ovarian tissues and as inhibitors of dihydrofolate reductase of *Galleria mellonella*. DAMP and amethopterin were also tested as potential inhibitors of three other folate-metabolizing enzymes of this insect. 2. All three compounds inhibited with great potency dihydrofolate reductase. The inhibition of methylenetetrahydrofolate oxidoreductase and formyltetrahydrofolate synthetase by DAMP and amethopterin was very weak, and thymidylate synthetase was inhibited by amethopterin only. 3. All three compounds inhibited *in vitro* cell proliferation from the ovarian tissues of *G. mellonella*. DAEP appeared to be less toxic than DAMP, which in turn was less toxic than amethopterin. The higher toxicity of amethopterin than that of DAMP might be due to the fact that the former inhibits with high potency dihydrofolate reductase and thymidylate synthetase whereas DAMP inhibits strongly dihydrofolate reductase only.

Amethopterin, the 4-amino-10-methyl analogue of folate is widely used in cancer chemotherapy. The specific perturbations of cellular metabolism by this drug were therefore studied in different cell lines *in vitro*. Thus, it has been shown that the target enzyme for amethopterin in mammalian cell is dihydrofolate reductase (e.g. Hitchings, 1967; Blakley, 1969; Slavík & Slavíkova, 1969; Harrap *et al.*, 1971; Huennekens *et al.*, 1973). Another enzyme of mammalian cells which is inhibited to some extent by amethopterin is thymidylate synthetase (e. g. Borsa & Whitmore, 1969; Harrap *et al.*, 1971; Bertino & Johns, 1972; Huennekens *et al.*, 1973).

It has been also demonstrated (Hitchings, 1967) that amethopterin exhibits less species specificity in its interference with the cellular metabolism than antimetabolites derived from pyrimidines and triazins. For this reason adamantyl derivatives of 2,4-diaminopyrimidine are now extensively investigated (Jonak *et al.*, 1971, 1972a, 1973; Ho *et al.*, 1972, 1973; Souček *et al.*, 1972).

This paper presents the results of an investigation of the effects of two 6-substituted adamantylpyrimidines and amethopterin on the proliferation of cells from the insect ovarian tissues *in vitro* and on folate-metabolizing enzymes isolated from insects. The biological and biochemical studies of related topics have been already reported (Manteuffel-Cymborowska & Grzelakowska-Sztabert, 1970; Saska *et al.*, 1972; Zielińska & Saska, 1973; Manteuffel-Cymborowska & Zielińska, 1974).

MATERIALS AND METHODS

Source of tissues. Last instar larvae (about 130 mg weight) and pharate adults (4 - 5 days after larval-pupal ecdysis) of *Galleria mellonella* were selected as the sources of tissues for investigation. In enzyme assays the larval fat body and in bioassays the pharate adult ovaries were used.

Bioassays. A modified hanging-drop technique was used to investigate drug toxicity on insect tissues and cells *in vitro*. The details of this technique were described earlier (Saska *et al.*, 1972; Zielińska & Saska, 1973). The ovariole fragments (1-2 mm long) were cultivated in triplicate in a drop of the basal medium (control) or in a drop of the medium containing the drug being investigated. The pH of the medium was controlled and if necessary adjusted to 6.4. At least 5 parallel cultures of sister ovarioles were used in each of the five experiments.

Preparation of the enzyme. Prior to homogenization in a Potter-Elvehjem homogenizer the fat body was suspended in: 0.05 M-potassium phosphate buffer, pH 5.5, for dihydrofolate reductase assay, 0.1 M-potassium phosphate buffer, pH 7.5, for methylenetetrahydrofolate oxidoreductase assay, 0.05 M-Tris-HCl buffer, pH 8.0, for formyltetrahydrofolate synthetase assay and 0.05 M-Tris-HCl buffer, pH 7.0, for thymidylate synthetase assay. The resulting homogenates were centrifuged at 20 000 *g* for 20 min at 4°C, the supernatants were filtered through cheese-cloth to remove lipids, and used as sources of enzymes.

Enzyme assays. Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP oxidoreductase, EC 1.5.1.3) activity was determined by a spectrophotometric method based on the decrease of absorbance at 340 nm caused by the enzymic oxidation of NADPH (Scrimgeour & Huennekens, 1966; Grzelakowska-Sztabert *et al.*, 1970; Grzelakowska-Sztabert & Manteuffel-Cymborowska, 1971). The standard reaction mixture in a total volume of 0.4 ml contained: 26.4 nmoles of dihydrofolate, 26.4 nmoles of NADPH, 20 μmoles of Tris-HCl buffer, pH 7.5, and enzyme extract, corresponding to about 500 μg of protein, and the reaction was carried out at 20°C for 8 min. The enzyme activity was expressed in nanomoles of dihydrofolate reduced/ /mg of protein.

Formyltetrahydrofolate synthetase (formate:tetrahydrofolate ligase (ADP), EC 6.3.4.3) activity was assayed by a spectrophotometric method (Rabinowitz & Pricer, 1956; May *et al.*, 1951) using the standard reaction mixture (Šhejbal *et al.*, 1962) adapted for the enzyme of this insect (Zielińska & Grzelakowska-Sztabert, 1968). The standard reaction mixture in a total volume of 1 ml contained: 40 μmoles of formate, 0.66 μmole of tetrahydrofolate, 0.66 μmole of ATP, 4 μmoles of cysteine,

2.6 μmoles of $MgCl_2$, 33 μmoles of NH_4Cl, 100 μmoles of Tris-HCl buffer at pH 8.0, and enzyme extract, corresponding to about 200 μg of protein. The sample used as blank was acidified with perchloric acid immediately after addition of the enzyme but before incubation.

Methylenetetrahydrofolate oxidoreductase (5,10-methylenetetrahydrofolate:NADP oxidoreductase, EC 1.5.1.5) activity was estimated spectrophotometrically (Scrimgeour & Huennekens, 1966). The modified reaction mixture in a total volume of 1 ml contained 0.6 μmole of tetrahydrofolate, 0.4 μmole of NADP, 2.5 - 4 μmoles of cysteine, 3.3 μmoles of formaldehyde, 3 - 6 μmoles $KHCO_3$, 50 μmoles of phosphate buffer, pH 7.5, and enzyme extract corresponding to 100 - 150 μg of protein. The blank consisted of the full reaction mixture and the enzyme inactivated by boiling.

After incubation either for 15 min (formyltetrahydrofolate synthetase) or 30 min (methylenetetrahydrofolate oxidoreductase) at 37°C the samples were treated with perchloric acid, left standing for about 15 min, centrifuged and the absorbance at 355 nm was determined (May *et al.*, 1951; Zielińska & Grzelakowska-Sztabert, 1968). The activity of these two enzymes was then expressed in nanomoles of formyltetrahydrofolate formed/mg of protein.

Thymidylate synthetase activity was estimated by a modification of the isotopic method of Roberts (1966). This method is based on the release of tritium (as tritiated water) from the position 5 of the pyrimidine ring of deoxyuridine-5′-monophosphate when thymidylate is formed. The standard reaction mixture in a total volume of 250 μl contained: 4.3 nmoles of [5-^{3}H]deoxyuridine-5′-monophosphate as the substrate (7.8×10^6 c.p.m./μmole), 0.5 μmole of HCHO, 0.3 μmole of tetrahydrofolate, 88 μmoles of 2-mercaptoethanol, 4.7 μmoles of Tris-HCl buffer, pH 7.0, and enzyme extract, corresponding to about 1 mg of protein. The reaction was started by addition of the substrate and terminated after one hour incubation by addition of 100 μl of 45% trichloroacetic acid (TCA). The suspension was centrifuged at 2000 *g* for 10 min. In control sample the enzyme was inactivated by TCA before addition of the tritiated substrate. Prior to charcoal treatment, unlabelled deoxyuridine-5′-monophosphate was added. A sample of 200 μl was then removed, added to 10 ml of the scintillation mixture containing 7 ml of PPO-POPOP solution (4 g of PPO and 0.1 g of POPOP in 1 litre of toluene) and 3 ml of ethanol, and counted in the Packard scintillation counter model 2003. The activity of thymidylate synthetase was expressed in picomoles of thymidylate synthesized/mg of protein.

Inhibitors. 2,4-Diamino-5-(1-adamantyl)-6-methylpyrimidine (DAMP) and 2,4-diamino-5-(1-adamantyl)-6-ethylpyrimidine (DAEP) were synthesized at the Laboratory of Dr. S. F. Zakrzewski at the Roswell Park Memorial Institute (Buffalo, U. S. A.). DAEP, the free base, is hardly soluble under conditions necessary for biological and biochemical testing. We used therefore the ethylsulfonate salt instead. The biological activity of both compounds is similar (see Jonak *et al.*, 1973). Stock solution of the ethylsulfonate salt of DAEP in water was 10^{-2} M, and that of DAMP in 0.05 M-HCl, 5×10^{-3} M.

When DAMP was the inhibitor, the pH of the reaction mixtures was thoroughly controlled. Addition of the minute volumes of the solution of this inhibitor did not

change pH of the reaction mixture for dihydrofolate reductase. However, in experiments with other folate-dependent enzymes, addition of the inhibitor affected the pH value of the medium, and thus influenced the enzyme activity. This effect was especially pronounced in the case of formyltetrahydrofolate synthetase, the activity of which is largely dependent on the pH of the medium. Thus, pH decrease from the optimum 8.1 to 7.6 or 7.0 caused a fall of the activity at least by 25 and 50%, respectively. Therefore in experiments with formyltetrahydrofolate synthetase, with methylenetetrahydrofolate oxidoreductase and thymidylate synthetase the enzymic activity was estimated in sets of experimental samples with DAMP and in parallel sets of control samples containing corresponding volumes of 0.05 M-HCl. For evaluation of the inhibitory effect of DAMP the activity of the enzyme was expressed as percentage of the respective control.

Amethopterin (Lederlé) was dissolved prior to use either in 0.1% $KHCO_3$ or in water with addition of few drops of 0.1 N-KOH; its concentration was determined spectrophotometrically according to Seeger *et al.* (1949).

All the chemicals used were of analytical grade.

Protein concentration was estimated according to Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

RESULTS

Bioassays. Effects of DAMP and amethopterin on the *in vitro* cultivated ovarian tissues of *Galleria mellonella* are summarized in Table 1.

The survival of the explants in the presence of 10^{-8} to 10^{-5} M-DAMP or 10^{-8} to 10^{-6} M-amethopterin was at least four days, whereas 10^{-5} M-amethopterin killed the explants in a few hours (Table 1). In comparison with the control, the cell migration and outgrowth after four days as well as the time of cell survival in outgrowth were decreased even in cultures with 10^{-8} M-DAMP. It is also evident that the decrease caused by amethopterin was greater than that caused by DAMP.

The pilot experiments with DAEP showed that this adamantylpyrimidine did not inhibit cell proliferation up to a concentration of 10^{-5} M, and did not arrest cell migration from the explant up to 5×10^{-3} M. Thus, DAEP seems to be less toxic than DAMP for the insect tissues and cells tested. For this reason, in the further enzymic investigation, DAMP was the pyrimidine we mainly used as inhibitor in comparison with amethopterin.

Enzyme assays. Dihydrofolate reductase, when assayed in the extracts exhibited the activity of 5 - 10 nmoles of the reduced substrate/mg of protein (see also Grzelakowska-Sztabert *et al.*, 1970; Manteuffel-Cymborowska & Zielińska, 1974). The inhibitory effect of 2,4-diamino-5-(1-adamantyl)-6-substituted pyrimidines on the activity of dihydrofolate reductase is illustrated in Figs. 1 and 2. The values of $I_{0.5}$ for DAMP and DAEP were 2×10^{-8} and 5×10^{-8} M, respectively. The inhibition analysis of the enzyme at pH 7.5 by DAMP according to Lineweaver-Burk (Fig. 3) showed that the inhibition is of the competitive type and the K_i is 4×10^{-8} M. As

Table 1

Biological effects of 2,4-diamino-5-(1-adamantyl)-6-methylpyrimidine (DAMP) and amethopterin on in vitro *cultivated ovarian tissues of* Galleria mellonella

Explants of the ovaries of the pharate adults 4 - 5 days after larval-pupal ecdysis were cultivated for 4 days by the hanging-drop technique in basal medium alone (control) or in the presence of a drug under investigation (experimental). Other details in Material and Methods, see also Zielińska & Saska (1973).

	Control	Experimental with							
		DAMP (M)				Amethopterin (M)			
		10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-8}	10^{-7}	10^{-6}	10^{-5}
Explant survival, days (at least)	4	4	4	4	4	4	4	4	none
Cell migration and outgrowth after 4 days	abundant	moderate	poor	very poor	none	moderate	very poor	none	none
Cell survival, days	4 (at least)	4	3	3	1	2	1		

Abundant : a network of approximately 600 cells after 2 days in culture
Moderate : small network of 100 - 200 cells after 2 days in culture
Poor : about 50 cells outside the explants
Very poor : few cells outside the explants

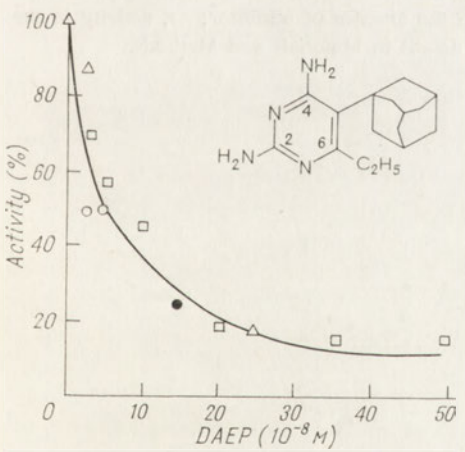

Fig. 1

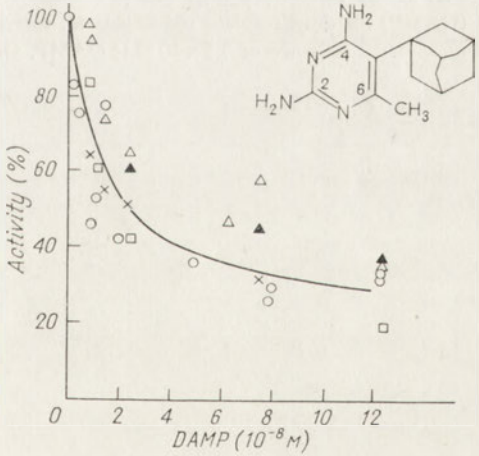

Fig. 2

Fig. 1. Inhibition of dihydrofolate reductase by 2,4-diamino-5-(l-adamantyl)-6-ethylpyrimidine (DAEP). Open and solid points of different shapes represent the results obtained in separate experiments. All details in Materials and Methods.

Fig. 2. Inhibition of dihydrofolate reductase by 2,4-diamino-5-(l-adamantyl)-6-methylpyrimidine (DAMP). Open and solid points of different shapes represenf the results obtained in separate experiments. All details in Materials and Methods.

shown earlier, the amethopterin inhibition of this enzyme is also of the competitive type under these conditions, and the K_i is 2×10^{-8} M (Manteuffel-Cymborowska & Grzelakowska-Sztabert, 1970).

Methylenetetrahydrofolate oxidoreductase activity in the extracts varied in the range of 80 to 120 nmoles of the product/mg of protein. Inhibition of this oxidoreductase by DAMP is presented in Fig. 4. It is evident that even 2×10^{-3} M-DAMP (i.e. at concentrations 3 times higher than that of the substrate tetrahydrofolate) did not inhibit this enzyme more than 30%. $I_{0.5}$ for inhibition by amethopterin was hardly achieved at 10^{-3} M.

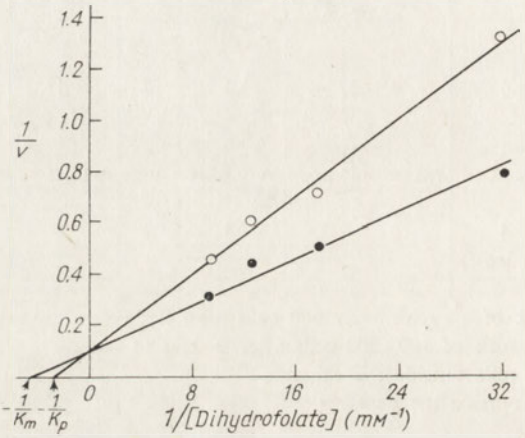

Fig. 3. The Lineweaver-Burk plot of the action of 2,4-diamino-5-(1-adamantyl)-6-methylpyrimidine (DAMP) on dihydrofolate reductase. ●, activity in the absence of inhibitor; ○, activity in the presence of 7.5×10^{-8} M-DAMP. Other details in Materials and Methods.

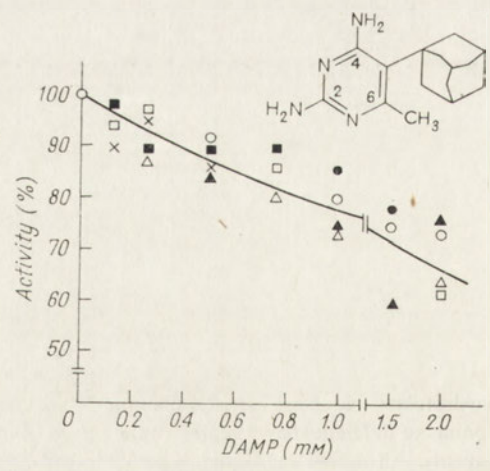

Fig. 4. Inhibition of methylenetetrahydrofolate oxidoreductase by 2,4-diamino-5-(1-adamantyl)-6-methylpyrimidine (DAMP). Open and solid points of different shapes represent the results obtained in separate experiments. All details in Materials and Methods.

Formyltetrahydrofolate synthetase activity varied in the range 70 to 150 nmoles of the product/mg of protein. Its inhibition by 30% was observed when 0.25×10^{-3} M--DAMP (pH 7.6) was present in the sample. Higher concentrations of DAMP could not be tested, even with the use of proper controls (see Materials and Methods) because of considerable decrease in the enzyme activity resulting from the change in pH of the reaction mixture. Amethopterin caused 30% inhibition of this synthetase at concentration 1.5×10^{-3} M, i.e. twice as high as that of either formate or tetrahydrofolate.

Thymidylate synthetase exhibited very low activity of 30 - 50 pmoles/mg of protein in the extracts of insect tissues. This enzyme was not inhibited by 0.3×10^{-3} M--DAMP, i.e. at the concentration one-fourth that of tetrahydrofolate and 18 times higher than that of dUMP in the reaction mixture. The effect of higher concentrations of DAMP could not be evaluated because of considerable decrease in the enzyme activity due to change in pH of the reaction mixture. Parallel examination of amethopterin showed that this folate analogue inhibited the insect thymidylate synthetase, and its $I_{0.5}$ value was 4.0×10^{-5}M.

DISCUSSION

Several pyrimidines and pteridines substituted with adamantyl moiety have been recently shown to interfere more or less with cell proliferation (Jonak *et al.*, 1970, 1971, 1972a,b, 1973; Ho *et al.*, 1972, 1973). Of all these compounds, 2,4-diamino-5--(1-adamantyl)-6-methylpyrimidine (DAMP) was the strongest inhibitor of cell proliferation. Its ID_{50} for the mouse mammary adenocarcinoma cells (TA3) was nearly equal to that of amethopterin (6 and 8×10^{-9} M, respectively; Ho *et al.*, 1972). On the other hand, amethopterin was a much more powerful inhibitor for the mammalian dihydrofolate reductase (K_i values 10^{-10} - 10^{-11} M, Werkheiser, 1961; Jonak *et al.*, 1973) than DAMP (K_i value 6×10^{-9} M, Ho *et al.*, 1972).

The results of our bio- and enzyme-assays with *Galleria mellonella* presented here indicate that DAMP is a strong inhibitor of the insect cell proliferation, being also a very strong inhibitor of the insect dihydrofolate reductase. Except of this reductase, none of other folate-metabolizing enzymes of this insect was appreciably inhibited by DAMP. Thus, in insect cells dihydrofolate reductase is the only target enzyme for this compound.

Amethopterin, which is a powerful inhibitor of the insect dihydrofolate reductase (Manteuffel-Cymborowska & Grzelakowska-Sztabert, 1970; Manteuffel-Cymborowska & Zielińska, 1974), inhibits also to some extent the insect thymidylate synthetase, $I_{0.5} = 4 \times 10^{-5}$ M. (The inhibition of insect thymidylate synthetase by amethopterin was reported by Carpenter, 1973, 1974). Since the K_i values for DAMP and amethopterin for dihydrofolate reductase are very similar (4×10^{-8} and 2×10^{-8} M, respectively) one can conclude that the inhibition of thymidylate synthetase by amethopterin might be responsible for the higher toxicity of this compound for insect cells *in vitro* in comparison with that of DAMP. The more so, as the permeability barrier to DAMP seems to be decreased due to its lipophylic adamantyl moiety (Ho *et al.*, 1972).

Our model of testing the proliferation of insect cells *in vitro* is different from that used by other authors in experiments with mammalian cells. We cannot therefore directly compare the sensitivity of insect cells and tissues to DAMP with that of mammalian cells. However, the proliferation of insect cells in the presence of 10^{-8} M--DAMP was diminished only slightly, whereas this compound in similar concentration (0.6×10^{-8} M) inhibited the proliferation of the TA3 cells by 50% (Ho *et al.*, 1972). Thus it seems that the sensitivity to DAMP of insect ovarian tissues and cells is lower than that of mouse mammary adenocarcinoma cells (TA3). This is in turn a new observation of the species selectivity in inhibition of cell proliferation by the same growth inhibitor.

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PORÓWNANIE NIEKTÓRYCH POCHODNYCH DIAMINOPIRYMIDYNY I AMETOPTERYNY, JAKO INHIBITORÓW PROLIFERACJI KOMÓREK OWADZICH ORAZ OWADZICH ENZYMÓW METABOLIZUJĄCYCH FOLIAN

Streszczenie

1. Porównano działanie dwóch nowych związków antyfolianowych: 2,4-dwuamino-5-(1-adamantyl)-6-metylopirymidyny (DAMP) i -6-etylopirymidyny (DAEP) z działaniem ametopteryny jako inhibitorów proliferacji komórek z tkanek jajnika *Galleria mellonella* oraz jako inhibitorów kilku enzymów folianowych izolowanych z tkanek tego owada.

2. Wszystkie trzy badane związki silnie hamowały reduktazę dihydrofolianową; DAMP i ametopteryna dopiero w wielokrotnie wyższych stężeniach nieznacznie hamowały oksydoreduktazę metylenotetrahydrofolianową i syntetazę formylotetrahydrofolianu; syntetazę tymidylianową zaś hamowała jedynie ametopteryna.

3. Wszystkie trzy związki hamowały *in vitro* proliferację komórek z tkanek jajnika *G. mellonella*. DAEP okazała się mniej toksyczna od DAMP, a ta z kolei mniej toksyczna od ametopteryny. Być może pozostaje to w związku z silnym hamowaniem przez ametopterynę aktywności reduktazy dihydrofolianowej i syntetazy tymidylianowej, podczas gdy DAMP hamuje silnie tylko aktywność reduktazy dihydrofolianowej.

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PREPARATION OF SOME *N*-METHYL ISOGUANINES *VIA* 6-METHYLTHIO-2-OXOPURINES, AND OF 8-METHYLISOGUANINE*

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A general procedure is described for the preparation of a number of *N*-methylated isoguanines, including an unequivocal synthesis of 3,9-dimethylisoguanine. The method is based on the amination of the corresponding *N*-methylated 6-methylthio-2-oxopurines, and also makes available the exocyclic N^6-methyl derivatives. Some spectral parameters and the p*K* values for the analogues obtained are reported. Contrary to previous generalizations, it was found that resistance to nitrous acid deamination of *N*-methyl isoguanines is conferred by substitution of the N_9 position. 8-Methylisoguanine was synthesized by condensation of 2-oxo-4,5,6-triaminopyrimidine with acetamide.

Isoguanosine (crotonoside) was isolated many years ago (Cherbuliez & Bernhard, 1932) from *Croton tiglium* L seeds; subsequently it was shown to be a constituent of the coloured pigments from butterfly wings (Purrmann, 1940). More recently a cytokinin which appears to be an isoguanine analogue has been isolated from corn (Letham, 1973). The possible biological functions of isoguanine and isoguanosine have been little studied, and neither appears to be a precursor in nucleic acid synthesis (Bendich *et al.*, 1950; Lowy *et al.*, 1952), but isoguanine is a potent inhibitor of xanthine oxidase (Leonard *et al.*, 1962) and IMP pyrophosphorylase (Hagen, 1973).

Isoguanine may be obtained by the nitrous acid deamination of 2,6-diaminopurine. Further deamination to xanthine occurs only in the presence of dilute HCl at elevated temperatures. However, an *E. coli* strain with a growth requirement for either xanthine or guanine as a source of purine, deaminates isoguanine to xanthine (Friedman & Gots, 1951). For some purine auxotrophs of *E. coli* B, either isoguanine or isoguanosine may partially replace adenine, guanine, hypoxanthine or xanthine, but at higher concentrations isoguanine inhibits growth.

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Syntheses of a number of *N*-methylated isoguanines have also been reported. The 9-methyl analogue was obtained by hydrolysis of 6-amino-2-ethoxy-9-methylpurine (Falconer *et al.*, 1939; Andrews *et al.*, 1949). The 7-methyl derivative was isolated in low yield following fusion of 4-amino-5-cyano-1-methylimidazole with urea (Shaw, 1962), and as a by-product in the preparation of 7-methylguanine by alkaline hydrolysis of 6-amino-2-chloro-7-methylpurine (Shaw, 1962). A recent report describes the preparation of 3-isomethylguanine by condensation of 1-methyl--2-oxo-4,5,6-triaminopyrimidine with formamide (Rogers & Ulbricht, 1971), as for the synthesis of the parent isoguanine. The 3,7-dimethyl derivative was reported by Fischer as long ago as 1897. Some N^6-alkyl analogues of isoguanine and isoguanosine were prepared by Yamazaki *et al.* (1968), by alkylamination of the 6-methylthio-2-oxo derivatives.

We present here a fairly general procedure for the synthesis of *N*-methylated isoguanines *via* the corresponding 2-oxo-6-methylthiopurines, which permits of the simultaneous preparation of N^6-methylated derivatives. All of these are presently being profited from to study the tautomerism of isoguanosine nucleosides and nucleotides, which may be obtained preparatively on a moderate scale by photolysis of the N_1-oxides of the corresponding adenosine analogues (Kazimierczuk & Shugar, 1973). A novel synthesis of 8-methylisoguanine is also described.

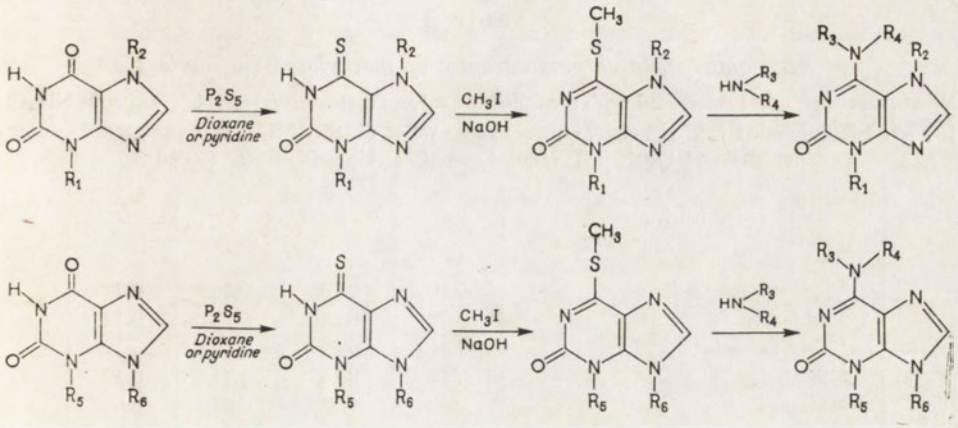
MATERIALS AND METHODS

Melting points were measured on a Boetius microscope hot stage. Absorption spectra were run on a Zeiss (Jena, D. D. R.) VSU-2 instrument and, in several instances, on a Cary Model 118. A Radiometer PHM-22 pH meter with glass electrode was employed for pH measurements. Spectral titrations to determine p*K* values made use of 0.01 M buffers; for pH values below 2 and above 12, the pH values were calculated from the acid and base concentrations. Paper chromatography was as described in Table 2.

RESULTS AND DISCUSSION

The general route for synthesis of the *N*-methyl isoguanines is shown in Scheme 1. The appropriate *N*-methyl xanthine was thiated in pyridine or dioxane with P_2S_5 to the corresponding *N*-methylated 6-thioxanthine. The latter was then methylated to give the analogous *N*-methylated 6-methylthio-2-oxopurine, which was treated with alcoholic ammonia at elevated temperatures to give the desired *N*-methylated isoguanine. Replacement of ammonia by methylamine or dimethylamine led to analogues methylated on the exocyclic amino group.

Some of the derivatives were isolated in crystalline form as the monohydrates (cf. Lister, 1971). However, since 7-methylisoguanine and 9-methylisoguanine analogues formed gels in concentrated solutions, similarly to isoguanosine (Ravindranathan & Miles, 1965), these derivatives were converted to the HCl salts prior to crystallization.



R_1, R_2, R_3, R_4, R_5 *and* $R_6 = H, CH_3$

Scheme 1

Spectral data and R_F values for the various isoguanine derivatives are presented in Tables 1 and 2.

Table 1

U.v. absorption maxima and pK *values for the neutral, cationic and anionic forms of isoguanine and its N-methylated derivatives*

Derivative	λ_{max} (nm)			pK_1	pK_2	pK_3
	Neutral form	Anion	Cation			
Isoguanine	287, 241	285, 291*	285	4.40	9.10	>13
N^6-Methyl-	281, 241	285, 290*	285	4.30	9.00	>13
N^6,N^6-Dimethyl-	285, 249	289, 295*	289.5	4.20	9.00	>13
8-Methyl-	289, 239	288, 292*	288	4.50	9.40	>13
3-Methyl-	284, 239, 215	285	289	4.55	8.6	—
N^6,3-Dimethyl-	283.5, 242, 219	286	290	4.40	8.45	—
N^6,N^6,3-Trimethyl	287, 253, 223	289, 248	294.5	4.10	8.45	—
7-Methyl-	282, 243	295	287	4.45	10.85	—
N^6,7-Dimethyl-	282,243	295	289	3.90	10.80	—
N^6,N^6,7-Trimethyl-	293, 263	304	303	3.50	10.80	—
3,7-Dimethyl-	285, 245, 206	—	292.5	4.30	—	—
N^6,3,7-Trimethyl-	285, 245, 206.5	—	293.5	3.75	—	—
N^6,N^6,3,7-Tetramethyl-	297.5, 262.5, 204	—	309	3.50	—	—
9-Methyl-	292.5, 247	285, 245	282, 233	3.85	9.90	—
N^6,9-Dimethyl-	293, 247	285, 251	283.5	3.90	9.90	—
N^6,N^6,9-Trimethyl-	289, 250	288, 228	288	3.90	9.90	—
3,9-Dimethyl-	280	—	286, 236	4.40	—	—

*Dianion

Table 2

Ascending paper chromatography of methylated isoguanines

Whatman paper no. 1 was used with the following solvent systems (v/v): (*A*) 3% aqueous NH_4Cl; (*B*) *tert*-butanol - ethyl methyl ketone - formic acid - water (40:30:15:15); (*C*) *n*-propanol - water - conc. NH_4OH (70:20:10); (*D*) *n*-butanol - acetic acid - water (50:30:20).

Derivative	*A*	*B*	*C*	*D*
Isoguanine	0.20	0.16	0.06	0.24
N^6-Methyl-	0.28	0.24	0.12	0.32
N^6,N^6-Dimethyl-	0.41	0.29	0.19	0.39
8-Methyl-	0.36	0.18	0.11	0.30
3-Methyl-	0.52	0.53	0.44	0.68
N^6,3-Dimethyl-	0.65	0.68	0.50	0.75
N^6,N^6,3-Trimethyl-	0.77	0.79	0.71	0.82
7-Methyl-	0.54	0.18	0.13	0.36
N^6,7-Dimethyl-	0.64	0.24	0.21	0.43
N^6,N^6,7-Trimethyl-	0.72	0.28	0.34	0.47
3,7-Dimethyl-	0.67	0.66	0.40	0.56
N^6,3,7-Trimethyl-	0.72	0.74	0.48	0.64
N^6,N^6,3,7-Tetramethyl-	0.77	0.79	0.58	0.71
9-Methyl-	0.49	0.27	0.11	0.40
N^6,9-Dimethyl-	0.61	0.36	0.21	0.46
N^6,N^6,9-Trimethyl-	0.70	0.45	0.29	0.52
3,9-Dimethyl-	0.70	0.22	0.23	0.30

Particular interest attaches to 3,9-dimethylisoguanine. Synthesis of this derivative was reported by Okano *et al.* (1967), by dimethylsulphate treatment of isoguanine in dimethylacetamide. The authors report a melting point > 300°C and the results of elemental analysis consistent with the 3,9-dimethylisoguanine formula. In our hands, however, this procedure gave a mixture of products which proved too complex to identify the presence of 3,9-dimethylisoguanine. Furthermore the u. v. absorption spectrum of the presumed 3,9-dimethylisoguanine presented by the foregoing authors is inconsistent with such a structure. The spectra at pH 2 and pH 11 are barely distinguishable from each other, while that at pH 7 differs radically from these. Fig. 1 exhibits the ultraviolet absorption spectrum of the product obtained in this investigation, over the pH range 1 - 13. It is readily seen that, in accordance with expectations, 3,9-dimethylisoguanine exhibits a single p*K* (4.40) corresponding to protonation of N_1 or N_7. Taken in conjunction with the results for elementary analysis and the unambiguous synthetic route, there appears to be no room for doubt as to this being the desired product.

As might be anticipated, methylamination of isoguanine derivatives with a methyl substituent on N_7 proceeded with a considerably reduced yield, as a result of steric hindrance by the N_7-methyl. In those instances where the ring nitrogens of the imidazole ring were not substituted, the existence of the N_7H tautomer would be expected to interfere as well with alkylation of the exocyclic N^6. This problem has

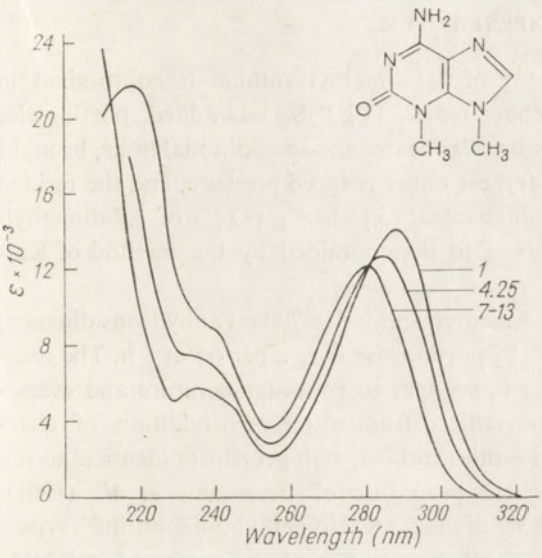
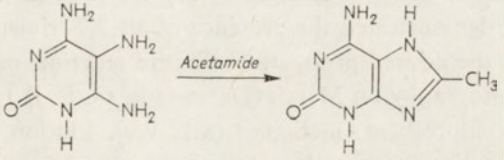


Fig. 1. U.v. absorption spectrum of 3,9-dimethylisoguanine over the pH range 1 - 13. Note the presence of only one ionic equilibrium (protonation of N_1 or N_7) with a pK_a of 4.40.

been previously encountered, and discussed in detail, in connection with alkylamination of the exocyclic N^6 of 5-alkyl cytosines and their glycosides, where the 5-alkyl substituent hinders such alkylation (Kulikowski & Shugar, 1971).

An examination of the behaviour of the various *N*-methylated isoguanine derivatives towards nitrous acid demonstrated that substitution on N_9 was of prime importance for the deamination reaction. On the basis of some literature data, Lister (1971) had suggested that substitution of the N_3 determined the behaviour towards this deamination reagent. However, under normally applied conditions, 3-methylisoguanine, 7-methylisoguanine and 3,7-dimethylisoguanine readily underwent deamination to the corresponding xanthine analogues. By contrast, 9-methylisoguanine and 3,9-dimethylisoguanine were quite resistant to this treatment, as is also isoguanosine (Davoll, 1951). All the foregoing compounds undergo hydrolytic deamination with concentrated HCl at elevated temperature.



Scheme 2

Finally, 8-methylisoguanine was obtained by fusion of 2-oxo-4,5,6-triaminopyrimidine with acetamide, as shown in Scheme 2. From Table 1, it will be noted that, as might have been anticipated, the u. v. spectrum differs only in minor respects from that for isoguanine; while the p*K* values for protonation and dissociation are also in line with expectations.

EXPERIMENTAL

3,7-Dimethyl-6-thioxanthine: To 9 g of 3,7-dimethylxanthine (theobromine) in 660 ml anhydrous dioxane, heated under reflux, 11 g P_2S_5 was added, portionwise over a period of 2 h. The reaction mixture was heated for an additional hour, brought to room temperature, evaporated to dryness under reduced pressure, and the residue crystallized from water with addition of charcoal to yield 9 g (93%) of 3,7-dimethyl--6-thioxanthine with properties identical to that obtained by the method of Kalmus & Bergmann (1960).

3-Methyl-6-thioxanthine: To 7.4 g 3-methylxanthine in 700 ml anhydrous dioxane, heated under reflux, was added 10 g P_2S_5 portionwise over a period of 3 h. The reaction mixture was further heated for 1 h, brought to room temperature and evaporated to dryness. The residue was crystallized from water with addition of charcoal to yield 6.25 g (77%) of 3-methyl-6-thioxanthine, with properties identical to the same product obtained according to the procedure of Bergmann *et al.* (1961).

3,7-Dimethylisoguanine: 1.5 g of 3,7-dimethyl-6-thiomethyl-2-oxopurine (Wooldridge & Slack, 1962) was treated with 50 ml anhydrous methanol saturated with NH_3 in a steel bomb at 130°C for 18 h. The reaction mixture was brought to room temperature, evaporated to dryness, and the residue crystallized from methanol-water with addition of charcoal to yield 1.13 g (80%) of 3,7-dimethylisoguanine monohydrate, m.p. > 300°C (sublimation). Fischer (1897) gives 350°C. Calculated: C, 42.41%; H, 5.54%; N, 36.03%; Found: C, 42.52%; H, 5.62%; N, 35.96%.

N^6,*3,7-Trimethylisoguanine*: 1.5 g of 3,7-dimethyl-6-methylthio-2-oxopurine was treated in a steel bomb with 40 ml of a 30% ethanolic solution of methylamine at 130°C for 18 h. The reaction mixture was cooled, brought to dryness, and the residue crystallized from methanol-water with addition of charcoal to yield 1.2 g (80%) of the monohydrate of N^6,3,7-trimethylisoguanine, m.p. 325 - 327°C (sublimation). Calculated: C, 45.30%; H, 6.12%; N, 33.78%; Found: C, 45.75%; H, 6.11%; N, 33.92%.

N^6,N^6,*3,7-Tetramethylisoguanine*: 1.5 g of 3,7-dimethyl-6-methylthio-2-oxopurine was treated with 40 ml of a 30% ethanolic solution of dimethylamine at 130°C for 18 h, cooled to room temperature and brought to dryness. Crystallization of the residue from alcohol-water demonstrated the presence of N^6,3,7-trimethylisoguanine, which co-crystallized with the desired product. The entire reaction mixture was therefore subjected to chromatography on Merck (Darmstadt, G. F. R.) PF_{254} silica-gel with the solvent system chloroform - methanol (8:2, v/v). Elution of the title product, followed by crystallization from methanol-water, yielded 0.53g (33%) of the monohydrate of N^6,N^6,3,7-tetramethylisoguanine, m.p. 242 - 243°C. Calculated: C, 47.93%; H, 6.66%; N, 31.05%; Found: C, 47.80%; H, 6.70%; N, 30.68%.

7-Methylisoguanine: A solution of 0.55 g of 7-methyl-6-methylthio-2-oxopurine (Lichtenberg *et al.*, 1972) in 40 ml methanol saturated with ammonia was brought to 130°C in a steel bomb and maintained at this temperature for 18 h. The reaction mixture was brought to dryness, the residue taken up in water and again brought to dryness, and the latter step repeated twice more. The product was converted to

the HCl salt by evaporation from aqueous HCl solution and crystallized from aqueous methanol to give the HCl salt of 7-methylisoguanine (0.40 g, 90%), m. p. > 300°C. Calculated: C, 35.73%; H, 3.97%; N, 34.73%; Found: C, 35.48%; H, 3.87%; N, 34.61%.

N^6,*7-Dimethylisoguanine*: A solution of 0.30 g of 7-methyl-6-methylthio-2--oxopurine in 35 ml of 30% methylamine in methanol was heated in a steel bomb for 15 h at 130°C. Following cooling and evaporation to dryness, the residue demonstrated the presence of side products and was therefore chromatographed on PF_{254} silica-gel with chloroform - methanol (7.5:2.5, v/v). Following elution, the product was converted to the HCl salt. Crystallization of this from ethanol-acetone gave 19 mg (8%) of N^6,7-dimethylisoguanine·HCl, m.p. > 250°C (decomp.). Calculated: C, 38.98%; H, 4.64%; N, 32.48%; Found: C, 38.86%; H, 4.57%; N, 32.29%.

N^6,N^6,7-*Trimethylisoguanine*: A solution of 300 mg of 7-methyl-6-methylthio-2--oxopurine in 35 ml of 30% ethanolic dimethylamine was heated at 130°C in a steel bomb for 18 h. The reaction mixture was brought to dryness and chromatographed on PF_{254} silica-gel with chloroform-methanol (7.5:2.5, v/v). The product was converted to the HCl salt and crystallized from ethanol-acetone to yield 25 mg (7%) of the desired compound, m.p. 224 - 226°C. Calculated: C, 41.83%; H, 5.23%, N, 30.50%; Found: C, 41.78%, H, 5.19%; N, 30.37%.

9-Methylisoguanine: 300 mg of 9-methyl-6-methylthio-2-oxopurine (Lichtenberg *et al.*, 1972) was treated with 20 ml anhydrous methanol saturated with NH_3 at 130°C for 12 h. The reaction mixture was brought to dryness, the residue taken up in water and brought to dryness, and the latter step repeated twice more. The final gelatinous residue was dissolved in 3 M-HCl and taken to dryness. Crystallization of the residue from aqueous methanol gave 210 mg (86%) of 9-methylisoguanine·HCl, m.p. > 300°C, Calculated: C, 35.73%; H, 3.97%; N, 34.73%; Found: C, 35.98%; H, 3.84%; N, 34.49%.

N^6,*9-Dimethylisoguanine*: 350 mg 9-methyl-6-methylthio-2-oxopurine was treated with 15 ml of a 30% ethanolic solution of methylamine for 13 h at 130°C. The reaction mixture was taken to dryness, the gelatinous residue converted to the HCl salt as above, and the latter crystallized from aqueous ethanol with addition of acetone to yield 190 mg (66%), m.p. > 270°C. Calculated: C, 38.98%; H, 4.64%; N, 32.48%; Found: C, 39.27%, H, 4.57%, N, 32.22%.

N^6,N^6,*9-Trimethylisoguanine*: 350 mg 9-methyl-6-methylthio-2-oxopurine was treated with 15 ml of a 30% ethanolic solution of dimethylamine for 13 h at 130°C. The mixture was brought to dryness, the gelatinous residue converted to the HCl salt as above, and then crystallized from anhydrous ethanol on addition of ether to turbidity to yield 305 mg (93%), m.p. > 250°C (decomp.). Calculated: C, 41.83%; H, 5.23%; N, 30.50%; Found: C, 41.61%; H, 5.17%, N, 30.27%.

3-Methylisoguanine: A solution of 1.2 g of 3-methyl-6-methylthio-2-oxopurine (Bergmann & Kleiner, 1963) in 30 ml methanol saturated with NH_3 was brought to 130°C and maintained at this temperature for 18 h. The reaction mixture was then brought to dryness and the residue crystallized from methanol-water with addition

of charcoal to yield 0.81 g (72%) of the monohydrate of 3-methylisoguanine, m. p. > 300°C. Calculated: C, 39.37%; H, 4.92%; N, 39.02%; Found: C, 39.80%; H, 5.05%; N, 38.80%. Rogers & Ulbricht (1971) obtained anhydrous 3-methylisoguanine by drying their product at 190°C at 0.01 mm Hg.

N^6,*3-Dimethylisoguanine*: A solution of 1.2 g of 3-methyl-6-methylthio-2-oxopurine in 30 ml of a 30% ethanolic solution of methylamine was heated in a steel bomb at 130°C for 18 h. The reaction mixture was brought to dryness and the residue crystallized from methanol-water with addition of charcoal to yield 0.72 g (60%) of the monohydrate of the title compound, m.p. > 300°C. Calculated: C, 42.62%; H, 5.01%; N, 35.56%; Found: C, 42.86%; H, 4.96%; N, 35.90%.

N^6,N^6,*3-Trimethylisoguanine*: A solution of 1.5 g of 3-methyl-6-methylthio-2--oxopurine in 30 ml of 30% ethanolic dimethylamine was maintained at 130°C for 18 h, cooled and brought to dryness. Chromatography demonstrated the presence of N^6,3-dimethylisoguanine. The mixture was therefore chromatographed on Merck PF_{254} silica-gel with chloroform-methanol (8:2, v/v). The required product was eluted with methanol, the eluate brought to dryness and the residue crystallized from aqueous ethanol to yield 0.22 g (17%) of the monohydrate of N^6,N^6,3-trimethylisoguanine, m.p. 314 - 316°C. Calculated: C, 45.48%; H, 5.68%; N, 33.21%. Found: C, 45.78%; H, 5.46%; N, 33.51%.

3,9-Dimethyl-6-methylthio-2-oxopurine: This was obtained by thiation of 3,9--dimethylxanthine in dioxane, followed by methylation as described by Lichtenberg *et al.* (1972) for other derivatives. Yield 70%, m.p. 225°C (decomp.).

3,9-Dimethylisoguanine: A mixture of 240 mg of 3,9-dimethyl-6-methylthio-2--oxopurine in 40 ml anhydrous methanol saturated with ammonia was heated for 15 h at 130°C. The reaction mixture was brought to dryness and the residue crystallized from aqueous ethanol following addition of charcoal, to yield 140 mg of the monohydrate (68%), m.p. > 300°C. Calculated: C, 42.64%; H, 5.58%; N, 35.53%; Found: C, 42.51%; H, 5.46%; N, 35.40%.

8-Methylisoguanine: A mixture of 0.43 g of 2-oxo-4,5,6-triaminopyrimidine (Bendich *et al.*, 1948) and 6 g acetamide was heated under reflux for 30 min. The reaction mixture was cooled, 20 ml water added, and the mixture decolorized with charcoal. The charcoal was removed by filtration and the filtrate cooled to give an amorphous precipitate, which was crystallized from 2 N-HCl to give 280 mg (46%) of 8-methylisoguanine·HCl, m.p. > 300°C. Calculated: C, 35.91%; H, 3.99%; N, 34.99%; Found: C, 35.90%; H, 3.78%; N, 34.82%.

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OTRZYMYWANIE *N*-METYLOWANYCH POCHODNYCH IZOGUANINY POPRZEZ 6-METYLOTIO-2-KETOPURYNY ORAZ OTRZYMYWANIE 8-METYLOIZOGUANINY

Streszczenie

Opisano ogólną metodę otrzymywania *N*-metylowanych pochodnych izoguaniny łącznie z jednoznacznie przeprowadzoną syntezą 3,9-dwumetyloizoguaniny. Metoda oparta jest na aminowaniu odpowiednich *N*-metylowanych pochodnych 6-metylotio-2-ketopuryny i umożliwia otrzymywanie również egzocyklicznych N^6-metylowanych pochodnych. Podano niektóre dane spektralne i wartości p*K* szeregu pochodnych izoguaniny. W przeciwieństwie do wcześniejszych uogólnień stwierdzono, że odporność na dezaminację kwasem azotowym *N*-metylowanych pochodnych izoguaniny zależy od podstawienia w pozycji N^9. 8-Metyloizoguaninę otrzymano przez kondensację 2-keto-4,5,6-trójaminopirymidyny z acetamidem.

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BIOCHEMICAL STUDIES ON SOME NEW ANALOGUES OF ADENOSINE-3′,5′-CYCLIC PHOSPHATE, INCLUDING ISOGUANOSINE-3′,5′-CYCLIC PHOSPHATE**

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1. A new analogue of adenosine-3′,5′-cyclic phosphate (cAMP) is described. It is isoguanosine-3′,5′-cyclic phosphate (iso-cGMP, 2-oxoadenosine-3′,5′-cyclic phosphate), which is also an isomer of guanosine-3′,5′-cyclic phosphate (cGMP). The analogue was prepared by a photochemical procedure from the N_1-oxide of cAMP (cNOAMP). The latter analogue was obtained in crystalline form as the free acid. Xanthosine-3′,5′-cyclic phosphate (cXMP) was also prepared directly by cyclization of 5′-XMP. 2. The three foregoing cAMP analogues were found to be substrates of rabbit brain cyclic phosphodiesterase, and their K_m and V_{max} values were determined relative to those for the natural substrates cAMP and cGMP. 3. None of the analogues was as active as cAMP in the *in vitro* activation of beef heart protein kinase. The one analogue, cNOAMP, which did exhibit activity comparable to that of cAMP, did not, however, show the normally observed decrease in stimulation of kinase observed with cAMP at higher concentrations.

A wide variety of cAMP analogues has now been synthesized comporting modifications of the heterocyclic ring or the sugar moiety (for review see Simon *et al.*, 1973). The interest in such analogues centres around their potential biological activity, including their ability to stimulate cAMP-dependent protein kinase, and their susceptibility to 3′,5′-cyclic phosphodiesterase as well as their possible inhibitory effects on the latter. It has also been shown that nucleoside-3′,5′-cyclic phosphates may serve as depot forms of known antiviral and antitumour agents, and more readily pass through cell membranes and subsequently undergo enzymic hydrolysis to the active 5′-phosphates (Sidwell *et al.*, 1973).

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We have recently demonstrated that iso-cGMP[1] may be synthesized by a photochemical procedure from the N_1-oxide of cAMP (Kazimierczuk & Shugar, 1973). Although isoguanosine has long been known as a constituent of croton seeds (Cherbuliez & Bernhard, 1932) and has also been prepared by chemical procedures (Davoll, 1951), its potential biological activity has been little studied. That this may be of interest is testified to by the recent isolation from corn of an isoguanine analogue of zeatin (Letham, 1973).

It consequently appeared of interest to examine the possible activity of iso-cGMP, which is not only a closely-related cAMP analogue (2-oxo-adenosine-3′,5′-cyclic phosphate), but also an isomer of cGMP. Preparation of iso-cGMP required the prior synthesis of the N_1-oxide of cAMP, and since reports on the activity of the latter are somewhat conflicting, the behaviour of this analogue, obtained in crystalline form, was also examined. We have also prepared cXMP directly from 5′-XMP, in place of deamination of cGMP (Miller *et al.*, 1973b) and included this derivative in our studies.

MATERIALS

Both 5′-AMP and 5′-XMP were products of Waldhoff (Mannheim, G. F. R.). [8-^{3}H]cAMP, specific activity 30 Ci/mmole, was purchased from The Radiochemical Centre (Amersham, England). We are indebted to Dr W. Jankowski and Prof. T. Chojnacki for the [γ-^{32}P]ATP, activity 6.7×10^4 c. p. m./pmole.

Crotalus adamanteus venom was obtained from Koch-Light (Colnbrook, Bucks, England), purified 5′-nucleotidase from *Cr. adamanteus* venom from Sigma (St. Louis, Mo., U. S. A.), calf thymus histone from the Molecular Biology Department of the Łódź Medical Academy.

Cyclic nucleotides: cAMP was obtained from Waldhoff (Mannheim, G. F. R.) and cGMP from Boehringer (Mannheim, G. F. R.).

Cyclic phosphodiesterase (EC 3.1.4.17) was prepared from rabbit brain as described by Drummond & Perrott-Yee (1961). The protein content of the preparation, determined according to Lowry *et al.* (1954) with albumin as standard, was 3 mg/ml.

cAMP-dependent kinase (EC 2.7.1.37) was prepared from beef heart according to the procedure of Miyamoto *et al.* (1969) up to, and including, chromatography on a DEAE-cellulose column.

N_1-Oxyadenosine-3′,5′-cyclic phosphate: To 300 mg cAMP in 20 ml water was added, portionwise, 280 mg of 85% *m*-chloroperbenzoic acid over a period of 3h during which the pH was maintained in the range 6.5 - 8.5 by addition of 2 N-NaOH. The mixture was stirred for an additional 2 h, then diluted with water to 50 ml and extracted with 2×50 ml chloroform and 50 ml ether. The aqueous phase was diluted sixfold and placed on a 32×1.5 cm column of Dowex 1X2 ($HCOO^-$). The column was washed with water and eluted with 0.1 N-formic acid. Non-reacted cAMP was found in the effluent between 2.5 and 3 litres, while the N_1-oxide of cAMP was eluted

[1] Unusual abbreviations employed: cXMP, xanthosine-3′,5′-cyclic phosphate; iso-cGMP, isoguanosine-3′,5′-cyclic phosphate; cNOAMP, N_1-oxide of cAMP; DTT, dithiothreitol.

between 5.4 and 6 litres. The combined eluates of the N_1-oxide were brought to dryness under reduced pressure at about 40°C, the residue taken up in a small volume of water and precipitated by addition of a mixture of alcohol and acetone, yield 208 mg (69%). The product was crystallized as the free acid by dissolving in a small volume of aqueous ethanol under reflux and then bringing to room temperature. The product crystallized in the form of tiny platelets, m. p. 245 -247°C.

Iso-cGMP was prepared from the N_1-oxide of cAMP by a photochemical procedure as elsewhere described (Kazimierczuk & Shugar, 1973).

Xanthosine-3′,5′-cyclic phosphate (cXMP): To 266 mg of xanthosine-5′-phosphate (free acid) in 5 ml water was added 260 mg of *N,N′*-dicyclohexylcarbodiimide--4-morpholidate, and the whole then brought to dryness. The residue was dried four times from anhydrous pyridine to remove traces of water, and dissolved in 100 ml anhydrous pyridine. To this solution under reflux was added, dropwise, over a period of 3 h, 420 mg *N,N′*-dicyclohexylcarbodiimide in 100 ml anhydrous pyridine. The mixture was heated under reflux for a further 2 h, following which 4 ml water was added and the whole brought to dryness. The residue was taken up in water and brought to dryness twice to remove traces of pyridine, then dissolved in 500 ml water and filtered. The filtrate was deposited on a 21×1.5 cm column of Dowex 1X2 (HCO_3^-) and eluted with triethylammonium carbonate (3 litres) using a gradient of 0.05 - 1 M. The desired cXMP was found in the eluates between 1.6 and 2 litres, which were pooled, brought to dryness and the residue converted to the sodium salt (59 mg, 21%). This compound was also prepared by Miller *et al.* (1973b) by deamination of cGMP in about 48% yield.

METHODS

cAMP-dependent kinase activity. This was determined by the procedure of Kuo & Greengard (1970), over a cyclic nucleotide concentration range of 10^{-8} - 10^{-3} M, and addition to incubated samples of 0.7 nanomoles of [γ-^{32}P]ATP with an activity of $4 - 6 \times 10^4$ c. p. m.

Cyclic phosphodiesterase activity was followed by two methods; (a) at substrate concentrations above 0.1 mM, the reaction being conducted in two stages, as follows: the incubation medium, 0.1 ml, contained 1×10^{-4} M to 30×10^{-4} M-cyclic nucleotide, 30 mM-Tris-HCl buffer, pH 7.5, 10 mM-$MgCl_2$; the reaction was initiated by addition of 0.03 mg of the phosphodiesterase preparation. Following incubation at 30°C for 10 min, the tube was brought to 100°C for 3 min to inactivate the enzyme, cooled to 37°C, 10 µl of snake venom solution (10 mg/ml) added, and incubation continued for 30 min. Liberated inorganic phosphate was estimated by the method of Chen *et al.* (1956). In several control runs, purified 5′-nucleotidase was used to verify complete dephosphorylation of the 5′-nucleotide by the crude snake venom; (b) the following procedure was employed to determine inhibition of cAMP hydrolysis by the analogues: The incubation medium, 0.05 ml, with the same buffer and Mg^{2+} concentrations as above, contained substrate concentrations ranging from 1×10^{-7} M to 1×10^{-3} M with addition of 1×10^{-1} µCi labelled cAMP. The reaction was ini-

tiated by addition of 0.005 - 0.03 mg protein of the enzyme solution (the stock enzyme solution was diluted with 0.05 M-Tris-HCl buffer, pH 7.5, containing 2 mM-EDTA and 0.1 mM-DTT). Incubation was for 10 min at 30°C, and the reaction terminated by heating at 100°C for 3 min. Cold carrier cAMP and 5′-AMP were then added, and the entire solution chromatographed on Whatman paper no. 1 (ascending) with ethanol - 1 M-ammonium acetate (7:3, v/v). The spots corresponding to cAMP and 5′-AMP were cut out and counted in a Nuclear Chicago scintillation counter. Control samples were brought to 100°C immediately on addition of the enzyme.

RESULTS AND DISCUSSION

Susceptibility to cyclic phosphodiesterase

With the partially purified rabbit brain enzyme at our disposal, all the cyclic nucleotides were active as substrates. With the use of method (a) for following hydrolysis to the 5′-phosphates, Lineweaver-Burk plots were employed to determine the Michaelis constants K_m and the maximal velocities of hydrolysis V_{max} (Fig. 1 and Table 1). It will be noted that all three analogues are somewhat poorer substrates than cAMP itself, but that there is no particularly pronounced difference in behaviour between them. Examination also showed that there was no significant difference in the optimal conditions for their hydrolysis to the 5′-phosphates.

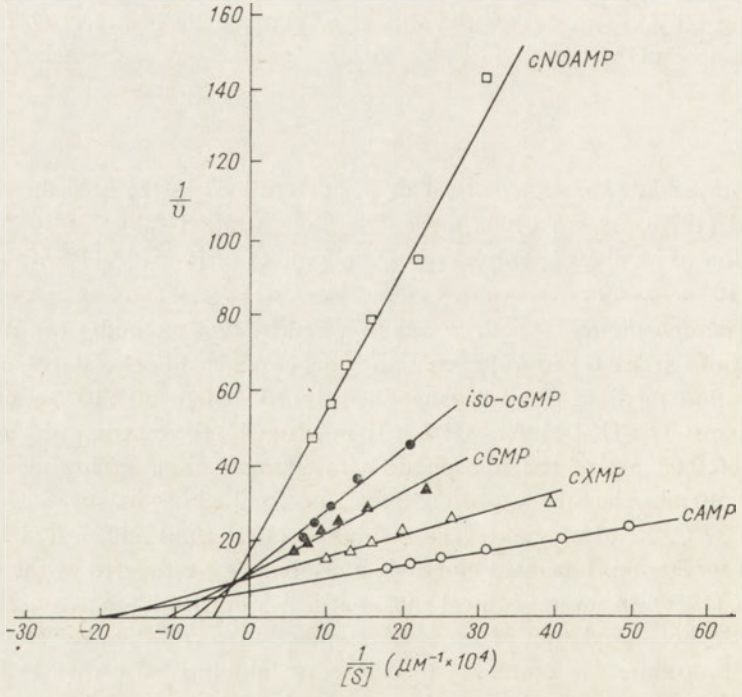

Fig. 1. Double reciprocal plots for the hydrolysis of cyclic nucleoside phosphate analogues by rabbit brain phosphodiesterase; v is in μmoles P_I/min/mg enzyme protein (see section (a) in Methods for further details).

Table 1

Michaelis constants and maximal velocities for hydrolysis of some cAMP analogues by rabbit brain cyclic phosphodiesterase

Analogue	K_m (M × 10^4)	V_{max} (μmoles $P_i \times 10^3 \times min^{-1} \times mg^{-1}$ enzyme protein)
cAMP	5	0.150
cXMP	7	0.108
cNOAMP	20	0.054
iso-cGMP	8	0.070
cGMP	8	0.100

Table 2

Relative rates of hydrolysis of cyclic phosphate analogues by partially purified rabbit brain cyclic phosphodiesterase

With the incubation conditions employed (see Methods), the absolute rate of hydrolysis of cAMP to 5′-AMP was 2.2 nmoles/min by 30 μg enzyme protein at 30°C. Cyclic phosphate concentrations were 0.5 mM, corresponding to the K_m value for cAMP determined in this study.

Analogue	Relative hydrolysis rate
cAMP	1.0
cNOAMP	0.15[a]
iso-cGMP	0.39
cGMP	0.44[b]
cXMP	0.64[c]

[a] With the rabbit kidney enzyme and a cyclic nucleotide concentration of 5 mM, Meyer *et al.* (1973) report a value of 0.39.

[b] With the dog heart enzyme, and a nucleotide concentration of 2 mM, Nair (1966) reports 0.33 for cGMP; with the rat liver enzyme, this value was 0.50.

[c] With the rabbit kidney enzyme, and nucleotide concentrations of 5 mM, Miller *et al.* (1973b) report values for cGMP and cXMP of 0.53 and 0.03 (see Discussion).

The data presented in Fig. 1 and Table 1 were used to formulate Table 2, which presents the relative hydrolysis rates of the various cyclic phosphates in a manner permitting of comparison with those of other observers. From the data and footnotes contained in Table 2, it will be seen that there is reasonably good agreement with other published values. The one exception is cXMP, where our value for the relative hydrolysis rate is 0.64, as compared to a value of 0.03 reported by Miller *et al.* (1973b). Such a large difference is difficult to interpret in terms of the fact that these authors used rabbit kidney as the source of their enzyme. It should be noted that the rates of hydrolysis of cAMP by the enzymes from rat brain and rat kidney are almost identical, although for other organs of the rat the rates are appreciably lower (Beavo *et al.*, 1971). We have checked the spectral properties of the cXMP preparation

of Miller *et al.* (1973b) against those of our own, which was prepared by a different procedure (see Materials), and find them to be identical. We subsequently subjected our cXMP preparation to exhaustive hydrolysis with our phosphodiesterase preparation and found that it was hydrolysed quantitatively to 5′-XMP, thus removing any possible doubts about its authenticity.

It is possible that the difference between our results and those of Miller *et al.* (1973b) may derive from the crude nature of the enzyme preparations employed in both cases (see also below). Resolution of this discrepancy will therefore require prior examination of the rates of hydrolysis with both enzyme sources under identical conditions; further discussion is probably not warranted until this has been done. However, Michal *et al.* (1970), using a commercially available preparation of cXMP, found that the susceptibility of this analogue to cyclic phosphodiesterase from beef heart was about 20% that of cAMP, hence comparable to our own results.

Inhibition of cAMP hydrolysis by the various analogues was examined with the aid of Method (b). The rate of hydrolysis of cAMP, at a concentration of 1 μM, was found to be unaffected in the presence of cXMP, iso-cGMP or cNOAMP when each of these was added to a concentration of 1 - 100 μM. This finding is consistent with the observation of Beavo *et al.* (1971), who noted that hydrolysis of cAMP (at a concentration of 1 μM) by the cyclic phosphodiesterase from the 20 000 *g* supernatant of rat brain was unaffected by the presence of cGMP at a concentration of 2 μM.

In view of the foregoing, and the fact that we did not have at our disposal labelled samples of the various cAMP analogues, it was decided to examine possible inhibitory effects at concentrations of the order of 1 mM. This was done with the aid of Method (b), the results being summarized in Table 3. Bearing in mind that Method (b) measures specifically the amount of cAMP hydrolysed (with the aid of labelled cAMP), it will be noted that both cXMP and cGMP appreciably inhibit cAMP hydrolysis, possibly because both are reasonably good substrates (Table 1) and

Table 3

Inhibition of cAMP hydrolysis by rabbit brain cyclic phosphodiesterase in the presence of various cAMP analogues

Concentration of cAMP in all instances 0.5 mM, with each analogue added to same concentration. Hydrolysis was followed by the appearance of labelled 5′-AMP, using Method (b). Enzyme protein added to each sample was 0.6 mg/ml.

Analogue	Hydrolysis of mixture containing labelled cAMP + analogue indicated, each at a concentration of 0.5 mM (nmole [^{3}H]5′-AMP liberated)	Inhibition (%)
cAMP alone	19	—
cXMP	11	44
cNOAMP	17	9
iso-cGMP	17	9
cGMP	2.5	87

readily compete for the enzyme. What is somewhat surprising is the lack of inhibition by iso-cGMP, which is at least as good a substrate as cGMP. Furthermore, when cNOAMP (which is a feeble inhibitor) was tested at a concentration of 1.0 mM in the presence of 0.5 mM-cAMP, the degree of inhibition did not exceed the 9% indicated in Table 3 for a concentration of 0.5 mM.

The foregoing results are not readily susceptible of interpretation on the assumption that the rabbit brain extract employed contains only one cyclic phosphodiesterase. It is, consequently, of interest to recall that rat liver contains at least two (Bevers *et al.*, 1974), and perhaps more (Russell *et al.*, 1973), distinct phosphodiesterases with differing specificities. Our own findings suggest that rat brain must also contain more than one type of cyclic phosphodiesterase and trials are now under way with a view to separating these activities.

In view of the known inhibition of cyclic phosphodiesterase by 3,7-dimethylxanthine and 1,3,7-trimethylxanthine (caffeine), the possible effect of some methylated isoguanines (Kazimierczuk & Shugar, 1974) was examined. However, concentrations of up to 10^{-4} M of 3-methyl-, 7-methyl-, 9-methyl-, 3,7-dimethyl- and N^6,3,7-trimethyl--isoguanines exhibited no detectable inhibition.

cAMP-dependent kinase activation

Figure 2 exhibits the activation profiles of bovine heart protein kinase by the three cAMP analogues, together with that for cAMP itself, which served as a reference standard. The profile for cAMP is similar to that reported by other observers. Table 4 presents the values of K_a (cyclic nucleotide concentration giving 50% of maximal activation) and $K_{a'}$ (relative activity *vs.* cAMP as 100). Of the three analogues tested, it will be seen that only cNOAMP exhibited activity comparable to that of cAMP. Furthermore, for both cXMP and iso-cGMP, optimal activation was also appreciably lower than that for cAMP.

It will be noted from Fig. 2 that, at elevated concentrations of cAMP ($> 10^{-6}$ M), kinase activation is appreciably decreased, a fact noted by other observers (Jones *et al.*, 1973). None of the three analogues tested exhibited such decreases in activation at higher concentrations. Such a decrease in activation might have been expected for cNOAMP, which exhibits activation comparable to that for cAMP. But, as will be seen from Fig. 2, cNOAMP activation shows no tendency to decrease even at a concentration of 10^{-3} M. It has been proposed that the decrease in cAMP stimulation at high concentrations is due to competition between cAMP and ATP for the kinase (Donnelly *et al.*, 1973b).

We are at a loss to interpret the low degree of activation reported by Meyer *et al.* (1973) for cNOAMP, as compared to the relatively high value found by us (Fig. 2 and Table 4). However, the foregoing authors employed the enzyme from beef brain, which may conceivably be the source of this difference (see Addendum).

It is possible that the low activity of cXMP could be due to the fact that at the incubation pH, 6.0, the xanthine ring is partially ionized (Cavalieri *et al.*, 1954). Attempts were therefore made to compare activities at pH 5.5, where the xanthine

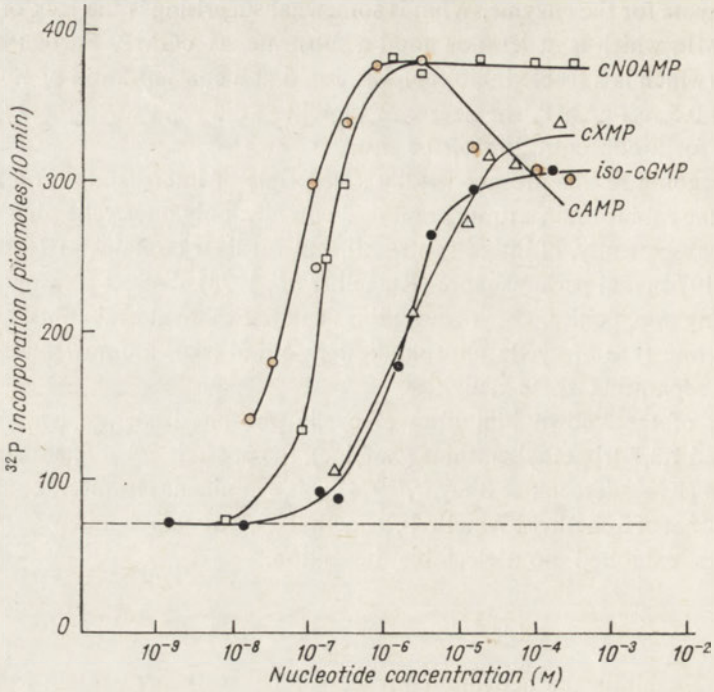

Fig. 2. Activation of bovine heart protein kinase by cAMP and its analogues. Results are expressed in picomoles of ^{32}P incorporated into 40 μg histone in 10 min by 5 μg kinase.

Table 4

Relative activities of cAMP analogues for activation of bovine heart protein kinase

cAMP analogue	K_a (M)	$K_{a'} = \frac{K_a \text{ (cAMP)}}{K_a \text{ (analogue)}} \times 100$
cAMP	8×10^{-8} [a]	100
iso-cGMP	2.5×10^{-6}	3.2
cNOAMP	2×10^{-7}	40[b]
cXMP	3×10^{-6}	2.7[c]

[a] 4×10^{-8} M (Kuo *et al.*, 1970); 7.3×10^{-8} M (Miller *et al.*, 1973a).

[b] Meyer *et al.* (1973) report a value of 4.5 for beef brain kinase; and with the same enzyme, 2.3 for cGMP.

[c] Miller *et al.* (1973b) report a value of 1.6 for beef brain kinase.

ring is virtually in the neutral form. However, at this pH the activities of both cAMP and cXMP were too low to permit of accurate measurements.

The general problem of the apparent "inhibition" of the enzymic hydrolysis of cAMP by some "substrate" analogues, and low inhibition by others, is undoubtedly worthy of further study. This is further underlined by the observation of Beavo *et al.* (1971) on the specific modulation of the activity of cAMP phosphodiesterase

from various organs by e.g. cGMP; at low concentrations, cGMP stimulates the hydrolysis of cAMP, while at high concentrations it inhibits. There is now no longer any doubt about the existence in a number of organs of enzymic systems which react in a differential manner (occasionally in opposing directions) to an increase or decrease in cAMP and cGMP concentrations (Kuo & Greengard, 1970; Russell *et al.*, 1973; Donnelly *et al.*, 1973a). In the light of the foregoing, it is possible that iso-cGMP may prove useful in the differentiation of cAMP- and cGMP-dependent enzymic systems. Trials in this direction are now in progress.

One final comment is called for at this point. In the present study we have treated cXMP as a cAMP analogue, largely because the appropriate enzymic systems had been prepared in this laboratory. However, strictly speaking, cXMP should really be considered a cGMP analogue, so that its potential activity should be measured with cGMP-dependent enzyme systems.

We should like to thank Mr. Piotr Naimski for assistance with the syntheses, Miss Dorota Haber for excellent technical assistance, and Doc. Dr A. Michałowski of the Institute of Oncology for making available counting facilities.

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BADANIA BIOLOGICZNE NOWYCH ANALOGÓW 3′,5′-CYKLICZNEGO FOSFORANU ADENOZYNY WŁĄCZNIE Z 3′,5′-CYKLICZNYM FOSFORANEM IZOGUANOZYNY

Streszczenie

1. W pracy opisano syntezę 3′,5′-cyklicznego fosforanu izoguanozyny (izo-cGMP), nowego analogu 3′,5′-cyklicznego fosforanu adenozyny (cAMP). Związek ten może być uznany za izomer 3′,5′-cyklicznego fosforanu guanozyny (cGMP). Fotochemiczną syntezę izo-cGMP poprzedziła synteza 3′,5′-cyklicznego fosforanu N_1-tlenku adenozyny (cNOAMP), otrzymanego w formie krystalicznej jako kwas. Opisano również syntezę 3′,5′-cyklicznego fosforanu ksantozyny z 5′-fosforanu ksantozyny.

2. Syntetyzowane analogi cAMP są substratami fosfodwuesterazy cyklicznych fosforanów wyodrębnionej z mózgu królika. W pracy oznaczono stałe Michaelisa (K_m) i wartości szybkości maksymalnej (V_{max}) enzymatycznej hydrolizy analogów w porównaniu z naturalnymi substratami enzymu, cAMP i cGMP.

3. Wszystkie badane analogi były gorszymi aktywatorami *in vitro* kinazy białkowej wyodrębnionej z serca wołu. cNOAMP, jakkolwiek aktywował kinazę w stopniu porównywalnym z cAMP, to jednak stosowany w wyższych stężeniach, w przeciwieństwie do cAMP, nie powodował obniżenia poziomu stymulacji kinazy białkowej.

Received 7 July, 1974.

Addenum (*added in proof*): Since submission of the foregoing, a paper has appeared (B. Jastorff & W. Freist, *Bioorganic Chemistry*, 3, 103 - 113, 1974) in which the activation of beef heart protein kinase by cNOAMP was measured and found to be approximately the same as that reported in this paper.

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RECENZJE KSIĄŻEK

VIRUS RESEARCH, collective volume edited by C. Fred Fox & William S. Robinson, Publication date February 8, 1974, Academic Press, New York and London, pp. XXV+599, price £ 9.00 or $ 19.50.

This volume presents the contributions delivered at the second of a series of conferences organized in 1973 by the Molecular Biology Institute of the University of California at Los Angeles, under the sponsorship of the International Chemical and Nuclear Corporation.

As stated in the preface by the editors, one of the major aims of this meeting was to "assist investigators... in bacterial and animal virology... in better establishing a dialog" between their respective fields. The extensive list of well-known participants in both fields testifies to the achievement of this objective, as does the subject matter itself, which is organized under the following general headings: I. Replication and Integration of Viral Genomes, II. Transcription and Translation of Animal Virus Genomes, III. Transcription, Translation, and Regulation in Bacterial Virus Genome Expression, IV. Structure and Assembly of Bacterial Viruses, V. Nucleic Acid Structure and Viral Oncogenicity, VI. Virulence, Latency and Molecular Pathology, VII. Genetics and Recombination, VIII. Sequencing of Viral Nucleic Acids.

Two of the contributions deal with the subject of anti-viral agents. One of these, by Levinson *et al.* of the University of California at San Francisco, describes the apparently specific inhibition of the RNA-directed DNA polymerase of Rous sarcoma virus, and several other oncogenic viruses, and the transforming ability of these viruses, by the well-known *N*-methyl isatin *β*-thiosemicarbazone. The other, by Simon *et al.* of ICN, Irvine, California, summarizes the results of several years of research by this team on the anti-viral activity of "Virazole" (1-*β*-D-ribofuranosyl-1,2,4-triazole-3--carboxamide), which at the moment appears to be the most promising wide-spectrum anti-viral agent against a number of RNA and DNA viruses, including the influenza viruses. Although this agent is shown to be a potent inhibitor of IMP dehydrogenase, it is perhaps premature to conclude that this is the sole basis of its molecular action, and further studies are both required and warranted in view of the promise held out by this important derivative.

The last section of this volume contains four contributions dealing with the sequencing of viral nucleic acids. Two of these present new approaches to this formidable problem. Salser *et al.*, of the University of California at Los Angeles show how to approach this by two pathways: (a) the *in vitro* synthesis of highly labelled *ribo*substituted DNA with the aid of DNA polymerase I in the presence of Mn^{2+}, based on the observation made more than 10 years ago by Berg and Chamberlin; the resulting RNA copy of the DNA template may then be subjected to sequencing by the procedures already established for RNA sequencing; (b) the application of reverse transcriptase to make a DNA copy of a messenger RNA, and use of the resulting DNA as a template for *in vitro* synthesis of labelled RNA, which again may be subjected to sequence analysis by standard techniques. Independently, Sanger of Cambridge also describes the use of Mn^{2+} to obtain an RNA copy of a specific DNA sequence in phage f1 DNA, followed by standard sequence analysis of the resulting RNA by techniques largely developed by himself originally for ribosomal 5S RNA. In the same contribution, Sanger reviews methods developed by other laboratories.

It is, however, symptomatic of the rapid rate of current developments in this field that, at the time the conference was organized, no separate presentation was included on the use of bacterial restriction nucleases for specific fragmentation of mammalian and viral DNA. The successes achieved

with this technique in the past two years, of which SV40 viral DNA is a striking example, are certain to be enlarged in the immediate future.

This volume is of interest not only to those working with bacterial and mammalian viruses, and with the development of anti-viral and anti-tumour agents. It presents considerable new fundamental information on virus-host interactions which should be of general interest also to those engaged in research in the fields of enzymology, molecular genetics, etc.

David Shugar

BACTERIAL MEMBRANES AND WALLS, Loretta Leive, ed., pp. 1 - 496. Marcel Dekker, Inc., New York, 1973; cena $ 38.00.

Od szeregu lat wzrasta w biologii molekularnej zainteresowanie badaniami nad budową i funkcją błon biologicznych. Jest ono wynikiem poznawania coraz nowych faktów, świadczących o doniosłej roli błon w bardzo różnorodnych zjawiskach biologicznych, jak kompartamentacja, przemiany energetyczne, regulacja procesów biochemicznych i fizjologicznych, recepcja bodźców, ruch, podział komórkowy itp.

Komórka bakteryjna, która jest od dawna używana jako nadzwyczaj dogodny model dla badań biologicznych, m.in. z uwagi na możliwość łatwej korelacji danych uzyskiwanych metodami genetyki i biochemii, jest przedmiotem szczególnie intensywnych prac również w dziedzinie biologii molekularnej błon.

Jak w każdej szybko rozwijającej się gałęzi nauki, tak i w problemie biologii i biochemii błon komórkowych odczuwa się brak dobrych opracowań syntetycznych, zwięzłych a jednak ukazujących wątpliwości lub pozostałe do zbadania zagadnienia, dających pogląd na całość zagadnienia a jednocześnie dość aktualnych aby zainteresować nawet badaczy aktywnie pracujących w danej dziedzinie.

Próbą takiego opracowania jest książka *Bacterial Membranes and Walls* pod redakcją Loretty Leive.

Zastosowano tu układ rozdziałów, opracowanych przez poszczególnych wybitnych specjalistów w danej dziedzinie, w którym poświęcając jednolitość stylu i ujęcia zyskuje się ogromnie na aktualności poruszanych problemów i wartości specjalistycznej materiału.

Całość podzielona jest na trzy części: Biosynteza, Współdziałanie ze Środowiskiem oraz Morfogeneza i Reprodukcja.

W pierwszej części wyczerpująco omówiono biosyntezę peptydoglikanu oraz lipopolisacharydów zewnętrznej warstwy błony. Stosunkowo mniej miejsca poświęcono niestety błonie cytoplazmatycznej, kładąc główny nacisk na omówienie biosyntezy lipidów bakteryjnych.

W drugiej części omówiono transport z punktu widzenia badań nad białkami wiążącymi substancje transportowane przez błony (L. A. Heppel) oraz badań nad pęcherzykami membranowymi jako modelem dla transportu (H. R. Kaback). W tej części znajduje się również artykuł o transformacji oraz o kolicynach.

Trzecia część zawiera dwa artykuły o roli błon w regulacji podziału komórkowego oraz w utrzymaniu określonego kształtu przez komórkę bakteryjną. Ten rozdział porusza najbardziej fascynujące zagadnienia całego problemu, mogące mieć implikacje wychodzące znacznie poza obręb biologii komórki bakteryjnej. Na przykładzie tego rozdziału jednakże widoczna jest nadzwyczajna szybkość postępu badań w tej dziedzinie, gdyż aktualizacja problemów poruszonych w tych dwu artykułach wypełniłaby zapewne miejsce wystarczające na dwa nowe artykuły.

W sumie należy stwierdzić, że *Bacterial Membranes and Walls* znajdzie zapewne czytelników zarówno wśród biochemików, jak i mikrobiologów oraz genetyków. Książka ta stanowi pierwszy tom całej serii wydawnictw pod wspólnym tytułem Microbiology Series i jakkolwiek wydawcy nie podają, co będzie treścią następnych tomów, ani nawet jakie będą ich tytuły, to pierwszy tom będzie stanowił solidną reklamę dla następnych.

Michał Bagdasarian

PROTIDES OF THE BIOLOGICAL FLUIDS. Proceedings of the Twenty-First Colloquium, Brugge, 1973. H. Peeters, ed. Pergamon Press, Oxford, New York, Toronto, Sydney, Braunschweig 1974; str. 677, cena £17.00.

Kolejny 21 tom z serii *Protides of the Biological Fluids*, wydany z okazji Międzynarodowego Sympozjum w Brugge (Belgia), poświęcony jest trzem zagadnieniom: najnowszym osiągnięciom w badaniach błon komórkowych, proteinurii oraz nowym metodom stosowanym w biochemii klinicznej.

W części poświęconej białkom błon komórkowych znajdujemy liczne prace dotyczące ich izolowania z różnych tkanek, głównie z krwinek czerwonych, limfocytów i płytek krwi. Nie brak jednak prac o białkach z błon komórkowych mięśni i innych tkanek. Szeroko omówiona jest także rola białek i lipidów w budowie błon komórkowych oraz wzajemne powiązanie tych składników. Z artykułów omawiających enzymy i hormony powiązane z błonami do ciekawszych należy zaliczyć ten, który omawia badania nad komórkowymi receptorami insuliny i ich ilościowe oznaczanie.

Druga część książki zawiera kilkadziesiąt prac na temat białkomoczu, a o ważności tego tematu może świadczyć fakt, że w ostatnich kilku latach odbyły się trzy międzynarodowe konferencje poświęcone temu zagadnieniu. Ta część książki będzie bez wątpienia bardzo ciekawa dla lekarzy i personelu laboratoriów analitycznych, ponieważ można tam znaleźć artykuły dotyczące etiologii i patogenezy białkomoczu, jego klasyfikacji i oznaczania. Oznaczanie pewnych białek, a szczególnie enzymów występujących w moczu, może być bardzo pomocne w diagnostyce.

Tradycyjnie już trzecia część książek z tej serii zajmuje się nowymi metodami biochemicznymi. Tym razem opisane są dwie metody immunologiczne ilościowego oznaczania białka. Bardzo interesującą metodą jest automatyczne, nefelometryczne oznaczanie białka oparte na tworzeniu się nierozpuszczalnego kompleksu antygen-przeciwciało. Druga — wprawdzie nie najnowsza ale stale udoskonalana — to metoda immunologiczna z zastosowaniem znakowanych antygenów.

Książkę otwiera wykład znakomitego specjalisty chemii białek, F. E. Putnama, dedykowany pamięci Arne Tiseliusa i poświęcony rozwojowi badań nad białkami surowicy krwi i ich dalszymi perspektywami.

Recenzowany tom, podobnie jak wszystkie poprzednie z tej serii, jest wydany niezwykle starannie i może być dużą pomocą dla lekarzy specjalizujących się w chorobach nerek, jak również dla fizjologów i biochemików.

Marek Ombach

Cena zł. 25.–

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