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STUDIES ON PROTEIN SYNTHESIS IN THE CELL NUCLEI

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Allfrey *et al.* [1] demonstrated that isolated nuclei of thymus cells could incorporate radioactive amino acids into their proteins in the presence of glucose. Similar results were obtained by Logan *et al.* [7] with isolated nuclei of liver. It was also shown that in the soluble protein fraction of nuclei enzymes activating amino acids were present [5]. Rendi [13] has proved recently that amino acids could be incorporated *in vitro* into various nuclear fractions obtained by means of desoxycholate and lubrol.

In the present paper the distribution of radioactivity in the nuclear fractions was investigated after incorporation of ^{14}C-amino acids. The incorporation was studied *in vivo* and *in vitro*. The experiments *in vitro* were performed with intact and desintegrated nuclei, and with nuclear fractions.

Besides this, the occurrence of nucleopeptides similar to those isolated by Szafrański *et al.* [16, 19] from cytoplasmic supernatant was studied.

MATERIALS

^{14}C-amino acids were obtained by acid hydrolysis of radioactive protein of *Chlorella*. Details concerning the culture growth and the method of isolation had been described previously [15].

The adenosinetriphosphoric acid (ATP) was prepared from rabbit muscles by the Szent-Györgyi method [20], and potassium salt was used for the experiments.

Phosphocreatine was obtained from creatine and phosphorus oxychloride [3], the purity of the preparation as regards phosphorus was about 90 per cent.

Creatine kinase was obtained from rabbit muscles by the method of Noda *et al.* [9]. Its activity was tested by chromatographic demonstration of formation of ATP from ADP.

Diethylaminoethylcellulose (ECTEOLA) was prepared according to Peterson & Sober [12].

METHODS

The nuclei were prepared from perfused guinea pig liver by the method of Hogeboom *et al.* [4]. To separate the undisrupted cells from the nuclei an additional centrifugation at 60 *g* for 5 min. was applied. Microscopic examination of the obtained preparations showed no more than 2-3 per cent of undisrupted cells.

Isolated nuclei were desintegrated in 0.25 M-saccharose in a glass homogenizer at 0° for 4 min. at 1500 r.p.m., then three fractions were obtained by centrifugation. The first sedimented for 10 min. at 600 *g* contained the unbroken nuclei and larger fragments, the second consisted of particles sedimented for 1.5 hour at 40 000 *g*, the third fraction was the supernatant. The first two fractions were suspended in water. Trichloracetic acid (TCA) was added to all fractions to a final concentration of 5 per cent. The precipitates of protein were purified according to the procedure described by Simkin & Work [14], suspended in 10 per cent ammonia, and samples were examined for radioactivity and protein. Radioactivity was determined by means of G. M. window counter after drying on plexiglass discs 2 cm. diameter in an infinitely thin layer.

Protein was determined by the method of Lowry *et al.* [8] and ribonucleic acid (RNA) according to the method of Ogur & Rosen [10].

Incorporation of ^{14}C-amino acids into nuclear fractions in vivo

Guinea pigs, 6 - 9 months old, starved for 24 hours, were given intracardial injections of about 2 ml. of hydrolyzate of ^{14}C-*Chlorella* protein, the amount being 45 μc per kg. of body weight. After 30 min. the animals were decapitated, the liver perfused with cold 0.25 M-saccharose and nuclei isolated. The nuclei obtained from 12 g. of liver were suspended in 12 ml. of 0.25 M-saccharose, desintegrated, fractionated, and radioactivity of proteins was determined. As it was shown in Table 1, fraction 2 exhibited the highest specific activity. It contained particles fairly visible in the optical microscope. Its RNA to protein ratio was 0.22. The Feulgen reaction was negative, and in the extract made with hot 5 per cent TCA,DNA was not detectable spectrophotometrically. The particles of fraction 2 were referred as ribonucleoprotein (RNP) particles.

Table 1

Incorporation in vivo *of ^{14}C-amino acids into fractions of liver nuclei*

The animals were killed 30 min. after intracardial injection of radioactive amino acids

No.	Nuclear fraction	Imp./hr/mg.protein
1	Sediment at 600 *g*	213
2	Sediment at 40000 *g* (RNP)	900
3	Supernatant	507

Incorporation of ^{14}C-amino acids into nuclear fractions of isolated intact and desintegrated nuclei

The incorporation of ^{14}C-amino acids into nuclei *in vitro* was studied on experiments of two types. In one of them the intact liver nuclei were incubated for 30 min. with radioactive amino acids, and then desintegrated and fractionated. In the second one the nuclei were desintegrated prior to incubation. The incubations were carried out in the presence of glucose as the source of energy [1]. The specific activities of proteins of individual

Table 2

Incorporation in vitro *of ^{14}C-amino acids into nuclear fractions in intact or desintegrated nuclei*

The composition of incubation mixture: 0.5 ml. of suspended nuclei (intact or desintegrated) obtained from 0.5 g. liver, in 0.25 M-saccharose; 0.25 ml. 0.1 M-phosphate buffer pH 7.3, in 0.25 M-saccharose; 0.2 ml. 0.1 M-glucose containing 0.75 mg. NaCl; 0.05 ml. ^{14}C-amino acids (0.675 μc). Incubation 30 min., at 37°, in open tubes

No.	Nuclear fraction	Incubated nuclei	
		intact	desintegrated
		Imp./hr./mg. protein	
1	Sediment at 600 *g*	70	962
2	Sediment at 40000 *g* (RNP)	254	1978
3	Supernatant	680	2753

fractions were presented in Table 2. The highest radioactivities after incubation of intact or desintegrated nuclei were found in the supernatant as contrasted with experiments *in vivo* (Table 1), where fraction RNP was bearing the highest label. Incorporation into desintegrated nuclei was found to be more intense than into intact nuclei.

Incorporation of ^{14}C-amino acids into isolated nuclear fractions

The experiments with isolated fractions were performed in order to verify whether the presence of all the three nuclear fractions was indispensable for the incorporation of amino acids.

Table 3

Incorporation of ^{14}C-amino acids into isolated RNP particles in the presence of nuclear supernatant, and into the supernatant alone

The composition of incubation mixture: 1.5 ml. of RNP particles suspension in 0.1 M-phosphate buffer, pH 7.3 (11 mg. protein); 5.4 ml. of supernatant (6.5 mg. protein), or in Expt. 2: 6.9 ml. of supernatant (8.3 mg. protein), RNP particles being omitted; 2.1 ml. 0.1 M-glucose containing 7.9 mg. NaCl; 1.0 ml. ^{14}C-amino acids (13.5 μc). Incubation at 37° in open tubes.

In Expt. 1: after the indicated times of incubation, samples were withdrawn, diluted up to 3-fold volume with 0.25 M-saccharose with an addition of non-radioactive amino acids and RNP particles centrifuged. In the RNP sediment the radioactivity and protein were determined. In Expt. 2 the protein was precipitated with TCA at a final concentration 10 per cent

Expt.	Fraction	Incubation time (min.)			
		1	10	20	40
		Imp./min./mg. protein			
1	RNP particles	48	85	113	148
2	Nuclear supernatant	276	301	508	407

RNP particles (fraction 2) were suspended in 0.1 M-phosphate buffer, pH 7.3, and incubated with labelled amino acids, glucose and the nuclear supernatant obtained at 40 000 *g* (fraction 3). The addition of the supernatant was indispensable, since it contained enzymes activating amino acids [5]. After various times of incubation the samples of RNP particles were centrifuged and the specific radioactivities of RNP protein determined. It was also investigated whether the incorporation of amino acids could occur in the supernatant alone. The nuclear supernatant was incubated with radioactive amino acids with addition of glucose. From

the incubation mixture 3 ml. samples were withdrawn, transferred to 3 ml of 10 per cent TCA, and after purification the specific radioactivity of the proteins was determined.

The results (Table 3) showed that addition of supernatant is sufficient for the incorporation of amino acids into RNP particles. Moreover, the supernatant alone incorporates amino acids into proteins at high rate. It is noteworthy that the incorporation of amino acids into proteins of nuclear supernatant was the highest after 20 min. incubation, then radioactivity decreased.

Incorporation of ^{14}C-amino acids into isolated nuclear RNP particles in the presence of cytoplasmic supernatant

Experiments were performed to see whether the nuclear supernatant could be replaced by the cytoplasmic one for the incorporation of amino acids into RNP particles. The cytoplasmic supernatant was prepared on 1.5 hr. centrifugation of liver homogenate at 40 000 *g*, at 0°. The RNP particles from nuclei were incubated in a system used in experiments on protein synthesis of microsomes. The incubation mixture was added with ATP and ATP-generating system. After incubation time as indicated in Table 4, samples were centrifuged and radioactivity and protein of

Table 4

Incorporation of ^{14}C-amino acids into isolated nuclear RNP particles in the presence of cytoplasmic supernatant

The composition of incubation mixture: 2.15 ml. of RNP particles suspended in 0.25 M-saccharose (14.5 mg. protein); 3.85 ml. of cytoplasmic supernatant (25 mg. protein); 2.3 ml. 0.1 M-tris buffer, pH 7.5, containing 4.48 mg. ATP, 34.2 mg. KCl and 0.92 mg. $MgCl_2$; 0.78 ml. of water solution of phosphocreatine sodium salt (35.9 mg.), and 2.3 mg. of creatine kinase; 0.92 ml. of ^{14}C-amino acids (12.3 μc). Incubation at 37° in open tubes. RNP particles were isolated as previously (Table 3) and specific activity of protein determined

Incubation time (min.)			
1	10	20	40
Imp./min./mg. protein			
56	95	155	201

RNP particles were determined. Results of these experiments showed that RNP particles incorporated ^{14}C-amino acids in the presence of the cytoplasmic supernatant. Thus it appeared that incorporation of amino

acids into nuclear RNP particles could proceed in a system necessary for protein synthesis in microsomes.

It should be mentioned that the cytoplasmic supernatant used in our experiments was slightly contaminated with microsomes residues. The incorporation of amino acids into nuclear RNP particles cannot, however, be attributed to these microsomes since it lasted for 40 minutes, whereas it is well known that microsomes lose their ability of incorporation already after 15 to 20 minutes.

Isolation and some characteristics of the nucleopeptides from cell nucleus

The fact that nuclear supernatant can be replaced by cytoplasmic one would point to a similarity of both protein synthesising systems. Therefore it was interesting to see whether there are some nucleopeptides in the nucleus similar to those isolated from cytoplasm [16, 17].

The same procedure was applied to isolate nucleopeptides from nuclei as for cytoplasmic material [19]. The suspension of desintegrated nuclei deriving from 24 g. liver was deproteinized by ammonium sulphate saturation, centrifuged in cold at 32 000 *g*, and the precipitate discarded. Ammonium sulphate was precipitated with ethanol at final concentration of 85 per cent, and supernatant evaporated to about 1.5 ml. The samples containing 700 - 800 μg. RNA (as estimated by absorption at 260 mμ) were made up with water to 4 ml. and dialyzed overnight against water, at 2°. Absorption at 260 mμ measured both in the solution surrounding the dialysis bag and inside it, showed similar values indicating that the compounds under investigation were dialyzable.

Other samples were subjected to the column fractionation. Since the nuclear material was not adsorbed on the triethylaminoethylcellulose, as the cytoplasmic one, another adsorbent was used, namely ECTEOLA. The material from ECTEOLA column (12 × 1.5 cm.) was eluted subsequently with 0.01 M-tris buffer, pH 7.4, 1M-NaCl in 0.01 M-tris buffer, pH 7.4, and 1.5 per cent NaOH. Thus three fractions were obtained. A typical course of elution is presented in Fig. 1. The obtained column fractions were tested for the presence of nucleopeptides, as described previously [19].

Paper electrophoresis revealed one spot absorbing ultraviolet light in each of three fractions. These spots were excised, eluted and subjected to chromatography in Partridge's system [11]. Each electrophoretic fraction was separated into 2 - 4 spots absorbing ultraviolet light. The same sites on the chromatograms gave the positive hydroxamic reaction, thus indicating the presence of activated groups, may be of amino acids or peptides. The total hydrolysis performed after elution of these spots

gave an increase of amino acid contents indicating that they contained peptides.

The different fractions from paper chromatography were investigated for the presence both of sugars and nucleic bases. Several chromatographic spots were eluted with water, treated with bromine water, and

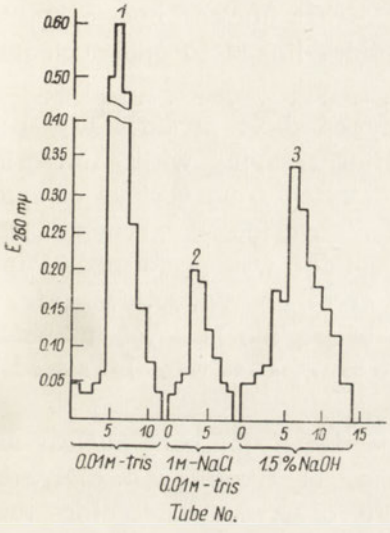

Fig. 1. Fractionation of protein free material of guinea pigs liver nuclei on diethylaminoethylcellulose (ECTEOLA) column. Volume of sample 3.8 ml., time of filling of single test tube: 4 min. in the case of fractions *1* and *2*, and 8 min. for the fraction *3*

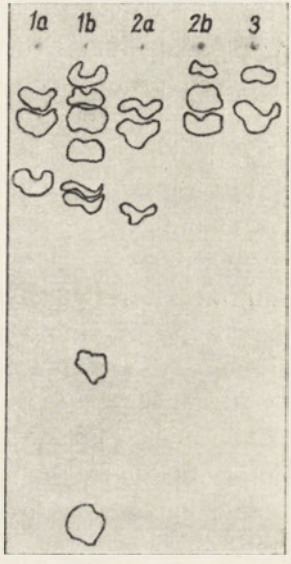

Fig. 2. Paper chromatogram of amino acids after total hydrolysis of short peptides (obtained on partial hydrolysis of peptides from hydroxamic acids). (*1*), (*2*), (*3*), fractions from column; (*a*), (*b*), eluted short peptides (see in the text)

paper chromatography was carried out in butanol - acetic acid - water (4 : 1 : 5) to detect ribose. Diphenylamine reaction for deoxyribose made both on filter paper and in eluates was negative.

Nucleic bases were tested chromatographically [21] after hydrolysis of material eluted from other spots. Spots corresponding to standards of adenine, guanine, uracil and cytosine were detected, the most intensive were these of adenine and uracil. Spots corresponding to thymine were not found on the contrary to the case of cytoplasmic nucleopeptides.

The detection of activated peptides, nucleic bases and ribose in protein-free material indicates that compounds under investigations are nucleopeptides, indeed.

For further characterization of nuclear nucleopeptides, the same procedure was applied as described previously [19] for cytoplasmic ones. Paper chromatograms with separated nucleopeptides were treated with hydroxylamine and hydroxamic acids were formed, then eluted and separated chromatographically in butanol-acetic acid - water (9 : 1 : 1). After chromatograms had been sprayed with ferric chloride solution 2 - 3 pink-brown spots of complex salts of hydroxamic acids were detected in each nucleopeptide. This might indicate that nuclear nucleopeptides like cytoplasmic ones contained 2 - 3 peptides linked to one nucleotide part.

Partial hydrolysis and chromatography of short peptides was performed to examine the peptide parts. After spraying with ninhydrine various amounts of short peptides were detected from various complex salts of hydroxamic acids. The excess of ninhydrine was removed and after elution total hydrolysis of short peptides was performed. Paper chromatography of amino acids deriving from short peptides (Fig. 2) showed that the amino acid composition of peptide parts of individual nuclear nucleopeptides was not always the same, as it was also stated in cytoplasmic nucleopeptides.

Results obtained point to the general similarity of both nuclear and cytoplasmic nucleopeptides — the presence of ribose, and dialyzable character, though some differences were observed concerning their absorption on TEAE column, as well as nucleic bases composition.

DISCUSSION

Investigations on the function of the subcellular fractions indicate that synthesis of cytoplasmic protein may be controlled by RNA of nuclear origin. This nuclear RNA synthesised in the presence of DNA would be a link between chromosomes and cytoplasm in transmitting genetic features. As RNA is an important part of microsomes well known for their intensive synthesis of protein, it has been suggested by Bonner [2] that microsomes are produced in the nucleus and then transmitted to the cytoplasm. If this is true particles of a composition and properties similar to those of microsomes should be found in nucleus. Our investigations showed that in desintegrated nuclei it was possible to obtain, by means of differential centrifugation, particles capable of achieving synthesis of proteins. These particles, however, are much larger than microsomes and well visible in an optical microscope. They are deprived of reticulum and their ratio of RNA to protein is 0.22, nearly twice that found in microsomes. The incorporation of amino acids into these nuclear RNP particles

is sustained during a 40 minutes incubation, whereas in microsomes synthesis of protein ceases already after 15 to 20 minutes. It is also to be noted that after the desintegration of nuclei the incorporation of amino acids seems to be more intensive than in the case of intact nuclei. It may be due to elimination of the barrier furnished by the nuclear membrane. Besides this, protein synthesis in the intact nucleus may be controlled by other metabolic processes.

The incorporation of amino acids into proteins of the nuclear supernatant took place even in absence of other fractions. It was analogical to results obtained by Kalf & Simpson [6] with mitochondrial supernatant. These authors stated that the supernatant obtained by centrifugation for 8 hrs. at 105 000 *g* of desintegrated mitochondria incorporated radioactive valine during several hours and then there appeared a decrease in protein radioactivity. This would point to the presence as well in mitochondria as in nuclei of a soluble system with an ability to achieve protein synthesis in the absence of particles.

We tried to show that nucleopeptides isolated from nuclei could be considered as identical with those found in cytoplasm. In our experiments we found some features common for both but some differences, too.

SUMMARY

The incorporation of ^{14}C-amino acids into ribonucleoprotein (RNP) particles obtained by centrifugation at 40 000 *g* of desintegrated guinea pig liver nuclei, as well as into the nuclear supernatant was investigated *in vivo* and *in vitro*.

Protein synthesis in isolated RNP particles occurred in the presence of nuclear supernatant and glucose, as well as in the presence of cytoplasmic supernatant and the ATP regenerating system. The nuclear supernatant alone showed itself very intense incorporation.

Nucleopeptides similar to those of cytoplasm described previously [19] were detected in nuclei.

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BADANIA NAD SYNTEZĄ BIAŁEK W JĄDRZE KOMÓRKOWYM

Streszczenie

Zbadano włączanie ^{14}C-aminokwasów *in vitro* i *in vivo* do cząstek rybonukleoproteidowych (RNP) otrzymanych przez wirowanie przy 40 000 *g* rozbitych jąder komórek wątroby świnki morskiej oraz do supernatantu jądrowego.

Synteza białka w izolowanych cząstkach RNP przebiegała w obecności supernatantu jądrowego i glikozy, jak również w obecności supernatantu cytoplazmatycznego i układu regenerującego ATP. Sam supernatant jądrowy wykazywał bardzo intensywne włączanie.

W jądrach komórkowych stwierdzono obecność nukleopeptydów podobnych do opisanych poprzednio [19] nukleopeptydów cytoplazmatycznych.

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BINDING OF ATP BY HUMAN SERUM ALBUMIN IN SOLUTION

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It has been shown in the previous papers [7, 9] that the binding of ATP by various proteins precipitated by alcohol or by heat denaturation resembles the adsorption isotherm. The present paper is concerned with the binding of ATP by serum albumin in solutions. Some results of this work have been presented earlier [8].

MATERIAL AND METHODS

Human serum albumin, V-th fraction of Cohn, was kindly supplied by the Institute of Haematology in Warszawa. Its concentration in solutions was determined according to Gornall *et al.* [11]. ATP (Light) was purified on Dowex-2 column, and did not contain other chromatografically detectable nucleotides. Its concentration was calculated from the optical density at 259 mμ (pH 7.0) using the molar extinction coefficient 15400 [3].

Equilibrium dialysis was carried out at 2° in bags made from dialyzing tubings (Kalle & Co., Wiesbaden-Biebrich, Germany) purified according to Hughes & Klotz [12]. Usually the protein dissolved in 10 ml. buffer was placed inside the bags and dialyzed against 10 ml. of ATP solution in the same buffer. If ATP and protein were put together the same final results were obtained. Final ionic strength of buffers was 0.05 - 0.1 μ. Because of the presence of comparatively high concentrations of electrolytes and low concentration of the protein the Donnan effect was negligible [13, 14].

When the equilibrium had been attained the concentration of ATP outside the bags was determined spectrophotometrically at 259 mμ after buffering to pH 7.0. Inside the bags, i.e. in the protein solution, the optical density at 259 and 280 mμ was measured after buffering to pH 7.0, and

the nucleotide concentration was calculated as in the previous paper [9]. The amount of protein-bound ATP was calculated in two ways: (1) from the difference of the concentration of the nucleotide inside and outside the bag multiplied by the volume of the fluid inside the bag; (2) from the difference between concentration of the nucleotide in the control (without protein), and in the tested sample (with albumin) multiplied by the total volume (inside and outside the bag).

Ultrafiltration with centrifugation was carried out according to the procedure elaborated in this laboratory [21]. 2 - 3 ml. samples containing ATP, serum albumin and buffer solution (of the same ionic strength as used in dialysis experiments) were centrifuged 3 - 4 hours at 6000 r.p.m. with the layer of liquid paraffin (usually 5 ml.) through a membrane filter in a vessel made of Plexiglass [21]. In the control samples ATP solution without albumin was filtered by the same procedure. The amount of protein-bound ATP was calculated from the difference in concentrations of the nucleotide between the sample with albumin and the control.

Paper electrophoresis was carried out on Macherey-Nagel No. 224 filter paper in the moist chamber, using the voltage about 3.5 V/cm. Veronal buffer [22] or 0.1 M-glycine buffer, pH 8.6, and 0.1 M-acetate buffer, pH 3.6 - 5.8, were used.

Nucleotides were detected on the paper using a mercury lamp with a filter containing $NiSO_4 + CoSO_4$ and Cl_2 according to Markham & Smith [17]. Staining with Light Green [22] was used for the detection of the albumin.

RESULTS

Dialysis and ultrafiltration

The influence of pH on the distribution of ATP after dialysis is shown in Table 1. As can be seen the concentration of the nucleotide is much greater on the protein-side of the membrane than on the opposite side. This indicates the existence of binding between ATP and protein. Maximum difference was observed below the isoelectric point; it decreased with the increase in pH, and was very small at pH 5.8.

Using equilibrium dialysis experiments were performed to check the ability of binding of ATP by native and modified serum albumins. Some results are shown in Table 2. The character of changes in the binding ability, dependent on the kind of modification, was the same as had been observed in the previous work [9] using precipitated proteins. Acetylated albumin binds less ATP, whereas esterified albumin binds more ATP than the native protein. Moreover, the experiments with dialysis enabled

Table 1

Equilibrium dialysis of ATP and human serum albumin. The effect of pH on the binding

Expt. 18/D. Inside the dialyzing tubes 5 ml. ATP solution, outside 15 ml. protein solution. Acetate buffer. The equilibrium was attained in 72 hours.

pH	ATP (μmoles per ml.)		
	calculated	found after dialysis	
		inside the tubes	outside the tubes
3.6		0.198	0.360
4.7		0.290	0.332
5.8		0.312	0.317
Control (without protein)			
5.8	0.325	0.320	0.315

Table 2

The equilibrium dialysis of ATP and esterified or acetylated serum albumin

Expt. 19/D. Inside the dialyzing tubes 5 ml. protein solution, outside 20 μmoles ATP in 15 ml. acetate buffer. (Acetylation and estrification were carried out as in the previous paper [9]).

	pH	Protein in the sample (mg.)	Initial relation of ATP to protein (μmoles/g.)	ATP found outside the tubes after dialysis (μmoles/ml.)	Calculated amount of protein bound ATP (μmoles/g. protein)
Control (without protein)	3.8	—	—	1.07*	—
Native serum albumin	3.8**	40.5	446	0.74	163
Esterified albumin	3.8**	38.1	524	0.65	220
	4.6**	38.1	524	0.70	193
	5.3**	38.1	524	0.71	189
Acetylated albumin	3.8**	43.3	460	0.94	60
	4.9	43.3	460	1.04	13

* After dialysis the amount of ATP found in the control sample inside the tubes was 1.11 μmoles per ml.

** In these samples, as a result of high concentration of ATP, a small amount of protein precipitated during the dialysis.

the estimation of the binding ability at pH values at which no coagulation of some of the modified protein occurred, e.g. in the case of esterified serum albumin at pH 3.6.

Table 3

Ultrafiltration of the solution of ATP and serum albumin. The effect of pH on the binding

4.82 mg. serum albumin in 0.25 ml., 0.7 μmoles ATP in 1 ml., 0.25 ml. 0.4 M-acetate buffer at various pH, 0.5 ml. distilled water. (The control sample contained distilled water instead of the protein solution) Initial relation of ATP to the albumin was 144 μmoles per g

pH	ATP found in the ultrafiltrate (μmoles per ml.)		Calculated amount of protein bound ATP	
	samples without protein (a)	samples with protein (b)	μmoles/ml. (a - b)	μmoles/g. protein
3.54	0.337	0.125	0.213	88.6
4.54	0.337	0.230	0.107	44.4
5.74	0.337	0.325	0.012	5.0

Dialysis is, however, rather inconvenient for the investigations of ATP binding, because of a very slow penetration of ATP ions through semi-permeable membranes [18, 21]. A long period of dialysis can cause, even at low temperature, some cleavage of ATP, which, according to our previous observations [7], decreases its binding ability with proteins.

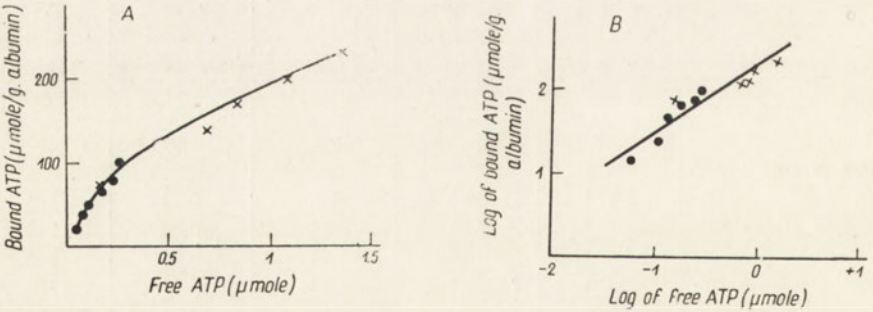

Fig. 1. Ultrafiltration of the mixture of ATP with serum albumin at pH 3.6 (●), expt. 21/U; (×), expt. 22/U. *(A)* The relationship betwen the concentration of free and bound ATP. *(B)* The logarithmic relationship between the concentration of free and bound ATP. For experimental conditions and data see Table 5.

Therefore, experiments concerning the binding of ATP by serum albumin were performed by the ultrafiltration method [21]. As it is shown in Table 3 ultrafiltration gives similar results on the pH dependence of ATP binding as dialysis.

Table 4

Ultrafiltration of the solution of ATP and serum albumin at pH 3.6. The relationship between the concentration of ATP and its binding by protein

0.25 ml. protein solution, 0.25 ml. 0.4 M-acetate buffer, variable amounts of ATP, distilled water to 2 ml.

Initial relation of ATP to protein μmoles per g.	ATP found in the ultrafiltrate (μmoles per ml.)		Calculated amount of protein bound ATP	
	samples without protein (a)	samples with protein (b)	μmoles/ml. (a - b)	μmoles/g. protein
Expt. 21/U, 6.26 mg. protein in the samples				
30.5	0.095	0.044	0.051	16.6
61.0	0.191	0.075	0.116	37.2
91.5	0.286	0.107	0.179	57.2
122.0	0.382	0.164	0.218	69.8
152.5	0.476	0.224	0.252	81.0
183.0	0.572	0.255	0.319	101.0
Expt. 22/U, 5.8 mg. protein in the samples				
126.0	0.365	0.153	0.212	73.0
378.0*	1.095	0.692	0.403	139.0
465.0*	1.350	0.835	0.500	173.0
577.0*	1.680	1.090	0.590	204.0
697.0*	2.030	1.350	0.680	235.0

* In these samples a small amount of protein was precipitated as a result of high concentration of ATP.

Table 5

Ultrafiltration of the solution of ATP and native or denaturated serum albumin at pH 3.6

Expt. 19/U. 6.1 mg. protein in 0.25 ml., 0.25 ml. 0.4 M-acetate buffer, ATP in samples (I) 0.39 μmoles, and in (II) 0.78 μmoles, distilled water to 2 ml. The denaturation was performed by heating in the acetate buffer at pH 3.6 during 10 min. on boiling water bath.

Albumin	Initial relation of ATP to protein (μmoles/g.)	ATP found in the ultrafiltrate (μmoles/ml.)		Calculated amount of protein-bound ATP (μmoles/g. protein)
		samples without protein	samples with protein	
I. Native	64	0.184	0.136	20.0
Denaturated	64	0.184	0.134	20.1
II. Native	128	0.390	0.209	59.5
Denaturated	128	0.390	0.206	60.5

Table 4 shows the relationship between protein-bound and free ATP. Data from this Table are presented in Fig. 1*A*, as a relation between the amount of bound ATP (in μmoles per g. albumin), and the concentration of free ATP in mM. The logarithmic dependence of these values is shown in Fig. 1*B*. As indicated by the character of the curves, the binding of ATP by serum albumin in the solution resembles the isotherm absorption, a situation similar to the binding of this nucleotide by coagulated protein observed previously [9]. The comparison of the present and the earlier results shows that the binding ability of ATP by serum albumin in solution is only insignificantly smaller than by the coagulated protein.

Using the ultrafiltration method, it was possible to establish the influence of denaturation on the binding ability. Table 5 shows that there is no difference between the extent of binding of ATP by the native albumin and by albumin previously heated to 100° at pH 3.6 (at which pH this protein does not coagulate).

Spectrophotometric measurements

As it is shown in Table 6, at pH 7, as well as at pH 3.6, only insignificant differences were found between the extinction at 259 mμ for the mixture of ATP and several proteins and the sum of the extinctions of these components determined separately. We were also unable to demonstrate any difference in extinction values if the mixture of ATP and protein was preincubated during 30 min. at pH 11, and adjusted afterwards to pH 7.6, according to the procedure of Anina & Wendt [1], used by these authors in their studies on binding of nucleotides by actin. Similarly, we could not detect any changes in ATP spectrum after incubation with tyrosine (Table 6).

In experiments shown in Table 6, the ratio of ATP to protein was 90 - 120 μmoles per g. It was shown earlier [7] that the greater was the concentration of ATP in the protein solution the greater was the amount of the protein bound nucleotide. It seemed, therefore, advisable to perform experiment with much higher concentrations of ATP, about 350 μmoles per g. protein. However, also in this case no changes in the spectrum of the nucleotide at pH 7.0 in the presence of serum albumin were observed.

At pH 3.6 and when the ATP to protein ratio amounted to 240 μmoles per g. there was a small decrease (up to 6%) in the extinction at 260 mμ. However, no change could be observed in the extinction at 280 mμ. It appeared impossible to use higher concentrations of ATP at pH 3.6, because of a partial precipitation of serum albumin.

Table 6

Optical density at 259 mμ of ATP solution, protein solution, and ATP in protein solution

All data are mean values from 3 to 6 analyses ± standard deviation

Substance	pH	Optical density at 259 mμ			
		found			calculated as the sum of (a) and (b)
		protein solution (a)	ATP solution (b)	the mixture of protein and ATP	
Serum albumin	3.6	0.171±0.004	0.509±0.007	0.670±0.010	0.680±0.011
Serum albumin	6.5	0.189±0.003	0.537±0.014	0.739±0.007	0.726±0.017
Serum albumin	3.6	0.229±0.004	0.454±0.014	0.680±0.021	0.670±0.018
Serum albumin	6.5	0.249±0.002	0.469±0.016	0.691±0.015	0.718±0.018
γ-globulin	3.6	0.233±0.001	0.532±0.003	0.768±0.013	0.765±0.004
γ-globulin	6.5	0.250±0.010	0.544±0.013	0.795±0.013	0.784±0.023
Ovoalbumin	7.6*	0.171±0.006	0.500±0.007	0.644±0.011	0.671±0.013
Ovoalbumin	7.6*	0.184±0.007	0.500±0.007	0.672±0.010	0.684±0.014
Tyrosine	7.6*	0.149±0.013	0.500±0.007	0.634±0.006	0.649±0.020
Tyrosine	7.0**	0.141±0.010	0.488±0.014	0.626±0.008	0.641±0.024

* The mixture of ATP and serum albumin, or ATP and tyrosine, was incubated 30 min., 50°, at pH 11, and then adjusted to pH 7.6.

** The mixture of ATP and tyrosine was incubated 30 min., 50°, at pH 11, afterwards it was kept at 0° during 48 hrs., and then adjusted to pH 7.6.

Paper electrophoresis

To elucidate the mutual influence of ATP and serum albumin on their migration in the electric field the paper electrophoresis of this nucleotide or protein alone, or the mixture of both compounds was carried out.

Fig. 2 shows electropherograms of ATP, serum albumin and their mixture at pH 8.6 and 5.4. It is obvious that there is no difference in the migration velocity of the protein in the presence and in the absence of ATP at both pH values. However, near the isoelectric point of serum albumin some influence of the nucleotides on the protein migration could be observed. As it can be seen in Fig. 3*A* albumin alone migrated slowly to the cathode, whereas in the presence of ATP it remained at the start line. On another electropherogram (Fig. 3*B*) albumin in the presence of

ATP migrated slightly to the anode, whereas in the absence of nucleotides it moved slightly to the cathode.

Greater differences in the behaviour of the albumin in electric field were observed below the isoelectric point (Fig. 4). In this case a decrease in the mobility of albumin was observed in the presence of nucleotides. The efforts, however, to detect the presence of nucleotides in the same places where protein was located, were unsuccessful. The decrease of the mobility of serum albumin, as well as the change of the shape of its spots, showed marked dependence on the initial ratio of ATP to the protein. At low concentrations of ATP, about 250 μmoles per g. protein, no influence of ATP on the migration of albumin could be shown. The decrease of the mobility of the protein was observed only at high concentrations of ATP, above 700 μmoles per g. protein. In this case a distinct dependence on the ratio between the concentration of ATP and the mobility could be observed (Picture C, Fig. 4).

In the whole investigated pH range no influence of serum albumin on the nucleotide migration was observed (Fig. 5). Preparations of ATP used in the electrophoretic experiments contained usually some amounts of ADP. Using acetate buffer in the pH range 3.6 - 5.5, a good separation of ATP from ADP could be obtained. However, the duration of the electrophoresis had to be longer than in the experiments concerned with the ATP-albumin interaction.

DISCUSSION

In the present paper the formation of the binding between ATP and protein in solution was demonstrated. Existence of a relationship was established between the binding ability and pH of the medium, and between the amount of bound ATP and the concentration of the free nucleotide. This was in agreement with previous experiments [7, 9] performed with the coagulated protein.

Earlier study from this laboratory [19, 20] showed that during the extraction of the muscle pulp with buffer solutions at pH 4.6 more than 50% of nucleotides remained in the residue together with proteins. On the other hand, ATP added during the extraction was under these conditions completely soluble, i.e. it did not bind with proteins. It is reasonable to assume that under these conditions muscle proteins were practically saturated with nucleotides originally present in the muscles.

The results of the present investigation confirm the earlier observations [9] concerning the role of the protein cationic side chains in the formation of the combinations with nucleotides. The results are also in agreement with absence of a difference between the binding ability of

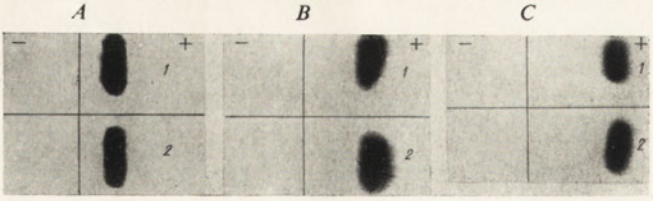

Fig. 2. Paper electrophoresis of ATP and serum albumin above the isoelectric point. *(A)* 0.05 M-veronal buffer, pH 8.6; 4 hrs. *(B)* 0.1 M-glycine buffer, pH 8.6; 13 hrs. *(C)* 0.1 M-acetate buffer, pH 5.4; 8 hrs. *(1)* serum albumin; *(2)* serum albumin in the presence of ATP

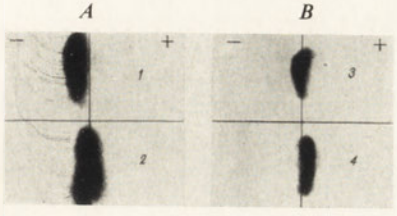

Fig. 3. Paper electrophoresis of ATP and serum albumin near the isoelectric point. 0.1 M-acetate buffer, pH 4.7. *(A)* 17.5 hrs., (B) 5 hrs. *(1)* serum albumin; *(2)* serum albumin in the presence of ATP

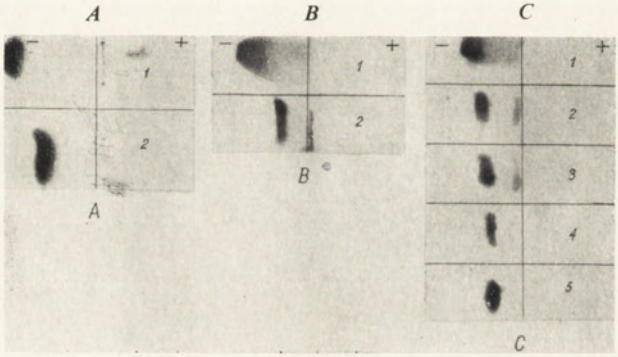

Fig. 4. Paper electrophoresis of ATP and serum albumin below the isoelectric point. *(A)* 0.1 M-acetate buffer, pH 4.0; 5 hrs., *(B)* 0.2 M-acetate buffer, pH 3.4; 7 hrs., *(C)* 0.1 M-acetate buffer, pH 3.8; 4 hrs. *(1)* serum albumin; *(2 - 5)* serum albumin in the presence of increasing concentrations of ATP. The initial ratio of ATP to the protein: *(2)* 950 μmoles per g. *(3)* 1900 μmoles per g. *(4)* 3800 μmoles per g. *(5)* 5700 μmoles per g.

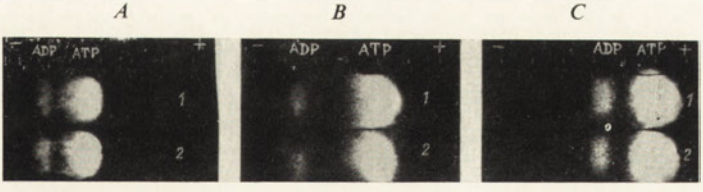

Fig. 5. The influence of the protein on the mobility of adenine nucleotides. Pictures taken at UV. 0.1 M-acetate buffer: *(A)* pH 4.0, 8 hrs.; *(B)* pH 4.0, 15 hrs.; *(C)* pH 5.4, 17 hrs. *(1)* ATP in the presence of serum albumin; *(2)* ATP alone

the native and denatured serum albumin observed previously in the case of coagulated proteins [7].

Several authors [cf. 14] demonstrated earlier that the light absorbing substances showed, after combination with proteins, a decrease of the extinction coefficient and/or a shift of the absorption maximum. Concerning natural combinations of proteins with various nucleotides or nucleic acids the results of various authors are not always in agreement with each other. Some of them show [2, 4, 16] that the extinction coefficient of nucleoproteins is lower than that of nucleic acids. On the other hand, Chargaff [6] found that the spectrum of nucleoproteins did not differ from that of free nucleic acids.

Madsen & Cori [15], relating the binding of AMP by phosphorylase. and Theorell [23], relating the combinations between DPN and alcohol dehydrogenase, showed changes in the spectra of these nucleotides in the presence of the enzyme. On the other hand, Velick [24] was able to demonstrate that in the complex of DPN with 3-phosphoglyceraldehyde dehydrogenase the absorption near 260 mμ was additive. Anina & Wendt [1] showed that the extinction coefficient of adenine nucleotides bound by actin was lower than that of the free nucleotides. These authors, as well as Fermin [10], showed also that the incubation of the tyrosine with adenine nucleotides led to the formation of combinations characterized by the decrease of the extinction coefficient. However, in the present investigation we were not able to confirm this phenomenon.

The experiments described in the present paper on the influence of the proteins on the UV spectrum of ATP showed, only at pH 3.6 and at higher concentration of ATP, a small decrease in the absorption coefficient in the presence of serum albumin. These results confirm previous observations [9] that the phosphate groups of the nucleotide molecule are of principal importance in the formation of the complexes with proteins, whereas the role of the purine ring is probably not more than a secondary one.

The fact that the extinction values of ATP and serum albumin were at pH 7.0 fully additive enabled the determinations of the nucleotide concentrations in the presence of proteins. This could be calculated according to the equation presented in the previous work [9] from the data provided by the estimations of the optical densities at 259 and 280 mμ.

The character of the investigated combinations of ATP with proteins resembles the adsorption isotherm both in the case of coagulated proteins and proteins in solution. This is in agreement with earlier observations [5, 14] concerned with the character of combinations of various ions with

proteins in the solution which also showed a resemblance with the adsorption isotherm.

The amounts of bound ATP found in the present paper, using ultrafiltration method, were only slightly lower than those bound by coagulated proteins. These results suggest that the binding of nucleotides by the boundary surface of the solid phase, which in this case is a precipitated protein, does not take a significant part in the case of investigated complexes.

Paper electrophoresis showed that below the isoelectric point of the albumin ATP had a marked effect on the electrophoretic mobility of this protein. Unsuccessful efforts to detect the presence of the nucleotides, in places where the protein was located, suggest, perhaps, that the investigated complexes, according to their ionic character, may be split in the electric field.

SUMMARY

Binding of ATP by serum albumin in solutions has been investigated by means of ultrafiltration and equilibrium dialysis. It has been found that the amount of ATP bound by albumin decreases with increasing pH of the medium and increases with increasing concentration of ATP, similarly as in the case of binding of nucleotides by precipitated proteins.

The presence of albumin in the solution has no influence upon the ultraviolet absorption spectrum of ATP, which indicates that the adenine ring is not involved in the linkage.

Below the isoelectric point ATP affects the electrophoretic mobility of albumin.

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WIĄZANIE ATP PRZEZ ALBUMIN SUROWICY W ROZTWORACH

Streszczenie

Przy zastosowaniu ultrafiltracji i dializy wyrównawczej stwierdzono powstawanie połączeń ATP z albuminem surowicy w roztworach. Zdolność wiązania ATP spada wraz ze wzrostem pH, a wzrasta ze wzrostem stężenia ATP, podobnie, jak w przypadku połączeń nukleotydów ze strącanymi białkami.

Badania spectrofotometryczne mieszaniny ATP i albuminu surowicy wykazały, że obecność białka nie wpływa na widmo ATP w ultrafiolecie, co potwierdza brak udziału pierścienia adeniny w tworzeniu badanych połączeń.

W pH poniżej punktu izoelektrycznego zaobserwowano wyraźny wpływ ATP na ruchliwość elektroforetyczną albuminu na bibule.

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SOME PROPERTIES OF COMPLEXES OF MUSCLE PROTEINS WITH THE NUCLEOTIDES PRESENT IN THE MUSCLE

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It has been often observed the presence of adenine nucleotides and of orthophosphate in preparations of muscle proteins and isolated myofibrils, and a binding of added nucleotides and orthophosphate by muscle proteins [cf. 7, 24, 28]. In general, only a small fraction, usually no more than 10% of the total amount of muscle nucleotides, have been detected as complexes with proteins. However, Niemierko *et al.* [27, 29] and Lange [19] have found that much greater quantities of muscle nucleotides, i.e. about 50%, may form combinations with proteins. These large discrepancies seem to be due to a high lability of complexes of nucleotides with muscle proteins as pointed out by Niemierko *et al.* [28, 29]. It has been shown by these authors that these complexes are rather stable in acetate buffer of pH 4.6 - 5.5 but are split in distilled water and in 60% ethanol.

As it has been described in a preliminary communication [10] there is a relationship between the amount of bound nucleotides and pH of the extracting medium. When frog muscles were extracted with a mixture of 0.2 M-acetate buffer, pH 3.6, and ethanol (1 : 1) as much as 90% of total nucleotides remained unextractable, i.e. presumably bound to muscle proteins. However, at pH 5.8 the greater part of total nucleotides was extractable and only 25% remained in the residue. It has been found that these changes in extractibility can be attributed to both the formation of new combinations between nucleotides and proteins during the extraction procedures and to the breakdown of the existing complexes. This has been shown by the following experiments. Two symmetric muscles were separately homogenized in a buffer of pH 6.0 and one of these two homogenates was brought to pH 4.4. It was found that the

amount of bound nucleotides was much higher in the acidified homogenate. In another experiment, muscles were homogenized at pH 4.4 and one of the two homogenates was brought to pH 6.0. This resulted in a diminution of the amount of bound nucleotides.

The ability to form complexes with nucleotides is not a specific property of the structural muscle proteins. A formation of such complexes and its dependence on pH was observed already in 1932 by Przyłęcki & Grynberg [31] with egg albumin. Recently Niemierko and his coworkers [10] found that the proteins in frog liver, kidneys and lungs homogenates may contain the bound nucleotides, while Drabikowski [7] observed a binding of adenine nucleotides, and its dependence on pH, by various purified protein preparations (egg albumin, serum albumin, serum γ-globulin, casein, myosin).

At the present state, our knowledge on the properties of complexes of nucleotides with muscle proteins is rather poor. It seemed, therefore, interesting to examine more carefully the formation of these complexes within a broad range of pH and to investigate the effect of some inorganic salts thereupon. In connection with criteria of Przyłęcki [32] it could be expected that such an investigation might furnish some new data concerning the nature of binding between muscle proteins and nucleotides.

MATERIAL AND METHODS

All experiments were carried out with the acetone-dried muscle powder from the frog (*Rana esculenta*). The powder was prepared according to the procedure described earlier [28]. In spite of a partial denaturation of proteins, this powder, as a uniform material, proved to be more convenient for this investigation than fresh muscles. Its nucleotide composition was stable for several months if stored *in vacuo* at 0°. Molar ratio of labile nucleotide P to ribose in the used muscle powder preparations amounted from 1.0 to 1.7 (on the average 1.3) which indicated that nucleotides of various phosphorus content were present. Paper chromatography of nucleotides from the acetone powder (performed according to [37]) and spectrophotometric analyses of the eluted spots revealed the presence of ATP and ADP and only minute amounts of AMP.

The muscle powder preparations contained orthophosphate originally present in the fresh muscles and orthophosphate split from the phosphocreatine which appeared to be unstable during preparation of the powder.

In experiments on the effect of pH on the binding of nucleotides by muscle proteins the following procedure was used. 200 - 250 mg. of the

acetone powder was extracted during 10 minutes at 0° with 10 ml. of buffer solution in 48% ethanol. At this concentration of ethanol practically all the protein remained undissolved. Final concentration of the buffers was usually not higher than 0.05 M in order to diminish the possibility of competition between ions of the buffer and the nucleotides for the protein. The extract was filtered on a Schott funnel G4 and washed three times with the buffer solution. All the filtrates were combined and analysed for the amount of nucleotides.

It has been assumed according to Niemierko *et al.* [27] that those nucleotides which, during the described procedure, are not extractable and remain in the protein residue are protein bound. These nucleotides were determined after liberation with 10% trichloracetic acid at 0°.

In experiments on the effect of salts, the acetone powder was extracted with ethanol-buffer solutions, as described above, supplemented with various salts. In order not to introduce additional factors the buffers used in these experiments contained very little or none of metal ions.

Nucleotides were determined by the amount of ribose and of labile nucleotide phosphorus liberated by 15 minutes hydrolysis in 1 N-H_2SO_4 at 100° (ΔP_{15}). Phosphate was determined according to Fiske & Subbarow [13] and ribose according to the method of Mejbaum [23] with modification of Niemierko and Strzelecka-Gołaszewska (in preparation); the time of hydrolysis was 40 min, according to Albaum & Umbreit [1]. The pH was measured by means of a glass electrode. All values given in the text and in figures indicate final pH of the extracting media, i.e. the mixtures of buffers, ethanol and salt solutions.

All data concerning the amount of bound nucleotides are means of at least two parallel experiments performed on samples of the same muscle powder preparation. The results were reproducible within a few per cent. The data are expressed as per cent of the total amount of nucleotides in the muscle powder.

RESULTS

Fig. 1 and 2 demonstrate the relationship between pH of the extracting medium and the amount of protein bound nucleotides. The highest amount of bound nucleotides was found at pH 2.20 - 2.78. With 0.05 M-citrate buffer, pH 2.20, about 90% of nucleotides (as determined by the labile phosphorus) remained bound to proteins (Fig. 1) and with 0.05 M-glycine buffer, pH 2.40 and 2.78, practically all the nucleotides remained unextracted (Fig. 2). In more acidic solutions, the amount of bound

nucleotides decreased rapidly; so it was only a few per cent at pH 0.90 (Fig. 2). The degree of binding also diminished with increasing pH, although more slowly. At pH 6.8 as much as 40% of the nucleotides were still bound to proteins (Fig. 1). On further alkalization of the extracting media the degree of binding increased again, although very slightly.

As can be seen in Fig. 1 and 2, changes in the degree of binding are almost identical whether determined by ribose or labile phosphorus (ΔP_{15}). There are only a few discrepancies which may indicate that

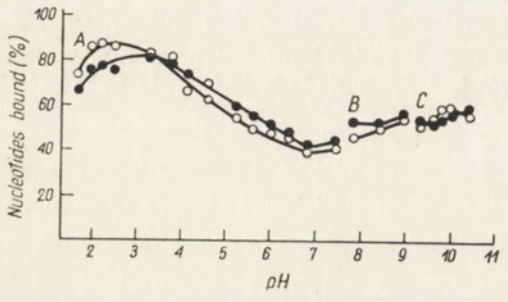

Fig. 1. Relationship between pH and binding of muscle nucleotides by muscle proteins. Muscle powder was extracted at 0° with the following solutions in 48% ethanol: (*A*) pH 1.70 - 7.46, 0.05 M-citrate buffer; (*B*) pH 7.85 - - 8.98, 0.05 M-barbital buffer; (*C*) pH 9.30 - 10.43, 0.05 M-glycine-NaOH buffer, (O), nucleotides determined by labile P; (●), by ribose. Two preparations (I and II) of muscle powder were used; (I) was extracted with solutions *A* and *B*, (II) — with solution *C*. Preparation I contained per g. 37.8 μmoles of labile nucleotide P, and 21,8 μmoles of ribose; preparation II — 52.1 μmoles of labile nucleotide P, and 36.2 μmoles of ribose

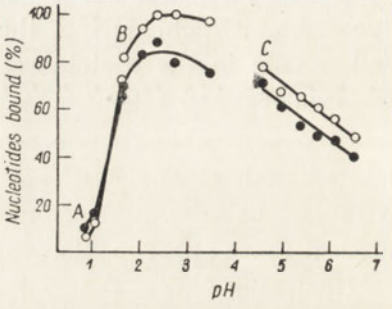

Fig. 2. Relationship between pH and binding of muscle nucleotides by muscle proteins. Muscle powder was extracted at 0° with the following solutions in 48% ethanol: (*A*) pH 0.9 - 1.65, HCl in concentrations 0.5 M, 0.25 M, and 0.05 M respectively; (*B*) pH 1.67 - - 3.48, 0.05 M-glycine-HCl buffer; (*C*) pH 4.59 - 6.58, 0.1 M-acetate buffer. (O), nucleotides determined by labile P; (●), by ribose. Muscle powder contained per g. 44.0 μmoles of labile nucleotide P, and 32.9 μmoles of ribose

nucleotides of different amount of phosphate moieties differ in their response to pH. However, it should be taken into account that a certain part of ribose, as determined in the present experiments, should not necessarily come from nucleotides.

In connection with the results obtained, indicating a different degree of binding of adenine nucleotides at different pH, experiments on the effect of inorganic salts ($CaCl_2$, $MgCl_2$, NaCl, and KCl) were performed at a few various pH values: 2.42, 6.96 and 8.98. It was found that at pH 2.42 (0.05 M-glycine buffer) the presence of 0.025 M-$CaCl_2$ or $MgCl_2$ considerably decreased the amount of bound nucleotides as compared

with buffer solution alone when no metal ions were present in the extracting medium (Fig. 3). A small but clearly detectable effect of $CaCl_2$

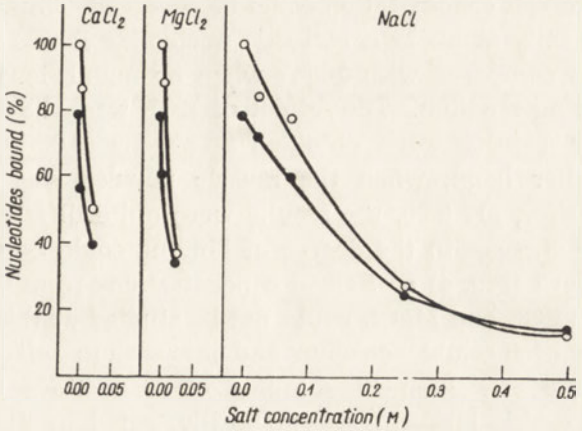

Fig. 3. Influence of some inorganic salts ($CaCl_2$, $MgCl_2$, NaCl, and KCl) on combinations of muscle nucleotides with muscle proteins at pH 2.42 ± 0.06. Muscle powder was extracted at 0° with solutions containing 0.05 M-glycine-HCl buffer, pH 2.42, inorganic salt in variable concentrations, and 48% of ethanol. (O), nucleotides determined by labile P; (●), by ribose. Muscle powder contained per g. 59,5 μmoles of labile nucleotide P, and 51,9 μmoles of ribose

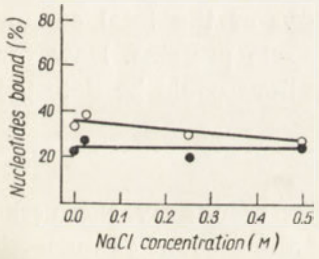

Fig. 4. Influence of NaCl on combinations of muscle nucleotides with muscle proteins at pH 6.96 ± 0.06. Muscle powder was extracted at 0° with solutions containing 0.05 M-Tris buffer, pH 6.96, NaCl in variable concentrations, and 48% of ethanol. (O), nucleotides determined by labile P; (●) by ribose. Muscle powder contained per g. 66.2 μmoles of labile nucleotide P, and 65.8 μmoles of ribose

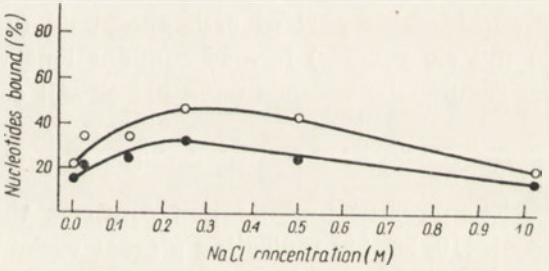

Fig. 5. Influence of NaCl on combinations of muscle nucleotides with muscle proteins at pH 8.98 ± 0.09. Muscle powder was extracted at 0° with solutions containing 0.05 M-Tris buffer, pH 8.98, NaCl in variable concentrations, and 48% of ethanol. (O), nucleotides determined by labile P; (●), by ribose. Muscle powder contained per g. 65.2 μmoles of labile nucleotide P, and 64.7 μmoles of ribose

and $MgCl_2$ was observed at concentration as low as 0.005 M, which corresponds approximately to the concentration of Mg^{2+} and Ca^{2+} ions in muscle tissue. The effect of NaCl at pH 2.42 could be observed only

at concentrations much higher than those of $MgCl_2$ and $CaCl_2$ (Fig. 3). However, in this case also a decrease of the amount of bound nucleotides with increasing salt concentration could be observed. Thus, in the presence of 0.5 M-NaCl only about 12% of labile nucleotide P was found as bound by proteins as compared with 100% when no metal ions were present in the extracting medium. The effect of KCl was the same as that of NaCl. Similar results were obtained at pH 1.9 (0.05 M-citrate buffer).

On the other hand, when the muscle powder was extracted with 0.05 M-Tris buffer, pH 6.96, the results were quite different. In this case no substantial changes in the degree of binding could be observed in the presence of NaCl (Fig. 4) or KCl in concentrations from 0.025 M to 0.5 M. The effect of $CaCl_2$ and $MgCl_2$ could not be studied at this pH because of the formation of insoluble calcium and magnesium salts of nucleotides.

The effect of NaCl on the binding of nucleotides in the alkaline medium (0.05 M-Tris buffer, pH 8.98) is illustrated in Fig. 5. As can be seen, an increase in the concentration of sodium chloride up to 0.25 M is accompanied by an increased binding of nucleotides by proteins. A further increase in NaCl concentration results, however, in a gradual decrease of bound nucleotides.

In all the experiments described above it was observed as well that a considerable part of orthophosphate (up to 30% of the total amount in muscle powder) formed combinations with muscle proteins. However, no regular dependence upon pH or salt concentrations could be detected.

DISCUSSION

The present investigation confirms the former results by Niemierko *et al.* [10, 28, 29] indicating a great variability of the amount of nucleotides bound by muscle proteins at different conditions. It has been shown that pH and the concentration of salts in the extracting medium are the most important factors influencing the extent of binding.

Przyłęcki [32] describes several kinds of linkages between macromolecular substances, like proteins, and compounds of low molecular weight. According to the criteria given by Przyłęcki, the results of the present investigation indicate that at different pH of the medium different kinds of linkages between muscle proteins and muscle nucleotides may exist. Electrostatic (salt type) linkage between muscle proteins and nucleotides in acidic medium is demonstrated by the effect of inorganic salts and of changes of pH upon the degree of binding. As nucleotides form anions at all pH values above 2 [22, 2] and as the isoelectric point of most of muscle proteins is in pH range from 4.6 to 5.4 [9] it can be supposed that electrostatic binding exists between phosphate moieties of

nucleotides and positively charged groups of proteins. A similar conclusion has been expressed by Drabikowski [7] who has found that an increase in the amount of phosphate groups in the nucleotide molecule increases its affinity towards proteins at, and below the isoelectric point of the protein. By blocking functional groups of the protein it has been shown also that ε-amino groups of lysine and guanidine groups of arginine are the main sites of binding between serum albumin and ATP [8].

At pH below 2 the dissociation of phosphate groups is decreased and the nucleotides exist as cations [22, 2]. This is probably the reason why below pH 2.2 the amount of bound nucleotides decreases abruptly, as observed in the present investigation. This decrease in the binding of nucleotides by proteins in strongly acid medium may be also partly explained by a competition of chloride ions (from HCl present in the extracting medium) with the nucleotides. The affinity of chloride ions towards muscle proteins increases with a decrease in pH, as it has been shown by Nanninga [26] with H- and L-meromyosin. The competition of Cl^- with nucleotides may also explain a decrease in the extent of binding when NaCl, KCl, $MgCl_2$ or $CaCl_2$ are added to the extracting medium at pH 2.42 and 1.9. As this decrease in the presence of $CaCl_2$ and $MgCl_2$ is much higher than in the presence of NaCl and KCl it is evident that Mg^{2+} and Ca^{2+} may have some additional effect. The nature of this effect needs, however, a further examination. A decrease in the binding of nucleotides in the presence of inorganic salts has been also observed by Drabikowski [7] with egg albumin.

As inorganic salts have no distinct effect on the extent of nucleotide--protein binding at pH 7 it may be concluded that in this case a binding

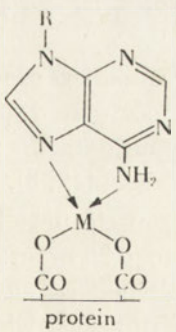

Fig. 6. A possible kind of binding of adenine nucleotides through a metal to the carboxyl rests of the protein

of some other type than electrostatic exists. It may be supposed that a chelate complexing of adenine moiety of the nucleotide with carboxyl groups of the protein is formed by a metal ion (Fig. 6), as suggested by Kirby [16] for complexes of deoxyribonucleic acid and proteins. It has been already shown by Epp *et al.* [11] that ATP and ADP form chelate

compounds with Mg^{2+} by the amino group attached to 6-carbon atom and the nitrogen atom in the position 7 of the purine ring. It is also known [26] that Mg^{2+} may combine with proteins.

Chelate binding of nucleotides with muscle proteins may exist side by side with the binding of the salt type. At neutral pH salt type binding may occur between phosphate groups of the nucleotide and positively charged groups of the protein, which at pH 7 are still rather numerous, or carboxyl groups of the protein and magnesium, or calcium ions. The affinity of Mg^{2+} and Ca^{2+} to muscle proteins [26] and to phosphate groups of nucleotides [33, 36, 21, 6] at neutral pH is well known. It can be supposed that the acetone-dried muscle powder used in the present investigation contained enough magnesium and calcium to make such type of binding possible.

A participation of calcium in the formation of binding between adenine nucleotides and actin has been already suggested by Straub & Feuer [34]. It has been also postulated recently [17, 35, 25, 18] that the well known effect of bivalent cations on the hydrolysis of ATP by myosin and actomyosin and on transphosphorylations which occur with the participation of nucleotides is due to the formation of an enzyme-metal-substrate complex. Hasselbach [14], however, does not agree with the conception of participation of bivalent cations in binding of nucleotide by actin. He has demonstrated that AMP and orthophosphate, which appear during the hydrolysis of actin-bound ADP by apyrase, are bound by the protein and that the amount of actin-bound calcium and magnesium remains unchanged. However, these results may be explained assuming that Ca and Mg form a chelate complex with actin and the nucleotide and that, at the same time, the nucleotide is attached to actin by its phosphate groups.

A binding of the tioester type between SH-groups of proteins and phosphate moieties of nucleotides has been also postulated [3, 4, 30]. However, in the case of myosin and actomyosin some data do not agree with the assumption of existence of such kind of binding [12, 5].

An increase in the binding of nucleotides by muscle proteins in the presence of NaCl and KCl (in concentrations up to 0.25 M) at pH 9, as observed in the present investigation, may be explained as an effect of binding of Na^+ and K^+ by carboxyl groups of the proteins, which shifts the isoelectric point towards more alkaline pH and extends the range at which a binding of the salt type between nucleotides and muscle proteins takes place. Such an interpretation is in accordance with the results of Lewis & Saroff [20] who found a violent increase of affinity of Na^+ and K^+ towards muscle proteins at pH above 7. Drabikowski [8]

blocked the carboxyl groups of serum albumin by esterification and also observed an increased binding of ATP by the protein. This modification of the protein molecule, like binding of sodium or potassium in the present investigation, increases the alkaline character of a protein.

On the other hand, higher concentrations (0.5 - 1.0 M) of NaCl had, at pH about 9, a slight decreasing effect on the amount of protein bound nucleotides. This effect might be explained as a competition of Cl^- ions with nucleotide ions for the binding sites in the protein molecule. Nanninga [26] did not find any binding of Cl^- by meromyosins at pH above 6, but the concentration of chloride ions in his experiments was not higher than 0.1 M. It seems quite possible that such a binding, even at pH 9, does occur at higher concentrations of Cl^- ions, as used in the present investigation, and that in this case chloride ions may compete with the nucleotides which are present in a far lower concentration.

It has been shown by Hermann [15] that the binding of ATP by myosin in the presence of KCl is accompanied by a simultaneous binding of potassium and that a low concentration (0.0005 M) of bivalent cations considerably increases the binding of ATP in the presence of potassium ions. These findings are related to our observations on the effect of low concentrations of inorganic salts at pH 9. A closer comparison is, however, difficult since no precise information concerning pH is available in Hermann's paper.

The present results indicate a great lability of complexes of muscle proteins with nucleotides. It should be taken into account that it is hardly possible to decide to what extent the amounts of bound nucleotides, as measured by various authors, correspond to the degree of binding which occurs in the intact, living muscle. One must realize that the amount of nucleotides present in the isolated protein is always strongly dependent on the isolation procedure. As was pointed out by Przyłęcki, already 20 years ago [32], the attempt to isolate natural complexes from biological material must be accompanied by a detailed study of artificial complexes, of their structure and of conditions of their formation and splitting.

SUMMARY

The effect of pH and of some inorganic salts (NaCl, KCl, $CaCl_2$ and $MgCl_2$) on the combinations of muscle nucleotides with muscle proteins was studied in acetone-dried powder prepared from the frog muscles. The extent of binding was dependent upon pH of the extracting medium. It was the highest at pH 2.2 - 2.8, when 90% to 100% of the nucleotides present in the material were in a protein-bound form. Inorganic salts had

a varying effect on the degree of binding, depending on pH and the salt concentration. A possibility of various types of chemical binding between proteins and nucleotides is discussed.

I wish to express my thanks to Prof. W. Niemierko for his interest, advice and helpful criticism in the course of this investigation.

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NIEKTÓRE WŁASNOŚCI POŁĄCZEŃ NUKLEOTYDÓW ADENINOWYCH Z BIAŁKAMI MIĘŚNIOWYMI

Streszczenie

Badano wpływ pH i niektórych soli nieorganicznych (NaCl, KCl, $CaCl_2$ i $MgCl_2$) na połączenia białek mięśniowych z zawartymi w mięśniu nukleotydami. Doświadczenia przeprowadzano na osuszonym acetonem proszku z mięśni żaby. Stwierdzono wyraźną zależność ilości nukleotydów związanych od pH. Najwięcej nukleotydów (do 90 - 100% ogólnej ich ilości w materiale) znaleziono w formie związanej przy pH 2,2 - 2,8. Dodatek soli nieorganicznych, zależnie od ich stężenia i od pH środowiska, powodował obniżenie lub zwiększenie ilości nukleotydów związanych, lub też pozostawał bez wpływu. W oparciu o uzyskane wyniki przedyskutowano możliwości istnienia między nukleotydami a białkami mięśniowymi różnego rodzaju wiązań w różnych warunkach.

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CHANGES IN PROTEINS OF YELLOW LUPIN *(LUPINUS LUTEUS L.)* DURING GERMINATION

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Ritthausen in 1811 [9] was the first who studied the typical protein of lupins and called it conglutin. Osborne & Campbell [8] separated it into conglutin α and β, that precipitate at 0.6 and 0.7 saturation with ammonium sulphate, respectively. Joubert [3] introduced a modification to the Danielsson's method for fractionation of peas globulins and separated lupin proteins into fractions A and B corresponding to the Osborne's fractions α and β. On further studies Joubert [4] separated proteins of yellow lupin into three components differing one from another by their sedimentation constants. One of these components showed dissociation, that depended upon pH of solution and salts concentration. Gerritsen [2] demonstrated that proteins derived from seeds of yellow lupin differed from one another by the presence of tryptophan and methionine. Wiewiórowski & Augustyniak [10] applied paper electrophoresis to separate proteins of yellow lupin as well as those of blue and white lupin. Moreover, Wiewiórowski *et al.* [11] followed the metabolism of protein in ripening seeds of white and blue lupin.

In this paper some attempts were undertaken to follow the changes in proteins during germination of seeds of yellow lupin.

EXPERIMENTAL

Studies were carried out on seeds of yellow lupin (*Lupinus luteus* L.) [1], with strenght of germination 95 ± 3%. Germination and development of seeds was carried out in pot cultures from 17th April till 6th May, on garden soil of pH 6.5 and relative moisture of 61%. Aeration was perfor-

[1] Seeds were kindly supplied by Wojewódzka Stacja Oceny Nasion, Wrocław.

med through glass tubes reaching gravel on the bottom of the pot. Optimal moisture of the ground was achieved by watering the plants daily in the pots to constant weight. Seedlings were sampled at the first, third and fifth phase of development. In these experiments the *I* phase was within 2nd and 4th day of germination, the *III* between 7th and 9 th day while the *V* one from 16th to 20th day of germination. The morphological description of plants at individual phases of development had been presented in previous report [7].

Extracts were prepared of seeds and seedlings. After seed-coat had been removed seeds were ground in a mill. Seedlings were washed with distilled water, lyophilized and then disintegrated in a porcelain mortar The powdered material from seeds or seedlings was treated with ethyl ether in the Soxhlet's apparatus for 12 hr. The fat-free material in amount 0.1 to 0.2 g. was twice extracted with 2.5 or 5 ml. of 0.1 M-borate buffer, pH 8.8 and μ 0.13, at room temperature for 8 hr. Supernatants after centrifugation were combined and made up to 5 or 10 ml., respectively. This procedure allowed for practically complete extraction of protein soluble in borate buffer.

Conglutin α and β were isolated from salt extracts of seeds according to Osborne [8] while fractions *A* and *B* according to Joubert [3]. The obtained preparations were lyophilized, then dissolved for 3 hr. in borate buffer, pH 8.8, and subjected to paper electrophoresis.

Paper electrophoresis of proteins was performed in borate buffer, pH 8.8, μ 0.13. Samples were applied on strips (37 × 3 cm.) of Whatman No. 1 filter paper, 4 cm. from the middle toward the cathode. Time of duration of electrophoresis was 7 hr. at 230 V, and intensity 0.5--0.9 mA/cm. Electropherograms were stained and individual fractions estimated according to Mejbaum-Katzenellenbogen & Dobryszycka [6]. Protein was determined with the micro-Kjeldahl and the tannin methods [5].

RESULTS

Results obtained by means of the tannin method [5] were compared with the Kjeldahl method in order to determine the applicability of the former. Protein was precipitated out of 2 ml. of borate extract with the equal volume of 10% trichloroacetic acid (TCA). The precipitated protein was twice washed with 5% TCA, then dissolved in 0.1 N-NaOH and estimated with both methods. Besides that protein was estimated by means of tannin in borate extracts diluted with 0.1 N-NaOH. It may be concluded from Table 1 that protein of seeds and seedlings of yellow lupin may be estimated with the tannin method.

Table 1

Estimations of protein in borate extracts of yellow lupin seeds by means of the Kjeldahl and tannin methods

Results obtained in two different extracts are presented

Material	Method	Protein	
		(g./100 ml. of extract)	(g./100 g. of dry seeds)
Borate extract	tannin	0.625 0.613	43.75 42.91
Protein of borate extract precipitated with 5 % TCA, precipitate dissolved in 0.1 N-NaOH	tannin	0.620 0.606	43.40 42.42
	Kjeldahl (coef. 5.81)*	0.611 0.621	42.77 43.47

* The coefficient is the arithmetical mean value of the nitrogen contents in yellow lupin proteins, as reported by Gerritsen [2].

Table 2

Amounts of protein extracted from yellow lupin seeds by means of various solvents

Mean values of six estimations, ± standard deviation

Solvent	Protein	
	(g./100 g. of dry seeds)	(%)
Borate buffer, pH 8.8; μ 0.13	42.1 ± 1.3	100
Veronal buffer, pH 8.6; μ 0.06	38.5 ± 1.6	91.4
10% NaCl, pH 8.8	35.6 ± 1.8	89.5
H_2O	8.9 ± 2.0	21.1

The yield of extraction of proteins from the yellow lupin seeds with borate buffer is compared in Table 2 to those obtained by means of veronal, of 10% sodium chloride, pH 8.8, and of distilled water. The difference between the amount of protein passing into borate buffer and into 10% sodium chloride was about 10%. Moreover borate extracts could be subjected to paper electrophoresis without previous dialysis and lyophilization.

Borate extracts, preparations of α and β conglutin as well as of Joubert's fractions *A* and *B* were subjected to paper electrophoresis in order to identify proteins of yellow lupin seeds. The electrophoretic picture of borate extracts (Fig. 1) contained five fractions: the first — the

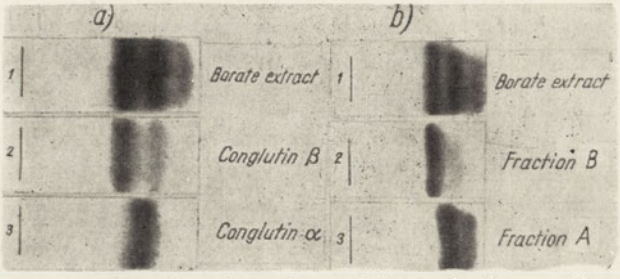

Fig. 1 *a, b*. Paper electrophoresis of proteins of the yellow lupin seeds. *a*) (*1*), borate extract of seeds; (*2*), conglutin β contaminated with conglutin α; (*3*), conglutin α. *b*) (*1*), borate extract of seeds; (*2*), Joubert's fraction *B* contaminated with traces of fraction *A* and with a fraction migrating slower; (*3*), Joubert's fraction *A* contaminated with albumin-globulin fraction migrating with the highest velocity towards anode

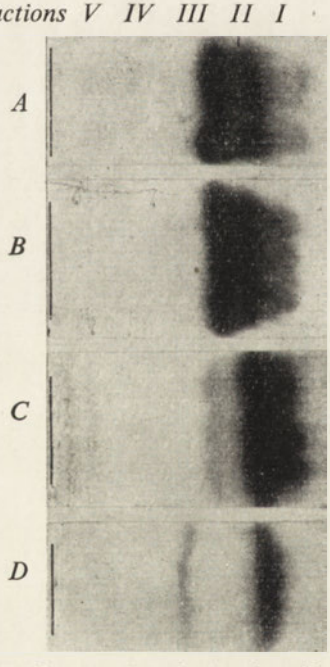

Fig. 2. Changes in electrophoretic pattern of proteins during germination of the yellow lupin seeds. Borate extracts of seeds: (*A*) not subjected to germination; (*B*) at *I* phase of development; (*C*) at *III* phase of development; (*D*) at V phase of development

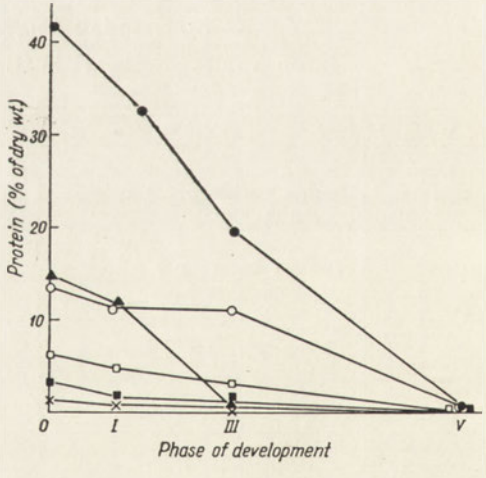

Fig. 3. Changes in the contents of total protein and of electrophoretic fractions during germination of the yellow lupin seeds, (●), total protein ; (□), first fraction — the albumin-globulin ; (○), second fraction — conglutin α; (▲), third fraction — conglutin β; (▣), fourth fraction ; (×), fifth fraction

most quickly migrating toward the anode, with a mobility of an albumin-globulin fraction; the second — corresponding in its mobility to conglutin α and fraction *A*; the third — corresponding to conglutin β and fraction *B*; the fourth and fifth fractions not identified precisely. The main proteins of yellow lupin seeds were conglutins α and β, which constituted about 73% of the total seed protein (Table 3).

Table 3

Protein fractions during germination of seeds of yellow lupin

The arithmetical mean values from three measurements are presented

Phase of development	Day of germination	Protein instilled (μg)	Protein recovered (%)	Distribution of electrophoretical fractions (%)				
				I	*II*	*III*	*IV*	*V*
O	0	640	100.8	14.8	34.9	37.6	8.9	3.8
I	2 - 4	872	98.7	15.1	36.3	37.7	6.3	4.6
III	7 - 9	385	103.4	18.2	59.5	8.1	0	14.2
V	16 - 20	368	100.7	0	59.8	0	29.6	10.6

Fig. 2 presents changes in electrophoreticel pattern of proteins of yellow lupin seeds during germination and early phases of development. Borate extracts deriving from *I* and *V* phase of development of the seeds were compared with freshly prepared extract of seeds not subjected to germination. No changes were found at the *I* phase of development, while at the *III* phase the amount of conglutin β was distinctly decreased. Conglutin β and albumin-globulin fraction disappeared at *V* phase of development and conglutin α remained as well as fractions fourth and fifth.

Fig. 3 presents changes in the contents of total protein as well as of individual fractions calculated per g. of dry weight of germinating seeds or plants. The percentage of total protein as well as that of all the individual fractions, except conglutin β decreases gradually during germination and development of the seeds. Conglutin β, however, disappears more quickly than other fractions and its level at *III* phase of development falls to 7.8% of the amount found in dry seed. Conglutin β and the albumin-globulin fraction disappear at *V* phase, while the level of total protein diminishes at this time from 40% to 1.3% of the plant's dry weight.

Table 3 presents the distribution of electrophoretic fractions of proteins deriving from the seeds of yellow lupin, expressed in per cent values.

No changes are found at *I* phase of development in comparison to seeds not subjected to germination, while at *III* phase of development the ratio of individual fractions is distinctly changed. At this phase conglutin α constitutes as much as 60% of total protein, and conglutin β 8% only, whereas the contents of both conglutins are equal in dry seed and amount to about 36% of total protein for each one. The ratio of other fractions remains at *III* phase similar to the initial one. Albumin-globulin fraction and conglutin β disappear at *V* phase thus the content of the other fractions, expressed in per cents rises correspondingly.

As seen from the data presented above conglutin β disappears first during germination of the yellow lupin seeds. This finding has been confirmed on fractionation of salt extract of 18-day old seedlings with increasing concentrations of ammonium sulphate (conglutin α precipitates at 0.6 and conglutin β at 0.7 saturation). About 60% of total protein has been precipitated at 0.6 saturation with ammonium sulphate, while the remaining protein has not been precipitated at 0.7 saturation.

DISCUSSION

The tannin micromethod developed by Mejbaum-Katzenellenbogen for animal proteins was applied for the determination of lupin proteins. The applicability of this method to the yellow lupin proteins was established by means of comparison of the results to those obtained with the Kjeldahl method. The results were consistent within the limits of experimental error of both methods.

Staining and elution of proteins from the electropherograms was performed according to the method of Mejbaum-Katzenellenbogen & Dobryszycka [6], which allowed for direct estimation of protein in each individual fraction, as opposed to other methods based on the determination of the dye adsorbed on the electrophoretic protein fractions.

Borate buffer was used to extract the proteins from seeds of yellow lupin, as it was found to be the most efficient reagent. Moreover, the borate extracts could be subjected directly to paper electrophoresis in the same buffer, without previous dialysis and lyophilization. Joubert demonstrated that one of the proteins of yellow lupin was characterized by sedimentation constant 11.6 S, which corresponded to conglutin β and which underwent complete dissociation to protein with sedimentation constant 7.2 S in borate buffer, pH 8.8 and ionic strength 0.1. Dissociation of this component depended upon the concentration of salts and pH of the solution. Borates induced dissociation of conglutin β while paper electrophoresis carried out in the same buffer prevented probably the secondary association to protein of higher molecular weight.

The general decrease of total protein during germination is accompanied by the decrease in the protein fractions under investigation. These changes, however, do not proceed at an equal rate in all fractions. The concentration of conglutin β falls more rapidly than that of other fractions and as early as between the *I* and *III* phase of the development of seedlings. Conglutin β and albumin-globulin fraction disappear at *V* phase of development and the level of total protein falls to 1.3% of the plant's dry weight. Thus conglutin β is the first and most quickly utilized protein by the growing plant. Gerritsen [2] has detected the presence of methionine and tryptophan only in this fraction. As proved by Wildmen [12] tryptophan is a precursor of β-indolylacetic acid — the plant heteroauxin. Thus, it seems possible that conglutin β is not only a source of energy and aminoacids as other fractions are, but also may be a source of precursors of growth substances.

SUMMARY

Proteins of borate extracts from seeds and seedlings of yellow lupin were separated into five fractions on paper electrophoresis. The behavior of individual fractions was followed during *I*, *III* and *V* phase of the seed development. During germination total protein diminishes from 40% of dry weigth of seeds to 1.3%. The amount of conglutin β falls rapidly between the *I* and *III* phase of development, and is not detected at *V* phase. The albumin-globulin fraction disappears at *V* phase, too.

The author wishes to express her very best thanks to Doc. Dr. W. Mejbaum-Katzenellenbogen for her kind advice and criticism.

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ZMIANY W BIAŁKACH ŁUBINU ŻÓŁTEGO (*LUPINUS LUTEUS L.*) PODCZAS KIEŁKOWANIA

Streszczenie

Białka wyciągów boranowych z nasion i siewek łubinu żółtego rozdzielono w elektroforezie bibułowej na pięć frakcji. Przebadano zachowanie się poszczególnych frakcji w *I*, *III* i *V* fazie rozwoju nasion. Podczas kiełkowania białko całkowite spada z 40% suchej masy nasion na 1,3%. Między *I* a *III* fazą rozwoju gwałtownie obniża się ilość konglutyny β, a w fazie *V* nie wykazano już jej obecności. Również w *V* fazie zanika frakcja albuminowo-globulinowa.

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INCORPORATION OF ^{32}P INTO THE PHOSPHORUS COMPOUNDS OF THE WAX MOTH LARVAE *(GALLERIA MELLONELLA)*

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It has been demonstrated earlier in this laboratory that phosphorus metabolism in the wax moth larvae (*Galleria mellonella* L.) shows some remarkable peculiarities. Thus, it has been established that pyro- and polyphosphates are formed in the insect body in great quantities and are found in the excreta as the principal phosphorus compounds [4, 4a, 5, 6, 12, 16]. During digestion of wax by the larvae large quantities of phospholipids are synthesized in the intestine; they disappear promptly during starvation and reappear during refeeding [9, 8, 17, 11].

It seemed that the application of isotopic tracers, especially of ^{32}P, could throw some light on the mechanism of formation of the mentioned phosphorus compounds. This could facilitate the approach to the problem of the possible interrelationship between the different features of phosphorus metabolism in *Galleria* and the unique ability of this insect to utilize wax.

The aim of the present investigation, which is the first of this series, was to follow the incorporation of ^{32}P into phospholipids, nucleic acids and different fractions of acid-soluble phosphorus compounds in the larval body and to follow the elimination of ^{32}P in the excreta during feeding and during starvation of the insects.

METHODS

Radioactive phosphorus was purchased from the Institute of Nuclear Research, Warszawa, in the form of the $Na_2H^{32}PO_4$ solution. Before use it was hydrolyzed for 10 minutes in 1 N-HCl at 100° to decompose pyrophosphate, and neutralized with alkali.

Small pieces of bee-comb, weighing about 200 mg. each, were sprayed with 0.1 ml. of the radioactive phosphate solution and dried using an infra-red lamp and a fan. The amount of radioisotope employed varied in different series of experiments from 5 μC to 44 μC.

Experiments were performed on groups of 10 full-grown wax moth larvae weighing about 150 mg. each, bred on bee-comb at 30°. They were placed in small glass vessels, 10 specimens in each, and supplied with 200 mg. of the comb containing ^{32}P. After the 20 hr. feeding and "labelling" period the whole portion of comb was consumed by the insects. The larvae were then transferred to another vessel, and either supplied with some non-radioactive comb or starved for several days. In the latter case the larvae were ligated just behind the head for preventing metamorphosis [6].

At the next and subsequent days of feeding with non-radioactive comb (normal feeding), or starvation, the larvae and the excreta were analyzed separately.

All determinations were duplicated. The phosphorus content and radioactivity were measured in various phosphorus fractions from the larval body and the excreta.

The phosphorus compounds were fractionated by the method of Niemierko *et al.* [7], the nucleic acids were separated according to Schneider [14]. The lipid phosphorus (P_{lipid}), nucleic acid phosphorus (P_{nucl}) and total acid-soluble phosphorus ($P_{t.a.s.}$) contents were determined by the method of Fiske & Subbarow [1] after digestion with nitric acid. The samples were evaporated to dryness in bakelite dishes, and their radioactivity was measured with an end-window Geiger-Müller counter (4 mg./cm^2.).

Among the acid-soluble phosphorus compounds the following fractions were determined: orthophosphate (P_i), compounds hydrolyzable after 10 min. heating at 100° in 1 $_N$-HCl (P_{10}), compounds hydrolyzable after 3 hr. heating (P_{180}) and the remaining phosphorus compounds resistant to 3 hr. acid hydrolysis (P_{nh}). The phosphorus content of each fraction was dettermined by the method of Martin & Doty [3], the corresponding radioactivity was checked in aliquots of *iso*butanol-benzene solution after evaporation of the solvent.

The excreta of the larvae were extracted with cold 5% trichloracetic acid (TCA). The content and the radioactivity of P_i, P_{10} and $P_{t.a.s.}$ were determined in the same way as in the larval body.

EXPERIMENTAL AND RESULTS

Incorporation of ^{32}P into the phosphorus compounds of the feeding larvae

The phosphorus contents of various fractions of the larval body, as established previously in this laboratory [4b] and in the present study, are indicated in Table 1.

Table 1

Phosphorus compounds in wax moth larvae

	mg % of fresh body weight
P_{lipid}	50 - 60
$P_{nucl.}$	40 - 45
$P_{t.a.s.}$	100 - 120
P_i	12 - 14
P_{10}	22 - 28
P_{180}	10 - 13
P_{nh}	50 - 60

The distribution of the incorporated ^{32}P among the various phosphorus compounds in the body of the feeding larvae is presented in Table 2. As mentioned above (see Methods) the radioactivity of the phosphate administered to the larvae was different in individual series of experiments (from 5 μc to 44 μc). Consequently, the radioactivity of the phosphorus fractions investigated varied greatly from one experiment to another. To enable comparison of the results, the radioisotope content of each fraction (total activity) was expressed as percentage of the total dose of ^{32}P ingested by the larvae. The radioactivity of the total fraction (counts per min.) expressed as percentage of the ingested dose and divided by the P content (mgs) of the corresponding fraction was designated as the "reduced" specific activity.

As can be seen from Table 2, at completion of the one day period of ingestion of the radioactive food the ^{32}P content of the larval body accounted for 60% (Exp. VI-1) up to 77% (Exp. III-2) of the ingested dose, the remainder having been excreted by the larvae. The distribution of the radioisotope among the fractions under investigation varied greatly. Although the insects were given ^{32}P in the form of orthophosphate, after cessation of ^{32}P ingestion merely 5 to 15% of the radioisotope administered was found in the P_i fraction; 28 to 58% of ^{32}P appeared in other acid-

Table 2

Distribution of ^{32}P in different phosphorus fractions from the fed larvae and in the excreta

(A) Content of ^{32} P expressed as percentage of the total ingested dose. (B) Percentage of the ingested dose (A) per 1 mg. P of the fraction (reduced specific activity)

The total dose of ^{32}P was given to the larvae during the first day of experiment; during the subsequent days larvae were fed unlabelled comb

Series No	Dose of ^{32}P (μC)	Duration of Exp. (days)	A					B			
			$P_{t.a.s.}$		P_{lipid}	$P_{nucl.}$	total P in excreta	$P_{t.a.s.}$		P_{lipid}	$P_{nucl.}$
			P_i	$P_{t.a.s.}-P_i$				P_i	$P_{t.a.s.}-P_i$		
II 1	5	1	6.5	57.5	7.5	3.0	25.5	64.0	46.2	15.0	7.5
2	5	1	5.2	54.0	9.5	3.6	27.7	51.0	45.5	14.4	8.2
3	5	2	4.5	25.0	14.2	6.3	50.0	35.6	29.2	24.0	17.5
4	5	2	5.2	35.0	16.6	8.2	35.0	41.5	35.0	27.0	16.5
5	5	3	2.6	12.2	17.3	8.0	60.0	24.0	15.2	26.6	17.2
6	5	3	4.2	16.7	20.0	10.0	49.0	28.0	21.0	32.2	21.0
III 1	8	1	15.0	44.5	10.7	6.5	23.3	77.0	34.0	12.2	9.0
2	8	1	9.1	48.5	12.5	6.0	24.0	53.5	30.4	12.5	7.0
3	8	2	4.1	22.5	18.6	7.8	47.0	25.8	25.0	27.0	13.0
4	8	2	3.6	20.0	21.4	9.0	46.0	20.0	20.6	31.2	15.8
5	8	3	3.5	13.5	17.1	8.0	58.0	19.0	18.0	26.2	15.5
6	8	3	3.0	13.1	18.0	6.5	59.5	18.5	15.0	25.0	15.0
7	8	4	3.3	13.0	12.0	6.1	65.5	24.0	18.0	25.2	16.5
8	8	4	2.4	8.0	9.3	6.5	74.0	16.8	14.4	19.5	12.5
IV 1	44	1	15.5	28.0	10.8	4.7	41.0	80.5	25.0	14.8	8.6
2	44	1	13.3	32.2	12.9	5.5	36.0	73.0	28.2	15.0	9.0
3	44	2	4.3	13.5	17.7	8.3	56.2	21.4	11.5	18.7	11.0
4	44	2	3.3	11.3	16.2	6.8	62.4	18.6	10.5	18.0	10.7
5	44	3	3.0	10.0	9.8	8.0	69.0	20.0	8.8	14.0	10.4
6	44	3	2.4	7.1	10.7	7.2	72.5	10.0	6.2	12.8	10.0

-soluble phosphorus compounds; 7,5 to 13% in the phospholipids and only 3 to 7% in the nucleic acids.

The highest specific activity amounting from 51 to 80% was found in the orthophosphate fraction, it was somewhat lower in the remaining acid--soluble compounds (25 to 46%) and still lower in the phospholipids and nucleic acids (12 to 15% and 7 to 9% respectively).

The subsequent feeding of the larvae with comb containing no radioisotope, which followed the „labelling" period, brought about remarkable changes in the distribution of ^{32}P among the various phosphorus fractions (Table 2 and Fig. 1). By far the greater part of the

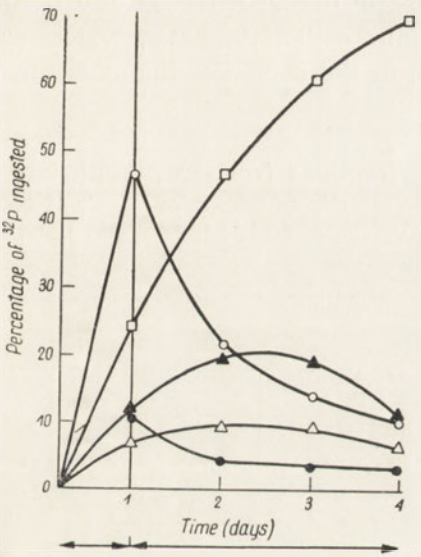

Fig. 1. ^{32}P content in various phosphorus fractions from feeding larvae and in their excreta. During the first day the larvae were fed with comb containing radioactive orthophosphate; during the subsequent days normal food was given (●), P_i; (○), $P_{t.a.s}$-P_i; (▲), P_{lipid}; (△), P_{nucl}; (□), $P_{excreted}$

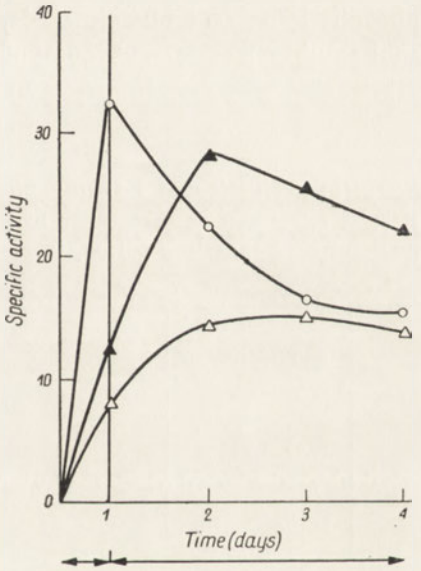

Fig. 2. Specific activity — time relations of the phosphorus fractions from feeding larvae. Specific activities expressed as percentage of the total ^{32}P ingested per 1 mg. P of the corresponding fractions. (○), $P_{t.a.s.}$-P_i; (▲), P_{lipid}; (△), P_{nucl}

radioisotope was excreted in the course of experiments (almost exclusively as pyro- and polyphosphates). The ^{32}P content of the acid-soluble fraction of the larval body decreased significantly (an abrupt fall in radioactivity was apparent already after the first day of normal feeding, but was much smaller on the second day). On the contrary, the ^{32}P content of the phospholipid fraction increased simultaneously and reached its maximum after

a 24 hr. period of normal feeding. The radioactivity-time relation of the nucleic acids resembled that of the phospholipids, the rise in ^{32}P content being less significant, however.

The changes in the specific activity of the fractions are illustrated in Fig. 2, which represents the results of a typical experiment. In each series of experiments some deviations were observed, but the general trend was the same. The most significant result was the abrupt fall in the specific activity of the acid-soluble phosphorus compounds accompanied by an increase in the specific activities of the phospholipids and nucleic acids within the period of normal feeding.

In Table 3 are presented the changes in specific activities of the phospholipids and nucleic acids, related to the specific activity of the acid-soluble phosphorus compounds (minus orthophosphate), which was arbitrarily taken as 100. As can be seen, in the course of the experiment

Table 3

Specific activities of various phosphorus fractions from wax moth larvae
Results expressed as percentage of the specific activity of $P_{t.a.s.}$ minus P_i at completion of ^{32}P ingesting period, taken as 100. Average of 6 experiments and standard deviation

Fraction	At completion of ^{32}P ingestion	Duration of subsequent normal feeding	
		one day	two days
$P_{t.a.s.} - P_i$	100	60 ± 13.6	39 ± 10.3
P_{lipid}	38 ± 4.8	72 ± 15.0	63 ± 10.3
$P_{nucl.}$	21 ± 3.7	39 ± 5.5	41 ± 7.1

the specific activities both of the phospholipids and the nucleic acids exceeded that of the acid-soluble P compounds, for the phospholipids this became evident on the second day and for the nucleic acids on the third day of the experimental period.

The distribution of the radioisotope among various acid soluble P fractions was also studied, and the results indicated that no uniformity of labelling occurred, at least within the period investigated. The rate of ^{32}P incorporation reactions seems to differ widely in the particular fractions. Figure 3 shows the results of a typical experiment, indicating the pronounced differences in ^{32}P distribution, which persisted throughout the experimental period. The phosphorus fraction, containing compounds resistant to acid hydrolysis (P_{nh}), accounts for more than a half of the $P_{t.a.s.}$, but its content of ^{32}P was comparatively small, whereas the P_{10} fraction revealed a rather high content of the radioisotope.

The lack of uniformity in „labelling" brought about considerable differences in the specific activities of the soluble P fractions, which were evident throughout the whole experimental period (Table 4). As indicated above, the specific activity of P_{nh} was always the lowest and the differences persisted to the end of the experimental period.

Table 4

Specific activities of the acid-soluble phosphorus fractions from wax moth larvae

Results expressed as percentage of the specific activity of $P_{t.a.s.}$, taken as 100. Average of 6 experiments, ± standard deviation.

Fraction	At completion of ^{32}P ingestion	Duration of subsequent normal feeding	
		one day	two days
$P_{t.a.s.}$	100	100	100
P_i	205 ± 25	126 ± 27	143 ± 26
P_{10}	170 ± 43	144 ± 22	150 ± 17
P_{180}	104 ± 23	125 ± 34	121 ± 26
P_{nh}	43 ± 22	70 ± 10	65 ± 5

The specific activity-time relation for each of the acid-soluble P fractions varied greatly. Although differences were observed in individual experiments, the general pattern remained similar. The results of one experiment are given in Table 5 (they will be discussed below).

Table 5

*Reduced specific activities * of the acid-soluble P fractions from wax moth larvae*

Average of duplicate determinations

Fraction	At completion of ^{32}P ingestion	Duration of subsequent normal feeding	
		one day	two days
P_i	77	20	15
P_{10}	76	15	14
P_{180}	32	19	11
P_{nh}	8	8	6

* Specific activities expressed as per cent of ingested ^{32}P per 1 mg. of the corresponding fractions.

Excretion of ^{32}P by the feeding larvae

The total P content of the excreta of the feeding wax moth larvae falls within relatively wide limits, depending upon the comb the insects are bred on. The comb used throughout our experiments contained about 0.4% P, and the content of P in the excreta of the larvae was 0.5 to 0.8%.

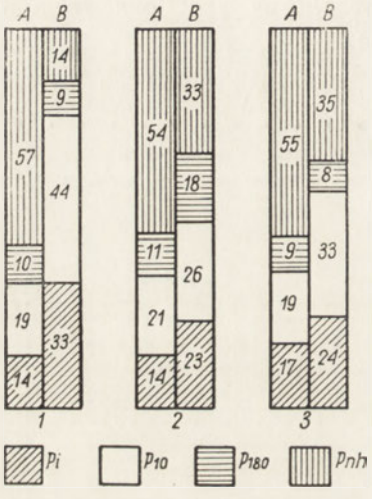

Fig. 3. Phosphorus content and ^{32}P distribution in the acid-soluble phosphorus fractions from feeding larvae. *(A)*, phosphorus content of the fractions as per cent of $P_{t.a.s.}$, *(B)*, ^{32}P content in the fractions as per cent of the ^{32}P content in $P_{t.a.s.}$ *(1)* at completion of the ^{32}P ingesting period; *(2)*, after one day *(3)* after 2 days of subsequent normal feeding

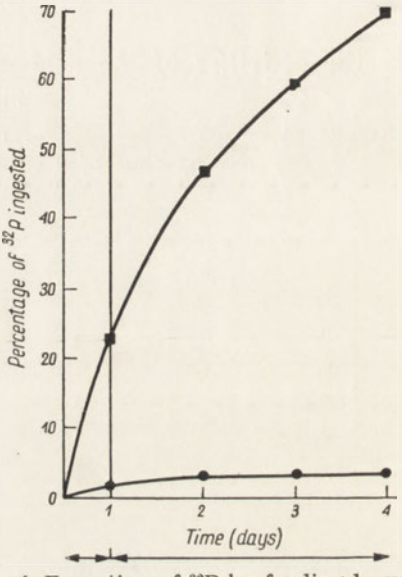

Fig. 4. Excretion of ^{32}P by feeding larvae. During the first day the larvae were fed with comb containing radioactive orthophosphate, during the subsequent days normal comb was given. (●), P_i fraction, (■), P_{10} fraction

The determinations of the P_i and P_{10} content of the excreta were in good agreement with the data reported earlier [4a] and were consistent with the view that nearly all phosphorus is present in the excreta as polyphosphates.

In the present study, the content of P_{10} accounted for 90 to 97% of the total P found in the excreta. The radioactivity measurements of both the P_i and P_{10} fractions indicated that practically all ^{32}P was confined to the polyphosphates, the radioisotope content of the orthophosphate constituting merely 3 to 10% of the total ^{32}P (Fig. 4). The amount of ^{32}P excreted increased during the experimental period and was attributed almost exclusively to the polyphosphates.

Incorporation of ^{32}P by starved larvae

The last series of experiments were undertaken in order to gain some information on the influence of a prolonged period of starvation upon the phosphorus metabolism. The results are presented in Fig. 5. In accordance with the results of the preceding experiments, after cessation of ^{32}P administration more that half of the ^{32}P ingested was found to be present in the acid-soluble P compounds, about 10% in the phospholipids, 5% in

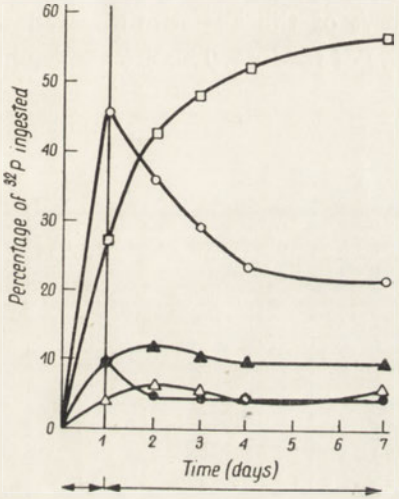

Fig. 5. ^{32}P content in the various phosphorus fractions from starved larvae and in the excreta. The larvae were fed with radioactive comb during the first day and starved throughout the subsequent period. Explanations as in Fig. 1

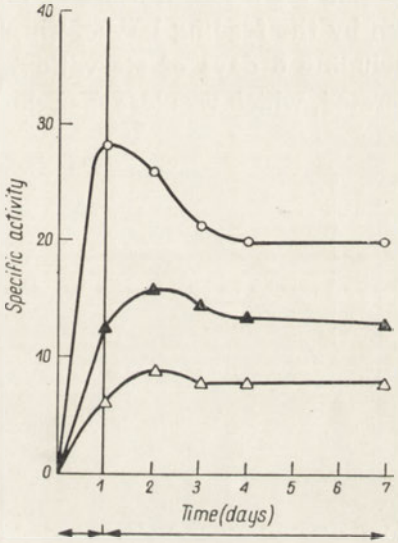

Fig. 6. Specific activity-time relations of the phosphorus fractions from starved larvae. The specific activities expressed as percentage of the total ^{32}P ingested per 1 mg. P of the fraction. The larvae were fed with radioactive comb during the first day and starved throughout the subsequent period. Explanations as in Fig. 1

the nucleic acids, the remainder having been excreted. Thoughout the period of starvation the radioisotope content of the phospholipids and nucleic acids underwent insignificant changes only, the ^{32}P content of the acid-soluble compounds declined less than in feeding larvae.

As indicated in Fig. 6, the changes in the specific activities of the various P fractions were less pronounced in the starved than in the fed larvae. Thus, the fall in the specific activity of the acid-soluble P fraction

was much slower than that observed in the feeding larvae. The specific activities both of the phospholipids and nucleic acids increased to some extent on the next day after ligation. Afterwards, they decreased and later did not undergo any significant changes, being steadily lower than the specific activity of the acid-soluble P fraction. It is noteworthy that the specific activity of the phospholipids never reached the high level observed in the feeding larvae.

Fig. 7 indicates that during starvation the ingested radioisotope was retained for a longer time in the larval body and excreted much slower than by the feeding larvae. Throughout the experimental period of 7 days (including 6 days of starvation) about 60% of the ^{32}P administered was excreted, which was less than during 3 days of feeding. The orthophosphate

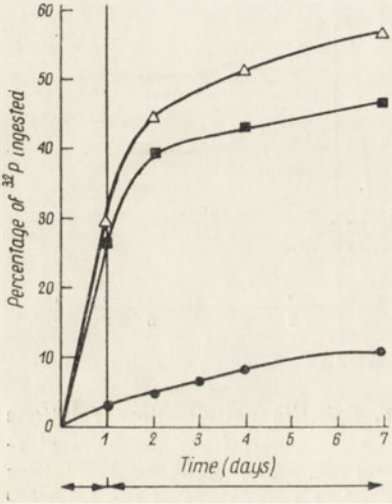

Fig. 7. Excretion of ^{32}P by starved larvae. Experimental conditions as in Fig. 5 and 6. (●), P_i fraction; (■), P_{10} fraction (△), $P_{t.a.s.}$

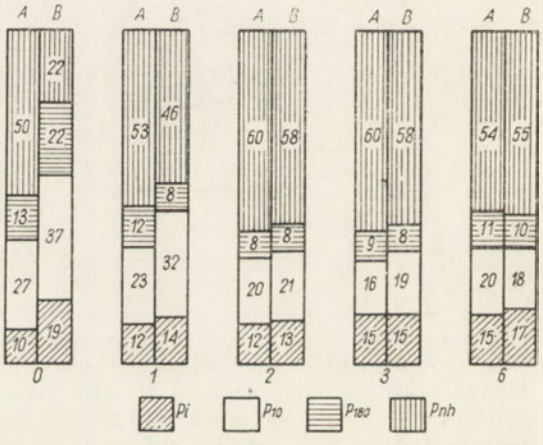

Fig. 8. The phosphorus content and ^{32}P distribution in the acid soluble phosphorus fractions from starved larvae. (*A*), phosphorus content of the fractions as per cent of $P_{t.a.s.}$; (*B*), ^{32}P content of the fractions as per cent of the ^{32}P content in $P_{t.a.s.}$ 0, at completion of ^{32}P ingesting period; 1, 2, 3, 6 — subsequent days of starvation

content was somewhat higher in the excreta of the starved than in those of the feeding larvae. The orthophosphate which accounted for 15 to 20% of the total P excreted contained about 25 to 30% of the total radioisotope, the remaining 70 to 75% were attributed to the polyphosphates.

It is evident from Fig. 8, which presents the distribution of ^{32}P among various acid-soluble P compounds, that the entire pool of the soluble compounds became uniformly labelled during starvation. Consequently, the specific activities of the soluble P fractions were approximately equal, which could never be found to occur in the fed insects.

DISCUSSION

The present study on the ^{32}P incorporation into various phosphorus fractions is thought to have thrown some light on the phosphorus metabolism in the wax moth larvae which, however, is still far from being elucidated.

The high incorporation rate of ^{32}P into the acid-soluble P compounds (let alone the orthophosphate) and the abrupt fall in ^{32}P content during the subsequent normal feeding seem to be indicative of a rapid turnover of this class of compounds. The P_i, P_{10}, P_{180} and P_{nh} fractions were analyzed separately. This procedure enabled to get some information on the rate of synthesis and breakdown reactions of each group of compounds. One must keep in mind, however, that each of these fractions, save the orthophosphate, still continued to be heterogeneous.

The changes in the specific activity of P_i are quite clear. The larvae were fed labelled orthophosphate, which resulted in high ^{32}P content of inorganic P compounds of the larval body. The specific activity of P_i fell abruptly during subsequent normal feeding, along with the transformation of the orthophosphate into other P compounds and with the simultaneous influx of unlabelled P from the comb.

It is noteworthy that at completion of ^{32}P ingestion the specific activity of P_{10} was as high as that of the orthophosphate and a similar abrupt fall was steadily observed. As has been mentioned already, the P_{10} fraction is highly heterogeneous: besides arginine-phosphate, ATP and ADP it contains pyro- and polyphosphates [4b, 12]. It was found that the specific activity of the P_{10} fraction was higher in the case when less of the ^{32}P ingested had been excreted, and it was lower when less ^{32}P was retained in the larval body. This suggests that the high content of ^{32}P of this fraction could be, partially at least, attributed to the polyphosphates already synthesized in the larval body and not yet excreted. As indicated above, most of the ^{32}P was excreted in the form of polyphosphates. The conclusion might be drawn, therefore, that the ingested orthophosphate is rapidly involved in metabolic reactions und undergoes transformations until finally phosphorus is excreted as polyphosphates. When, along with the subsequent normal feeding, the larvae were supplied with non radio-

active phosphate present in the comb, the ^{32}P content in the P_{10} fraction diminished rapidly. The polyphosphates have been previously reported by Niemierko & Niemierko [4a, 5] to be the chief phosphorus constituents of the excreta of the wax moth larvae. In the present study with the aid of ^{32}P the high rate of the reactions concerned with the synthesis of polyphosphates and the intensity of transformations could be evidenced. In one series of experiments (not described above) the administered $Na_2H^{32}PO_4$ contained large amounts of carrier (about 20 mg./1 ml.), which resulted in much higher orthophosphate content of the excreta (15 to 20% of the total P) accompanied by a high content of ^{32}P in the P_i fraction (25 to 30% of the total ^{32}P excreted). One might conclude, therefore, that the larva was not able to metabolize all of the phosphate, when given in too large a dose, and that a considerable part of it passed through the intestine unchanged.

Compounds resistant to acid hydrolysis (P_{nh}) had always the lowest specific activity as compared with other acid-soluble fractions, and this activity did not undergo any significant changes during subsequent normal feeding. As reported by Lenartowicz [2] the P_{nh} fraction of *Galleria mellonella* larvae consists chiefly of phosphoethanolamine and phosphoglycerol. One might suppose that the turnover rate of these compounds is much lower than that of the other acid-soluble P fractions. On the other hand, it is not to be excluded that phosphoethanolamine and phosphoglycerol might be continuously derived from the breakdown of the phospholipids. As the latter compounds were found in the present experiments to be rich in ^{32}P, this kind of transformation might result in an increase of the radioactivity of the P_{nh} fraction.

The fall in ^{32}P content of the total acid-soluble P fraction can be the result of the following processes: (1) excretion of ^{32}P, (2) inflow of unlabelled P from the honeycomb, and (3) transformation of some acid-soluble P compounds into phospholipids and nucleic acids.

The course of ^{32}P incorporation into the phospholipids is indicative for a high metabolic rate of these compounds. Conclusion may be drawn that a complete phospholipid synthesis takes place in the larval body, in which some acid-soluble compounds are utilized. The time-specific activity relation curves for $P_{t.a.s.}$ and P_{lipid} fractions seem to yield evidence on this point (Fig. 1 and 2). The phospholipid specific activity was increasing during the first day after cessation of ^{32}P ingestion, which points to the acid-soluble compounds of the larval body as the primary source of P for phospholipid synthesis. However, on the basis of the present experiments no evidence could be obtained suggesting which of the acid-soluble compounds might serve as phospholipid precursors.

The changes in the activity-time relation of the phospholipids led to the conclusion that the turnover rate of the phospholipids might be lower than that of the acid-soluble P fraction. In a previous study [17] it was found that the intestinal phospholipids of the wax moth larvae undergo a very rapid breakdown and resynthesis. The results of the present experiments seem to indicate that the bulk of phospholipids outside the intestine has a lower turnover rate than the intestinal phospolipids. Experiments performed by Perlman *et al.* [10] on the rat demonstrated that the ^{32}P content of phospholipids (of the whole animal body, not in a particular organ) reached its maximum in 20 to 24 hr. after ^{32}P administration, which is consistent with the results obtained for the wax moth. In the rat, however, only about 5% of the administered dose could be recovered in the phospholipids, as compared with about 20% found in the wax moth. This seems to indicate that the rate of phospholipid metabolism in the wax moth is higher than in the rat.

Attention was directed to the possibility that the intense metabolism of phospholipids in the wax moth larvae could be correlated with the peculiar food of the insect and influenced by the digestion and absorption of wax. The results of our experiments carried out on starved larvae seem to confirm this assumption. During starvation the amount of ^{32}P incorporated into the phospholipids was smaller and the fall in the radioactivity was less pronounced than during feeding of the insects. A small increase in the ^{32}P content in the lipids, observed the next day following ligation, was probably due to the fact that the intestine still contained a certain quantity of the comb and that no real starvation took place at that time. Throughout the following days of starvation the ^{32}P content in the lipids underwent insignificant alterations only.

There are good reasons also for assuming that the metabolism of the acid-soluble P compounds during feeding and during starvation is different to some extent. This was confirmed by the finding that during starvation some kind of „isotopic uniformity" was established in these compounds. The various acid-soluble fractions (P_i, P_{10}, P_{180} and P_{nh}) became uniformly labelled and their specific activities equalled each other, what could never be observed during feeding of the larvae. Uniformity of labelling has been previously claimed to occur in the various acid-soluble P fractions by Winteringham *et al.* [15], as well as by Samuels *et al.* [13]. No explanation is available so far for the fact that uniformity of labelling can be accomplished in the starved and not in the feeding larvae. The reasons for so pronounced differences are still obscure.

SUMMARY

1. With the aid of ^{32}P, given as orthophosphate, the phosphorus metabolism in the feeding and in the starved wax moth larvae was studied.

2. The ^{32}P content of the acid-soluble P fraction, which was very high at completion of the radioisotope ingestion, fell abruptly during the subsequent period of normal feeding. The fall in the specific activity of the acid-soluble P compounds was accompanied by an increase in the specific activities of the phospholipids and nucleic acids. The phospholipid specific activity reached its maximum 24 hr. after cessation of ^{32}P ingestion and exceeded the activity of the acid-soluble P fraction.

3. Practically all of the ^{32}P excreted by the larvae was found in the polyphosphates.

4. During starvation the fall in the specific activity of the acid-soluble P fraction was less pronounced than during feeding while the changes in the specific activities of the phospholipids and the nucleic acids were rather insignificant.

5. Uniformity of labelling was found to occur in the acid soluble P compounds of the starved but not of the fed larvae.

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WŁĄCZANIE SIĘ ^{32}P W ZWIĄZKI FOSFOROWE MOLA WOSKOWEGO *(GALLERIA MELLONELLA)*

Streszczenie

1. Przy pomocy ortofosforanu, znakowanego ^{32}P, badano metabolizm związków fosforowych u żerujących i głodzonych gąsienic mola woskowego.

2. Zawartość ^{32}P we frakcji kwasorozpuszczalnej bezpośrednio po zaprzestaniu pobierania izotopu jest wysoka i spada znacznie po przejściu na „normalne" żerowanie. Równocześnie ze spadkiem aktywności właściwej we frakcji kwasorozpuszczalnej stwierdza się wzrost aktywności właściwej fosfolipidów i kwasów nukleinowych. Aktywność właściwa fosfolipidów osiąga maximum po 24 godz. normalnego żerowania i przekracza aktywność właściwą kwasorozpuszczalnych związków fosforowych.

3. Cały niemal ^{32}P, wydalony przez gąsienice, zawarty jest we frakcji polifosforanów.

4. Podczas głodu gąsienic stwierdza się słabszy spadek aktywności właściwej frakcji kwasorozpuszczalnej niż podczas żerowania, a zmiany aktywności fosfolipidów i kwasów nukleinowych są nieznaczne.

5. W związkach fosforowych kwasorozpuszczalnych ustala się równowaga izotopowa już po 2 dniach głodu, natomiast nie stwierdza się tego zjawiska podczas żerowania.

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THYMIDINE PHOSPHORYLASE AND OTHER ENZYMES IN REGENERATING RAT LIVER

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It has now been reasonably well established that the immediate precursors involved in DNA biosynthesis are the nucleoside-5′-triphosphates [26, 30, 3, 24, 41] which may be synthesized by the appropriate enzymes from nucleosides or 5′-nucleotides. While incorporation of thymine into tissue DNA occurs only to a small extent, if at all [21, 37, 27], thymidine is readily incorporated in a number of biological systems [35, 15, 17, 39] following its transformation to the mono-, pyro- and triphosphate [4, 8, 42]. The principal reason for the lack of incorporation of thymine into DNA, or uracil into RNA, is the rapid degradation of pyrimidines in metabolizing tissue [10, 5, 16]. A detailed examination of the relationship between anabolism and catabolism of pyrimidines led to the suggestion that a decrease in degradation of tissue pyrimidines is accompanied by an enhanced probability of their incorporation into nucleic acids [6, 7]. Such a decreased rate of degradation of pyrimidines is usually encountered under conditions of rapid cell division as in regenerating rat liver where, in fact, a decrease in degradation rate of uracil is accompanied by its incorporation into RNA. The rate of degradation of thymine is likewise reduced in regenerating tissue [7], from which it might be expected that appropriate conditions exist for DNA incorporation of thymine; and both Holmes *et al.* [21] and Reichard [35] have actually demonstrated such incorporation of injected labelled thymine in regenerating rat liver.

Presumably such incorporation proceeds *via* the transformation of thymine to thymidine. Since the only enzyme known to synthesize thymidine from thymine is thymidine phosphorylase [13], it appeared of interest to study the activity of this enzyme in regenerating rat liver with a view to obtaining some concrete informations as to its metabolic role; the more so in that the same enzyme exhibits similar activity in the

synthesis of uracil deoxyriboside, which is also closely related to DNA synthesis inasmuch as it is a specific acceptor for methyl groups *via* an alternate pathway for thymidine synthesis [14, 2, 22, 34, 33].

The activity of thymidine phosphorylase has therefore been measured as a function of time of regeneration, in relation to protein and DNA synthesis, in the regenerating liver of the rat. For purposes of comparison, the activities of acid phosphomonoesterase and 5′-nucleotidase have been simultaneously estimated.

MATERIALS AND METHODS

Thymidine phosphorylase. Activity was measured according to the method of Friedkin & Roberts [12] *via* the rate of arsenolysis of thymidine; the thymine thus formed is estimated spectrally at 300 mμ in alkaline medium, under which conditions thymidine absorption is negligible. The optimal reaction conditions established as a result of preliminary trials were as follows: thymidine, 2.4 mg. (4.6 μM) and a volume of aqueous liver homogenate containing 20 mg. tissue in 0.05 M-succinate-arsenate buffer, pH 5.9 to a total final volume of 0.4 ml. Incubation time was 30 mins. at 37° and the unit of activity was the amount of thymine liberated during this interval.

Sample preparations. White male rats, 200 - 250 gms., were used. Hepatectomy was performed according to Higgins & Anderson [20] and the mice killed after predetermined periods of regeneration. The liver was immediately removed and homogenized in cold, redistilled water in a Potter-Elvehjem type of homogenizer; 1 gm. tissue in 10 ml. water for enzymatic determinations and 1 gm. in 5 ml. water for isolation of DNA.

Protein was determined by the method of Lowry *et al.* [28], using γ-globulin for the standard curve, with all measurements performed in duplicate.

Determination of DNA. DNA was isolated according to the method of Schneider [38] and estimated by the Dische reaction [9] as modified by Allfrey & Mirsky [1] and involving heating of the test samples for 20 mins. on the water bath. Acetic acid freshly distilled from dichromate was used for the Dische reagent.

5′-nucleotidase. This enzyme was determined according to de Lamirande *et al.* [25] using AMP as substrate with an incubation time of 30 mins. The unit of activity is the amount of inorg. P liberated by the entire liver or by 100 mg. protein.

Acid phosphatase. This was estimated as follows: 30 μMoles glycerophosphate, liver homogenate containing 10 mg. tissue, 0.05M-acetate buffer, pH 5, total volume 3 ml. Incubation time 30 mins. at 37°. The activity is expressed in terms of the amount of inorg. P liberated by the entire liver.

RESULTS AND DISCUSSION

The overall results have been brought together in Table 1 and are presented graphically in Fig. 1, in the latter of which are the figures for the individual components in the liver during the regeneration period. The initial mass of the liver was calculated on the assumption that the portion removed was 2/3 of the total weight. As will be seen from Fig. 1, both

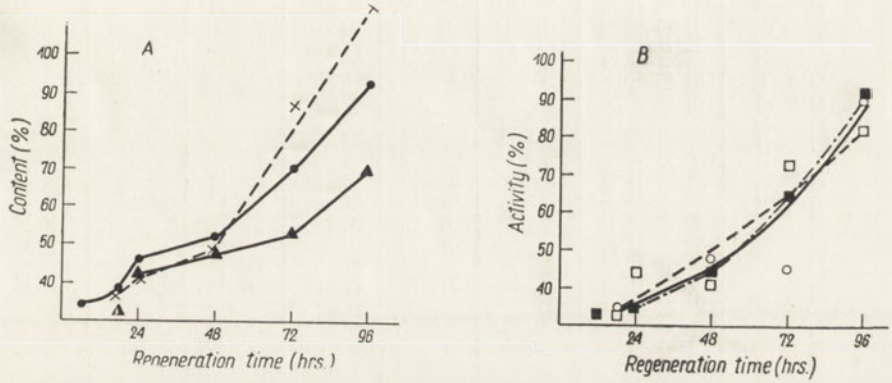


Fig. 1. Rate of regeneration of various components of the liver in percentages (initial values taken as 33%). (*A*); (×), DNA, (●), liver mass, (▲), protein. (*B*); (■), thymidine phosphorylase, (□), acid phosphatase, (O), 5′-nucleotidase

protein and DNA synthesis as well as an increase in liver mass, are noticeable about 18 hours following hepatectomy: but regeneration of the enzyme systems exhibits a pronounced lag. The initial increase in activity

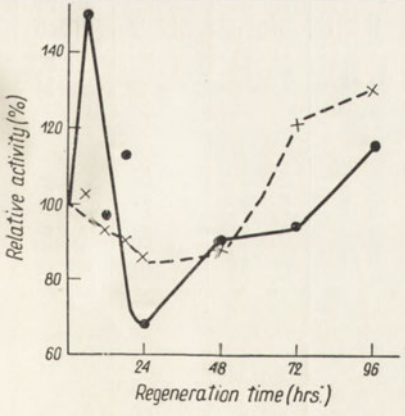


Fig. 2. Variations in enzyme activities per 100 mg. protein during regeneration. The 100% values are for the activities of the control animals (see Table 2). (x) thymidine phosphorylase, (●), 5′-nucleotidase

of the enzymes investigated was clearly lower than that of total protein and only after about 50 hours for nucleoside phosphorylase, and about 75 hours for 5′-nucleotidase, did regeneration of enzyme activity surpass

Table 1

Level of various components of rat liver as a function of time of regeneration

Figures in brackets refer to the number of animals used; and mean deviations calculated according to formula $\sigma = \sqrt{\frac{\varepsilon (x - z)^2}{n - 1}}$

Regeneration time (hrs.)	Liver weight (gms.)	Protein content (mg.)	DNA content (mg.)	Thymidine phosphorylase (in μMoles thymine liberated in 30 mins.)	Acid phosphatase (in mg. inorg. P liberated in 60 mins.)	5′-nucleotidase (in mg. inorg. P liberated in 30 mins.)
0	3.13±0.35(3)	655 ± 13(3)	7.2±0.52(3)	379 ± 32(3)	8.2± 1.6(3)	13.1±1.4(3)
6	3.23±0.46(3)	690 ± 65(3)	8.5±0.34(3)	343 ±104(3)	6.7± 1.3(3)	16.0±3.8(3)
0	3.45±0.21(3)	736 ± 69(3)	7.6±0.68(3)	351.6± 49(3)	14.3± 1.0(3)	13.9±1.6(3)
18	3.96±0.95(3)	710 ±206(3)	8.3±2.1(3)	297.6± 72(3)	14.2± 4.0(3)	14.2±4.0(3)
0	3.17±0.81(8)	573 ±107(8)	7.0±1.05(8)	348 ± 98(8)	14.5± 4.1(8)	16.2±3.1(8)
24	4.45±0.85(8)	736.7±133(8)	8.9±2.34(8)	385 ±108(8)	19.0± 1.6(8)	14.0±6.6(8)
0	3.04±0.49(8)	550 ±111(8)	6.5±0.3(5)	275 ± 55.8(8)	17.2± 7.0(5)	11.1±2.8(8)
48	4.82±0.71(8)	782 ±116(8)	9.7±0.78(5)	370 ±172(8)	20.9±10.0(8)	16.4±4.1(8)
0	3.27±0.74(6)	661 ±124(6)	7.3±1.2(3)	333.6± 98(6)	14.6± 8.7(6)	13.9±2.5(6)
72	6.97±1.05(6)	1075 ±156(6)	19.4±5.58(3)	657.5±161(6)	32.6±21.6(6)	19.1±6.7(6)
0	2.71±0.44(6)	583 ±107(6)	6.1±0.95(6)	303.6±129(6)	18.9± 7.4(6)	11.0±4.0(6)
96	7.7 ±0.96(6)	1251 ±210(6)	21 ±2.6(6)	838.6±212(6)	47.9±23.6(6)	30.1±8.2(6)

that of total protein. This relationship between rates of enzyme and protein regeneration is perhaps more clearly illustrated in Table 2 and Fig. 2, where enzyme activities per 100 mg. protein are presented as percentages of control values.

From Fig. 2 it will be observed that thymidine phosphorylase activity decreases during the period of most active DNA synthesis, i.e. between 20 and 30 hours after hepatectomy [32, 18]. Notwithstanding all the regulatory mechanisms the living cell disposes of for the channeling of various metabolic processes, the increase in the amount or activity of a given enzyme is the most convincing evidence for its participation in a given process or reaction. The decrease in activity of nucleoside phosphorylase during the synthesis of DNA argues, therefore, against its participation in DNA synthesis during regeneration. The course of regeneration of thymidine phosphorylase does not differ significantly from that

Table 2

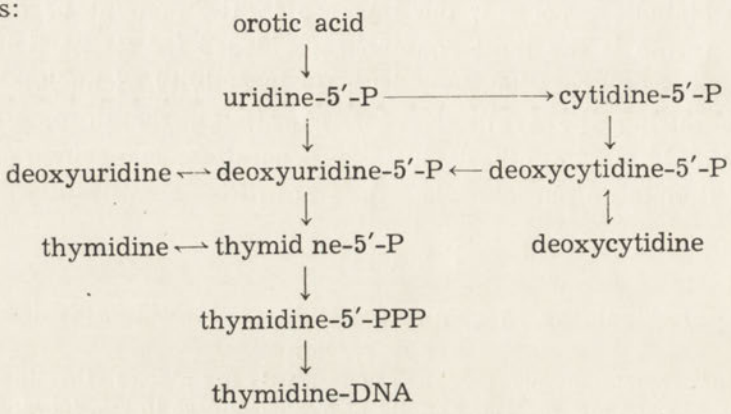
Activities of thymidine phosphorylase and 5′-nucleotidase during rat liver regeneration

Activity of thymidine phosphorylase expressed in μMoles thymine liberated in 30 mins. per 100 mg. protein. Activity of 5′-nucleotidase in mg. inorg. P liberated in 30 mins. per 100 mg. protein. Mean deviations calculated as in Table 1.

Number of rats	Time of regeneration (hrs.)	Thymidine phosphorylase	Activity (as % of control)	5′-nucleotidase	Activity (as % of control)
3	0	47.7±10		1.89±0.01	
	6	49.8±13	104	2.84±0.5	152
5	0	51.7±6.0		2.18±1.2	
	12	48.2±5.6	93	2.15±1.1	98
3	0	47.7±7.9		1.84±0.2	
	18	42.4±2.2	88	2.06±0.2	112
8	0	58.3±15		2.71±0.3	
	24	49.2±9	84	1.85±0.7	68
8	0	52.4±14		2.11±0.8	
	48	45.7±11	87	1.92±0.3	91
6	0	50.7±12		2.13±0.3	
	72	62.2±16	122	1.98±0.5	93
6	0	52.0±14		1.9 ±0.6	
	96	67.8±16	130	2.39±0.5	125

for acid phossphatase and 5′-nucleotidase, two enzymes the metabolic functions of which are not known.

Several other enzyme systems are known which have been correlated with DNA synthesis and which make their appearance, or exhibit increased activity, during liver regeneration. These include thymidine kinase [4, 8], thymidylate kinase [8, 30, 19, 42], thymidylic synthetase, deoxycytidylic acid deaminase [29], and polymerase [4, 40] so that the formation of DNA thymine in the cell may proceed by one or more of the following pathways:



Uridylic acid, formed from orotic acid, is aminated to cytidylic acid [23]. Both uridylic and cytidylic acids are transformed to the corresponding deoxy derivatives by reduction of the 2′-hydroxyls [36, 31]. Deoxycytidylic acid may be deaminated to deoxyuridylic acid in the presence of thymidylic synthetase to thymidylic acid [11], which is then phosphorylated by the appropriate kinases to mono-, di-and triphosphate for incorporation into DNA. While incorporation of pyrimidine deoxynucleosides is made possible by the existence of the appropriate kinases, their formation is undoubtedly only a by-pass from the general anabolic pathway. The results obtained here with thymidine phosphorylase, at any rate, suggest that it is concerned principally with the catabolism of thymidine.

Finally, mention should be made of the fact that Friedkin & Roberts [12], measuring thymidine phosphorylase activity at pH 7.5, observed an inhibitory effect of mitochondria on the rate of hydrolysis of thymidine. However at the optimal pH, 5.9, used in the above work, we could find no such inhibition.

SUMMARY

The level of thymidine phosphorylase during DNA synthesis in regenerating rat liver has been measured with a view to obtaining some information as to the metabolic role of this enzyme. Simultaneous

measurements were made of 5′-nucleotidase, acid phosphomonoesterase, DNA and protein contents, and liver growth. During the period of maximum DNA synthesis the activity of thymidine phosphorylase, in relation to total protein, decreases; its subsequent increase, based on activity measurements during further regeneration, does not differ markedly from that for the other enzymes.

The results suggest that thymidine phosphorylase is involved rather in the degradation, and not synthesis, of DNA.

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FOSFORYLAZA TYMIDYNOWA I INNE ENZYMY REGENERUJĄCEJ WĄTROBY SZCZURA

Streszczenie

W celu uzyskania wiadomości o roli fosforylazy tymidynowej przebadano zachowanie się tego enzymu w okresie syntezy DNA w regenerującej wątrobie szczura. Równocześnie oznaczano 5′-nukleotydazę i fosfatazę kwaśną, zawartość DNA i białka oraz przyrost wagi wątroby. W okresie największego nasilenia syntezy DNA aktywność fosforylazy tymidynowej w stosunku do białka całkowitego maleje; w następnych okresach regeneracji aktywność jej wzrasta, nie różniąc się od zachowania pozostałych enzymów.

Uzyskane wyniki przemawiają, że jest to enzym biorący udział raczej w rozpadzie niż w syntezie DNA.

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NUCLEOLYTIC ENZYMES OF *THIOBACILLUS THIOPARUS*. PURIFICATION AND PROPERTIES OF RIBONUCLEASE

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During studies of the metabolism of *Th. thioparus* carried out in this department [19, 27], the cell extract was found to contain a considerable amount of nucleic acids especially in the initial phase of growth, and also to display marked nucleolytic activity. The extract, obtained after disintegration of the cells in the presence of phosphate buffer at pH 7.0, degraded RNA [1] practically completely, setting free oligonucleotides, mononucleotides and nucleosides. With appropriate methods of fractionation protein mixtures, ribonuclease, nonspecific phosphodiesterase and 5′nucleotidase were shown to be present in the extract of *Th. thioparus*.

In this work a method of purification of ribonuclease free of phosphodiesterase and 5′-nucleotidase is reported and some properties of the enzyme are described.

MATERIAL AND METHODS

Microorganism. The strain of *Th. thioparus* was isolated and grown in previously described conditions [20]. Cells in the logarithmic phase of growth (72 hr. after inoculation) were harvested from the medium by centrifugation or filtration through Berkefeld filters. Elementary sulphur was then removed by differential centrifugation [20]. After washing with distilled water, the bacterial mass was stored in the frozen state at -12°.

Yeast RNA (Merck) and thymus DNA (Light & Co., London), were employed. Limit polynucleotide ("core") was obtained from yeast RNA

[1] Abbreviations used are: RNA, ribonucleic acid from yeast; DNA, deoxyribonucleic acid from thymus; RNA-Th, ribonucleic acid from the cells of *Th. thioparus;* NA, nucleic acids; Poly A, polyadenylic acid; Pu, purine; Py, pyrimidine; A, adenine; G. guanine; C, cytosine; U, uracil; tris, tris-(hydroxymethyl)-aminomethane.

by exhaustive hydrolysis with pancreatic ribonuclease according to Hilmoe [12]. Low-molecular products of hydrolysis were dialyzed against distilled water and the residue was lyophilized. Beef pancreas ribonuclease, three times crystallized, was obtained by the method of Mc Donald [17].

Determination of ribonuclease activity. Determinations were based on the method of Anfinsen *et al.* [2], modified as follows. Yeast RNA, which was first dialyzed against distilled water for 48 hr. at 0°, was used as substrate. Before use, a 0.6% solution in 0.1 M-citrate-phosphate buffer of pH 7.0 was prepared. In this buffer solution the original activity of non-specific diesterase was diminished to about one-third. To 0.5 ml. of the substrate solution, 0.5 ml. of enzyme solution was added, and the mixture was incubated at 37° for 60 min. At zero time, a 0.2 ml. sample was drawn, and 0.3 ml. of 2.5% solution of perchloric acid containing 0.25% uranyl acetate immediately added. A similar sample was prepared under identical conditions after the incubation period. The samples were set aside for 10 min. at 0° and the precipitate was centrifuged. To 0.2 ml. of the clear supernatant, 2.8 ml. distilled water was added and measurements were taken at 260 mμ with Uvispec spectrophotometer (Hilger, London) in 1 cm. cuvette. The increase of extinction in the incubated sample as compared with the control was calculated and expressed in Δ^{260} units for 60 min. and 1 ml. of undiluted enzyme solution. The amount of enzyme giving $\Delta^{260} = 1.00$ under the described conditions was assumed to represent one unit of ribonuclease activity. In all cases the average of two determinations with each sample was taken for calculation of enzymatic activity.

The specific activity of the ribonuclease was obtained as the ratio of activity, expressed in Δ^{260} units to the extinction of the sample at 280 mμ.

Determination of phosphodiesterase activity. Ca-[bis (*p*-nitrophenyl)-phosphate]$_2$ (Sigma, USA) was used as substrate [26]. To 100 μl 0.01 M-solution of substrate in 0.1 M-tris-HCl buffer of pH 8.5, 100 μl 2×10^{-3} M $MnSO_4$ as the activator was added and 10 μl of enzyme solution. The mixture was incubated at 37° for 60 min. The reaction was stopped by adding 2.8 ml. 0.1 N-NaOH and extinction was then determined at 400 mμ. The values of extinction served to calculate the amount of μmole *p*-nitrophenol liberated by 1 ml. of enzyme solution during 60 min. at 37°.

The activity of 5′-nucleotidase was determined using 5′-AMP (Light) as substrate. Adenosine was separated chromatographically in solvent 2 (*v. i.*) and localized under UV-lamp. The spots were then eluted with

0.1 N-HCl and extinction at 257 mμ measured. Adenosine was calculated quantitatively using the molar coefficient of 14 600 in 0.1 N-HCl.

Column chromatography. Cellulose ion exchangers CM, DEAE and ECTEOLA obtained from the Serva Co. (Entwicklungslabor., Heidelberg, Germany) with exchange capacities 0.66, 0.60 and 0.40 mequiv./g. respectively, were employed. Before using, CM and ECTEOLA were twice treated with 0.1 N-NaOH and 0.1 N-HCl alternately, washed in the chloride form with distilled water, and finally with appropriate buffer solutions [29]. DEAE-cellulose was treated first with 1 N-NaOH, washed with water and then with buffer solution [21]. All chromatographic separations were done in a cold room at about 4°.

Amberlite IRC-50 (XE-64), 200-400 mesh (Light) was treated before use according to the method of Hirs [13]. The protein content was estimated as the extinction at 280 mμ using Uvispec spectrophotometer. The concentration of protein in chromatographic fractions collected from IRC-50 column was determined by ninhydrin analysis [24a].

Paper chromatography. The following solvent systems were used: (1) HCl azeotrope-isopropanol-water (65 : 33 : ad 100) [33]; (2) 96% ethanol - 1 M-ammonium acetate, adjusted to pH 7.5 by addition of NH_3 (75 : 30) [6]; (3) *iso*propanol-water-NH_3 (70 : 10 : 20) [23]; (4) *n*-butanol saturated with saturated boric acid [2]. Whatman No. 1 or No. 3MM filter paper were used for separation by the ascending technique with solvents 2, 3 and 4 and by the descending technique with solvent 1. The spots of the separated substances on the paper were visualized by taking photographs of the chromatograms in UV light.

High voltage electrophoresis. Separation was carried out in a Wieland--Pfleiderer type apparatus [32] on Whatman No. 1 filter paper at a potential gradient of 35 V/cm. at 4°. An 0.1 M-acetate buffer solution at pH 3.5 served as electrolyte for separation of the mononucleotides [16], and 0.4 M-sodium tetraborate solution adjusted to pH 13.0 by means of NaOH for separation of the cyclic 2′ : 3′-phosphonucleosides from linear 3′-phosphonucleosides [11].

Isolation and characterization of RNA-Th. RNA-Th was isolated by the method of Kirby [15] by extraction with 90% phenol from bacteria ground with carborundum [3]. The precipitate which was formed by adding 2 volumes of ethanol in the presence of 1% sodium acetate was washed with alcohol then with ether, and dried *in vacuo*. The powder obtained was poorly soluble in water, but readily soluble in 0.2 M-NaCl

[2] Dr. D. Shugar, personal communication.

[3] The carborundum before use was first boiled with sulphuric acid then with water and washed until neutrality.

at pH 7.0. Further purification was achieved by chromatography on a DEAE-cellulose column. In Fig. 1 is shown the result of separation of 35 mg. RNA-Th in conditions as described under Figure. Fractions 50 - 70 were pooled and, after removing salt by dialysis, RNA was precipitated with 2 volumes of ethanol and dried as previously.

In UV light the preparation showed maximum absorption at 258 mμ, and minimum at 230 mμ (in 0.2 M-NaCl buffered with 0.03 M-tris-HCl of pH 7.0). The ratio of extinctions $D_{max}/D_{min} = 1.74$; $D_{max}/D_{280} = 2.00$; $D_{max}/D_{220} = 1.39$. Analysis of the composition of purine and pyrimidine bases after hydrolysis of the preparation with 12 N-$HClO_4$ gave the following results: G, 25.4; A, 23.2; C, 24.0; U, 27.4 molar per cent. Ratio

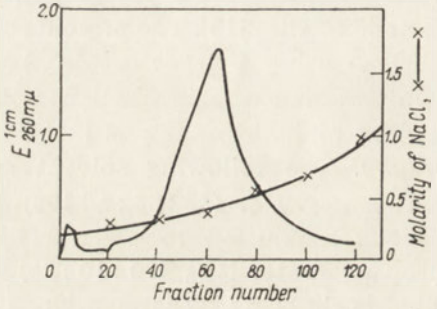

Fig. 1. Gradient elution of a sample of 35 mg. RNA-Th on a DEAE-cellulose column. The column (1.2 × 9.5 cm.) was equilibrated with 0.02 M-sodium phosphate buffer, pH 7.8, containing 0.2 M-NaCl. After adsorption of the sample (2 ml.) gradient elution was begun with a buffer solution composed of 0.2 M-phosphate of pH 5.5 containing 1.2 M-NaCl. The mixing chamber contained 500 ml. of starting buffer and the reservoir 500 ml. eluting buffer. Fractions (5 ml.) were collected at the rate of 60 ml./hr. The continuous line denotes extinction at 260 mμ; x, shows the change of gradient of NaCl

Pu/Py = 0.95, G/C = 1.06, A/U = 0.85. The preparation contained approx. 3% of DNA calculated as thymine. RNA-Th with above properties was used as the substrate for ribonuclease in some of the experiments further described in this paper.

RESULTS

Ribonuclease activity during growth of the microorganism

With the aim of studying the relation between the growth of the bacteria and the ribonuclease formation, 4-liter volumes of medium were inoculated with equal amounts of suspensions of bacterial cells in the log phase of growth. At 24-hours intervals the cells were harvested from

the medium and after grinding extracts were prepared in conditions identical with those described below (under "Purification of ribonuclease"). Assays were made in the extract of nitrogen by the micro-Kjeldahl method, and ribonuclease activity and nucleic acids content. The amount of NA was calculated on the basis of measurements of extinction at 280 and 260 mμ [31]. From Fig. 2 it can be seen that during exponential growth the activity of the ribonuclease and the NA content increase in proportion to the mass of bacterial nitrogen. In the stationary phase, in which partial

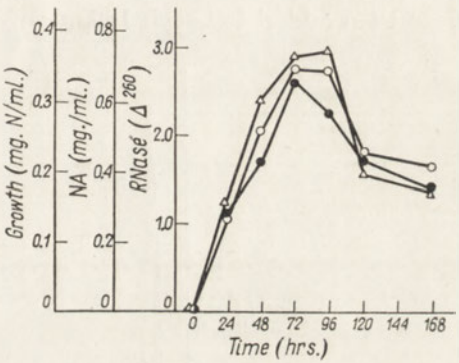

Fig. 2. Growth and course of ribonuclease formation and NA in cells of *Th. thioparus*. The culture was incubated at 25°; (●), bacterial nitrogen in mg./ml.; (O), ribonuclease activity per ml.; (Δ), NA in mg./ml. of extract

lysis of the cells occurs, the activity of ribonuclease and NA content are not diminished, but continue on the same level. This indicates that enzyme activity, as well as the amount of material absorbing at 260 mμ per cell, are still increasing.

The agreement between the rates of synthesis of ribonuclease and NA is interesting; we are inclined to accept the view of those authors according to whom this phenomenon can be explained by the participation of ribonuclease in hydrolytic processes as well as in the biosynthesis of NA in the bacterial cell [7, 24, 5].

Purification of ribonuclease

1st step. Preparation of the crude extract. All procedures connected with purification of the enzyme were carried out at approx. 4°. The wet mass of bacteria was ground up for about 2 hr. in a porcelain mortar with carborundum in the presence of 0.1 M-citrate-phosphate buffer, pH 7.0. After centrifuging at 15000 r.p.m. for 20 min. the sediment was washed twice with small portions of buffer. The pooled extracts were dialyzed overnight against distilled water, and then centrifuged at 1500 r.p.m. for 10 min. The sediment was discarded and the pinkish-colored solution was stored at -12°.

2nd step. Protamine fractionation. 1% aqueous solution of protamine sulphate (Light) — 75 mg. per 100 mg. nitrogen in the extract — was added slowly while mixing. The precipitate was centrifuged at 10 000 r.p.m. for 10 min. and discarded. The supernatant was then fractionated with ammonium sulphate.

3rd step. Ammonium sulphate fractionation. To each 100 ml. of solution obtained in step 2, 35 g. powdered ammonium sulphate was added and, after dissolving, the mixture was set aside for several hours at 0°. The precipitate was centrifuged and discarded; 20.5 g. ammonium sulphate were then added for each 100 ml. of the solution and kept overnight at 0°.

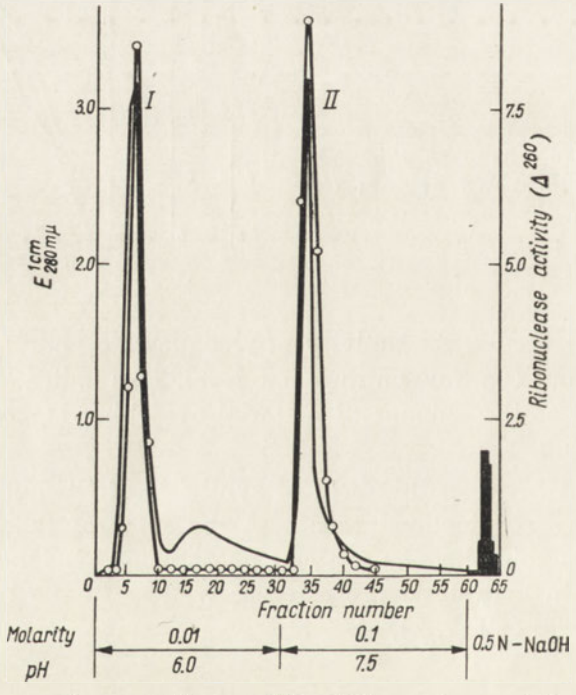

Fig. 3. Chromatographic separation on CM-cellulose column of the fraction obtained in step 3. The sample (250 E_{280} units) was dialyzed against 0.01 M-phosphate buffer pH 6.0, and adsorbed on a column (1.2 × 9.0 cm.) equilibrated with the same buffer. After washing the column with approx. 200 ml. of starting buffer, this was replaced by 0.1 M-phosphate of pH 7.5. The rate of elution was 45 ml./hr., fraction volume 4 ml. The continuous line denotes absorption at 280 mμ, (O), ribonuclease activity. The shaded area depicts the protein remaining on the column, which could be eluted with 0.5 N-NaOH.

After centrifuging at 15000 r.p.m. for 15 min., the supernatant was discarded. The precipitate was dissolved in a small volume of water and dialyzed against water for 24 hr.

4th step. Fractionation on CM-cellulose. The solution obtained in step 3 was next dialyzed against 0.01 M-sodium phosphate buffer, pH 6.0 [3] and transferred to a column previously equilibrated with the same buffer. After washing the column with approx. 200 ml. buffer solution, the former solution was replaced by 0.1 M-phosphate buffer of pH 7.5. The result of the separation is shown in Fig. 3. Two active peaks were obtained in conformity with the extinction of protein at 280 mμ. A small amount of protein remaining in the column could be eluted with 0.5 N-NaOH. A small protein peak emerging from the column in fraction 13 - 23 was pink coloured and contained cytochrome *s* [27]. On the basis of analysis of peaks *I* and *II* it was found that peak *I* is a mixture of at least three different enzymes: ribonuclease resistant to acids and elevated temperatures, nonspecific phosphodiesterase and 5′-nucleotidase not completely precipitated from the solution during fractionation with ammonium sulphate. The separation of the different enzymes in peak *I* will be reported in another paper. Peak *II* did not contain any appreciable amounts of diesterase or 5′-nucleotidase. The enzyme in this peak was submitted to further purification and called ribonuclease II.

Table 1

Isolation of ribonuclease II

Step No.	Fraction	Total E_{280} units	Total Δ^{260} units	Specific activity (Δ^{260}/E_{280})	Relative activity	Yield (%)
1	Crude extract	2170	900	0.42	1.00	100
2	Protamine	1720	880	0.51	1.23	98
3	Ammonium sulphate (0.55-0.85 satur.)	238	378	1.60	3.90	42
4	CM-cellulose	19.5	99	5.07	12.10	11
5	Heating (70°,5 min.)	4.6	71	15.50	37.40	8
6	Amberlite IRC-50 (peak *II-A*)	0.35	47	131.50	313.00	5

5th step. Heat denaturation. Ribonuclease II is resistant to elevated temperatures for prolonged periods of time, withstanding heating to 70° for 60 min. at pH 7.0 without appreciable loss of activity. The fractions of peak *II* were pooled, adjusted to pH 7.0 and heated to 70° for 5 min. After centrifuging, the precipitate was discarded. At this stage of purification the enzyme was concentrated about 40-fold (Table I). The subsequently described experiments were carried out with this preparation.

6th step. Fractionation on Amberlite IRC-50. The preparation obtained in step 5, concentrated by blowing the air and dialyzed overnight against 0.2 M-sodium phosphate buffer of pH 6.5 was adsorbed on a 0.9 × 30.0 cm. column of IRC-50 which had been previously equilibrated with the same buffer. Elution was carried out with 0.2 M-phosphate buffer, pH 6.5. Ribonuclease activity and intensity of the colour reaction with ninhydrin were determined in each fraction. The result of separation is shown in Fig. 4. Ribonuclease II was separated into two active peaks, *II-A* and *II-B*.

Table 1 contains data pertaining to typical purification of ribonuclease II, concentrated more than 300-fold in comparison with the activity of the crude extract.

Evidence for the multiple nature of ribonuclease of Th. thioparus

As can be seen from the results of purification of ribonuclease, at least three different heat-stable enzymes capable of depolimerizing RNA were indicated by fractionation on CM-cellulose and Amberlite IRC-50: ribonuclease I, II-A and II-B (cf. Figs. 3 and 4). The action of 0.25 N-H_2SO_4

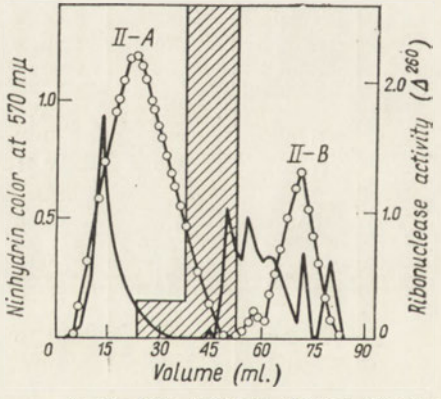

Fig. 4. Chromatography on Amberlite IRC-50 of the fraction obtained in step 5. The sample was poured on a column (0.9 × 30 cm.) equilibrated with 0.2 M-phosphate buffer, pH 6.5, and eluted with the same buffer at a rate of 20 ml./hr., volume of the fractions 0.75 ml. The continuous line denotes absorbancy of the color reaction with ninhydrin at 570 mμ; (O) shows ribonuclease activity. The dashed area shows the range of elution of crystalline pancreatic ribonuclease when 10 mg. of the enzyme was chromatographed in the same column under identical conditions

on enzyme *I* after separation on CM-cellulose of the buffer extract diminished the original nucleolytic activity by about one-half. The activity of fraction *II* in the same conditions was not changed. When the extract was prepared by grinding the cells with 0.25 N-H_2SO_4 at 0°, adjusted to pH 5.8, the precipitate removed by centrifugation, and the supernatant

separated on CM-cellulose, ribonuclease activity was found only in the fraction eluted with 0.1 M-buffer at pH 7.5, that is in peak *II*. If peak *I* from the buffer extract was rechromatographed after treatment with sulphuric acid, activity was obtained not in fraction *I* but in fraction *II* as shown in Fig. 5c. Obviously action of sulphuric acid on peak *I* inactivated diesterase, which is very unstable in acid medium [4] and the remaining activity, resistant to the low pH, belongs to ribonuclease. After treating with acid its adsorptive properties are changed and it is

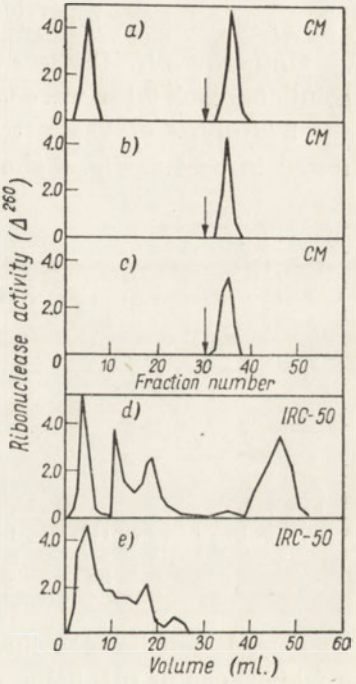

Fig. 5. Chromatography of buffer extract and extract obtained by means of 0.25 N-H_2SO_4. The buffer extract from the cells was prepared as described under "Purification". The acid extract was adjusted at 0° to pH 5.8 and the precipitate was centrifuged. Both extracts were dialyzed for 24 hrs. against water, concentrated about five-fold by blowing and then submitted to column chromatography. Segments (*a*), (*b*) and (*c*) represent separation on CM-cellulose under conditions as described in Fig. 3. (*a*), buffer extract; (*b*), acid extract; (*c*), rechromatography of fraction *I* from segment (*a*) after treatment with 0.25 N-H_2SO_4. Segments (*d*) and (*e*) represent fractionation of the buffer extract (*d*) and acid extract (*e*) on a column of IRC-50 under conditions as in Fig. 4. In all the diagrams the ribonuclease activity is presented by a continuous line. The arrows show the change of buffer solutions

eluted only by higher molarity of buffer solution and at higher pH. It is conceivable that acid splits off some association product or peptide fragment from the enzyme in fraction *I*, without affecting its activity, but alters its affinity for the adsorbent. Preliminary investigations showed that enzyme I possesses a different optimum pH as well as different optimum temperature of RNA hydrolysis as compared with enzyme II.

When the buffer extract and that obtained by means of sulphuric acid were subjected to analogous separation on Amberlite IRC-50, at least four active fractions were obtained from the buffer extract, but the

[4] Properties of phosphodiesterase isolated from the cells of *Th. thioparus* are described by one of us (W. O.) in separate paper, *Experientia*, 1961, in press.

fraction corresponding to peak *II-B* in Fig. 4 disappeared from the acid extract. The results of separation of the extracts on CM-cellulose and IRC-50 are shown diagrammatically in Fig. 5 *a-e*. The studies of other authors [14, 9, 8] pertaining to ribonucleases in animal tissues also indicate the presence of varying numbers of active fractions, depending on the method of extraction. This is probably connected with dissociation under the influence of acid products of association of ribonuclease with cytoplasmic proteins [9, 8].

Properties of purified ribonuclease II

Optimum pH. Citrate-phosphate, Tris-HCl and glycine-NaOH buffer solutions, each 0.1 M, were used to determine the optimum pH. Optimum pH for hydrolysis of yeast RNA, on the basis of three determinations, was found to be 8.5. Fig. 6 shows the effect of pH on the activity of the

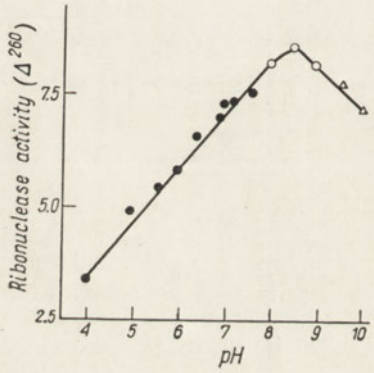

Fig. 6. Effect of pH on ribonuclease activity. Enzymatic activity was determined as described under "Methods" using a preparation of the enzyme with specific activity 15.5. (●), citrate-phosphate buffer; (O), tris-HCl buffer; (Δ), glycine-NaOH buffer

enzyme. It can be seen that the activity of enzyme II is maintained over a wide range of pH, being still appreciable at pH 4.0. If the crude extract was used as the source of the enzyme, three optima were obtained in the same conditions, at pH 5.5 - 6.0, 7.0, and 8.0 - 8.5.

Effect of temperature. Ribonuclease II heated to 70° at pH 7.0 for 60 min. retains its activity. Complete inactivation occurs only after heating to 100° for 30 min. The curve of the rate of hydrolysis of RNA in relation to temperature has a flat shape, with optimum hydrolysis between 40 - 50°. At 60° approx. 80% of the initial activity is still retained.

Effect of bivalent metal ions. Ribonuclease II is activated by Fe^{2+} ions at a concentration of 10^{-3} M (Table 2). The presence of versene (ethylenediaminetetraacetic acid) in the reaction mixture does not exert any effect on the activity of the enzyme. Other bivalent metals exhibit nonspecific action on the enzyme's activity first at higher concentra-

Table 2

Effect of Fe^{2+} ions on the activity of ribonuclease II

The reaction mixture contained 3 mg. yeast RNA in 0.5 ml. 0.1 M-triethanolamine. HCl-NaOH buffer, pH 7.0, enzyme II (specific activity 15.5) and Fe^{2+} in the form of Mohr's salt. The final volume was adjusted to 1.0 ml. with buffer solution. Incubation was carried out at 37° for 60 min.

Molarity of Fe^{2+}	Activity Δ'^{60}	Relative activity
0*	1.75	1.00
10^{-5}	1.71	1.00
10^{-4}	2.65	1.50
10^{-3}	5.32	3.04
10^{-2}	5.32	3.04
Versene (0.02 M)	1.70	0.97

* Enzyme before use was dialyzed against doubly distilled water for 24 hr.

tions (10^{-2} M), usually causing precipitation in the reaction mixture. Mn^{2+}, Cu^{2+}, Zn^{2+}, and Cd^{2+} in concentrations of 2×10^{-2} M diminished the activity of the enzyme by one-half to one-third. Mg^{2+}, Ca^{2+}, and Co^{2+} showed no effect on the enzymatic activity under the same conditions.

Table 3

Comparison of the activity of ribonuclease II in respect to different polynucleotides

The reaction mixture contained 8 mg. of each polynucleotide in 0.5 ml. of 0.1 M-citrate--phosphate buffer, pH 7.0, and 6.6 Δ^{260} units of ribonuclease II of specific activity 15.5. Final volume was 1 ml., and incubated at 37° for 60 min. Extinction of the acid soluble products of hydrolysis at 260 mμ was determined as described under "Methods".

Substrate	Relative activity
RNA (yeast)	1.00
RNA - Th	1.50
"Core"	0.40
Poly - A	0.04
DNA (thymus)	0.0

Specificity of ribonuclease II. Ribonuclease II digests yeast RNA, RNA-Th, "core" and Poly-A at various rates. RNA-Th is the most rapidly hydrolyzed, and Poly-A most slowly. Thymus DNA is completely resistant to the action of the enzyme. The synthetic diesters of phosphoric acid,

such as bis(*p*-nitrophenyl)-phosphate and the cyclic 2′ : 3′-phosphonucleosides, such as 2′ : 3′-uridylic acid also are not digested. Poly-A is hydrolyzed 25 times, and "core" 2.5 times more slowly than yeast RNA (Table 3).

In the products of hydrolysis no appreciable amounts of inorganic phosphate or nucleosides were detected. During hydrolysis of RNA about 25 - 50% of higher polynucleotides always remain (even after 72 hr. incubation), and can be precipitated with 70% ethanol, uranyl reagent or perchloric acid.

Separation on ECTEOLA-cellulose of hydrolysis products of RNA

Fifty mg. of yeast RNA in 8 ml. water was incubated for 48 hr. at 37° with 26.4 Δ^{260} units of ribonuclease II (specific activity 15.5); pH of the mixture was maintained at 8.5 by repeated additions of 0.1 N-NaOH.

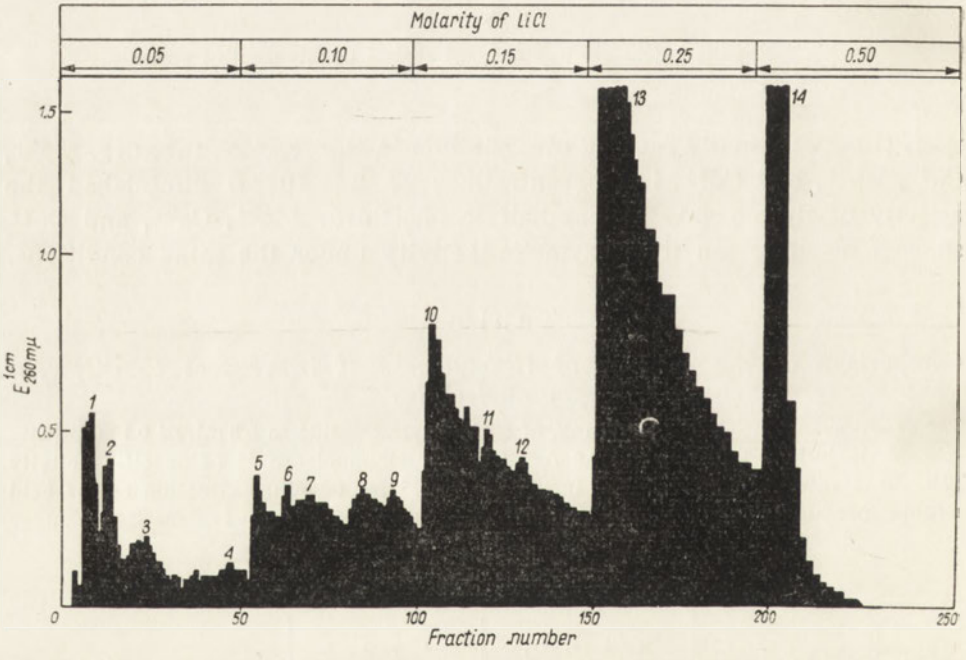

Fig. 7. Chromatographic separation on ECTEOLA-cellulose of the products of hydrolysis of yeast RNA obtained by the action of ribonuclease II. The column (1.9 × × 13.0 cm.) was prepared as described under "Methods". Stepwise elution was carried out by means of solutions of LiCl at concentrations shown in the upper part of the diagram. The rate of elution was 1.5 ml./min. fraction volume 10 ml. The absorption at 260 mμ was measured in each fraction

The solution was then adsorbed on a column and stepwise elution was carried out with LiCl solutions of increasing concentrations, as shown in Fig. 7. As can be seen, several fractions were obtained, and most of the

material absorbing at 260 mμ emerged from the column at 0.15 - 0.50 M concentration of LiCl. When 50 mg. RNA not subjected to enzymatic hydrolysis was fractionated in the same conditions, absorption at 260 mμ, not exceeding 0.2, was observed only in the range of 0.25 - 0.50 M-LiCl.

The fractions with highest extinction of the particular peaks were pooled and concentrated *in vacuo*. Lithium chloride was extracted with methanol [29] and the nucleotide sediment was dissolved in water. Preliminary experiments with paper chromatography in solvents 2, 3 and 4, and by means of paper electrophoresis indicate that all the fractions eluted from the column are chiefly mixtures of di- and trinucleotides. Fraction 1 contained 3′-cytidylic and 3′-uridylic acids in the proportion of 1 : 4. The total content of both mononucleotides in the hydrolyzate of RNA was about 2%. The pooled fractions were next submitted to hydrolysis in 12 N-$HClO_4$ during one hour at 100°. The solution was neutralized with KOH and after removing the precipitate by centrifugation, the hydrolysates were separated chromatographically in solvent system 1. After elution of the spots with 0.1 N-HCl, extinctions were determined and quantitative composition of bases in each fraction was calculated. Table 4 shows the results of the analysis. As can be seen, most of the fractions

Table 4

Quantitative analysis of the bases composition of chromatographic fractions from Fig. 7

Technical data in the text

Fraction No.	Guanine		Adenine		Cytosine		Uracil	
	(μmole)	(%)	(μmole)	(%)	(μmole)	(%)	(μmole)	(%)
1	—	—	—	—	0.005	20.2	0.018	79.8
2	—	—	—	—	0.022	28.4	0.054	71.6
3	0.036	27.2	0.039	29.3	—	—	0.057	43.5
4	—	—	0.015	21.7	—	—	0.055	78.3
5	0.064	30.1	0.063	29.9	0.055	26.0	0.029	14.0
6	0.020	17.2	0.031	26.8	0.032	27.2	0.033	28.7
7	0.031	15.8	0.076	38.3	0.047	23.8	0.044	22.2
8	0.048	25.7	—	—	—	—	0.138	74.3
9	0.098	40.1	0.049	20.0	—	—	0.097	39.9
10	0.062	19.5	0.057	17.6	0.057	17.6	0.147	45.5
11	0.036	12.6	0.046	16.1	0.037	12.9	0.167	53.4
12	0.031	20.4	0.049	32.1	—	—	0.072	47.1
13	0.166	33.8	0.139	28.6	0.183	37.6	—	—
14	0.165	12.5	0.362	27.0	—	—	0.812	60.5

display the presence of two or three bases. The fractions containing four bases, are probably mixtures of di- and trinucleotides, which can be concluded both from the results of chromatographic analysis in solvents 2 and 3, and from the percentage molar content of the different bases.

Although the sequences of oligonucleotides arising as hydrolysis products of RNA by ribonuclease II were not determined in the present study, the preliminary results shown in Fig. 7 and Table 4 suggest that the products belong to rather lower oligonucleotides containing 2 or 3 bases. In fractions containing purine and pyrimidine bases the former predominate quantitatively.

DISCUSSION

Intensive studies of the purification and properties of nucleolytic enzymes occuring in animal [25, 1, 2] and plant [10, 30] tissues and especially in microorganisms [7, 24, 5, 18, 22, 28] are being conducted at present in many laboratories. This is due to the possibility of making use of these enzymes in structural studies of polynucleotides participating in numerous biological cellular processes. The study of the occurence and properties of such enzymes in the cells of *Th. thioparus* possesses also a general biological aspect, in that this organism belongs to the obligative autotrophs, occupying a peculiar position in the evolution chain [4].

The results obtained in this paper indicate that extracts of *Th. thioparus* contain several ribonucleases which are resistant to acids and elevated temperature. Ribonucleases I, II-A and II-B appear to be endonucleases, liberating only slight amounts of mononucleotides. The ribopolynucleotides are hydrolyzed, whereas the deoxyribopolynucleotides are completely resistant to the action of these enzymes. Studies on the specificity of the different ribonucleases now under way already indicate that hydrolysis of RNA by each of them gives rise to different end-products, owing to different internucleotide bonds being split by each enzyme. Hence, a system of polyribonucleases in the cells of *Th. thioparus* is conceivable.

Ribonuclease II, as can be seen from Table 3, much more readily digests polynucleotides formed by both types of bases than those containing only the purine bases (Poly-A). Also, the products of hydrolysis of RNA subjected to prolonged action of the enzyme are chiefly of the type PyPy or PuPuPy (cf. Table 4). These facts suggest that the internucleotide secondary phosphate bonds most readily split by ribonuclease II are of the PyPy or PuPy type and not PuPu.

As a protein, ribonuclease II displays a number of properties similar to pancreatic ribonuclease, such as resistance to acids, thermostability and

range of precipitation by ammonium sulphate [17]. Adsorption on CM-cellulose of both enzymes is also characteristic [3]. The optimum pH of ribonuclease II lies somewhat higher than that of pancreatic enzyme. On the other hand, the optimum temperature of ribonuclease II is about 20° lower. A characteristic feature of ribonuclease II is its activation by Fe^{2+} ions during RNA hydrolysis.

Ribonuclease II may therefore be classified among the acid-resistant, thermostable, specific endonucleases causing hydrolysis of phosphodiester internucleotide bonds within the ribopolynucleotide chain and leading to liberation of probably 3′-phosphooligonucleotides composed of pyrimidine and purine-pyrimidine nucleotides in sequence. Studies of the further purification of ribonucleases from *Th. thioparus* and their characterization are under way.

SUMMARY

The presence of at least three ribonucleases hydrolyzing RNA to oligonucleotides in an extract of the cells of *Th. thioparus* was demonstrated. The fractionation of cell extract with protamine and ammonium sulphate and by chromatography on CM-cellulose and Amberlite IRC-50 resulted in a considerable degree of purification (more than 300-fold) of ribonuclease, which was resistant to elevated temperature and acid reaction. The optimum pH of isolated ribonuclease is 8.5, optimum temperature of hydrolysis of RNA approximately 45°, and the enzyme is activated by Fe^{2+} ions. The enzyme hydrolyzes yeast RNA, RNA isolated from the same cells of microorganism, and to a lesser degree the "core" of yeast RNA. Synthetic Poly-A is digested about 25 times more slowly as compared with RNA. The enzyme does not digest deoxyribopolynucleotides. Preliminary studies of the specificity of the enzyme indicate that linkages between pyrimidine and purine-pyrimidine nucleotides are more easily hydrolyzed than those between purine nucleotides. The main products of hydrolysis of RNA are oligonucleotides built of 2 or 3 nucleotides.

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ENZYMY NUKLEOLITYCZNE *TH. THIOPARUS*. OCZYSZCZANIE I WŁASNOŚCI RYBONUKLEAZY

Streszczenie

W ekstrakcie komórek *Th. thioparus* wykazano obecność conajmniej trzech rybonukleaz powodujących hydrolizę RNA do oligonukleotydów. Przez frakcjonowanie ekstraktu komórek siarczanem protaminy, siarczanem amonu, przez chromatografię na CM-celulozie i na Amberlicie IRC-50 uzyskano znaczny stopień oczyszczenia (ponad 300-krotny) jednej z rybonukleaz, odpornej na ogrzewanie w wyższej temperaturze i na działanie niskiego pH. Enzym posiada optimum pH przy 8,5, optimum temperatury hydrolizy RNA ok. 45° i jest aktywowany przez jony Fe^{2+}. Enzym powoduje hydrolizę RNA drożdżowego, RNA wyosobnionego z tych samych komórek oraz w mniejszym stopniu "core" z RNA drożdżowego. Ok. 25 razy wolniej w porównaniu z RNA trawi syntetyczny kwas poliadenilowy. Wyosobniona rybonukleaza nie trawi desoksyrybopolinukleotydów. Wstępne studia nad specyficznością enzymu wskazują, że łatwiej hydrolizuje wiązania między nukleotydami pirymidynowymi i puryno-pirymidynowymi niż między nukleotydami purynowymi. Głównymi produktami hydrolizy RNA są oligonukleotydy zbudowane z dwu lub trzech nukleotydów.

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SYNTHESIS AND PHYSICO-CHEMICAL AND ENZYMATIC PROPERTIES OF 5-BROMO DERIVATIVES OF URIDINE PHOSPHATES AND THEIR POLYMERS

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In a continuation of previous studies on the physico-chemical and enzymatic properties of N-methyluridylic acid and its polymers (MeUp and poly-MeU) [31, 32], we have examined the effect of bromination of the 5 position in the uracil ring. It has been shown by a number of observers [9, 33, 36] that 5-bromouracil and 5-bromouridine may be incorporated into the nucleic acids of certain microorganisms and mammalian cells. Furthermore d-BrUTP has been found to act as a substrate for deoxynucleotide polymerase [5], a fact which has been interpreted as supporting evidence for the role of this enzyme in DNA synthesis *in vivo*. On the other hand, it has been stated that BrUDP is not a substrate for polynucleotide phosphorylase [23]. It seemed worth while to check this finding, which we have done. Since it proved impossible to obtain high molecular weight polymers by enzymatic methods, it was decided to apply chemical procedures [20, 21, 22, 31] to obtain appropriate oligonucleotides of 5-bromouridylic acid (I) and N-methyl-5-bromouridylic acid (V) with a view to examining the properties of such polymers, as well as their behaviour towards enzymes attacking nucleic acids and their derivatives. It was also considered of value to investigate the ability of oligo-BrU to complex with poly-A. It would obviously have been more satisfactory to examine complex formation between poly-BrU and poly-A; since, however, the former cannot be prepared, the use of the oligonucleotide provides the only means of estimating the effect of bromination on formation of twin strand complexes. Such twin strand complexes obviously exist *in vivo* in view of the known incorporation of bromouracil into DNA. Complex formation between poly-A and oligo-U has already been reported upon [32, cf. 16].

5-bromouridine-2′(3′)-phosphate (I) was obtained by the action of N-bromosuccinimide on the tri-*n*-octylamine salt of uridylic acid as described by Michelson [8] for the bromination of UMP. 5-bromouridine-2′ : 3′-cyclic phosphate (II) was prepared from I by the action of dicyclohexylcarbodiimide (DCC) in dry dimethylformamide [28]. Compound II was found to be susceptible to pancreatic ribonuclease, so that substitution of the position 5 by bromine does not affect the enzyme specificity. In view of the previous observation that N-methylation of uracil ribotides effectively blocks against the action of RNase [31, 32], N-methyl-5-bromouridine-2′(3′)-phosphate (V) was prepared from I by the method previously used for the synthesis of N-methyluridylic acid [31]; this involves methylation of the cyclic phosphate with diazomethane (III), followed by opening of the cyclic phosphate ring and hydrolysis of the resultant methyl ester (IV). This procedure was found to be applicable also to the 5-bromo compounds, with the exception that alkaline conditions could not be employed to hydrolyze the methyl esters of the latter, due to the fact that compounds III, IV and V are extremely alkali labile even at pH 10 due to opening of the pyrimidine ring. At pH 12.5 the half-time for this reaction is about 60 mins., and is therefore much more rapid than for the corresponding N, N′ substituted uracils [26, 27, 12]; the reaction is also irreversible. Consequently, the hydrolysis IV → V was carried out at pH 1 and 37°. Compound V was also obtained by the action of diazomethane on 5-bromouridine-2′(3′)-phosphate (I), followed by hydrolysis of the resulting triester [1]. However, this hydrolysis is both tedious and inconvenient, and we have not used it on a preparative scale. As was to be anticipated, the cyclic phosphate of N-methyl-5-bromouridine was completely resistant to RNase.

Oligo-MeBrU (VI) was obtained according to the procedure of Michelson [21, cf. 31] but, contrary to expectations, polymerization was not quantitative as is the case for uridylic acid and N-methyluridylic acid [21, 31]. The resulting oligonucleotide was fractionated by dialysis against water and then 2 M-NaCl. The dialyzate and dialysis residue, from dialysis against 2 M-NaCl, and of mean chain lengths 3.2 and 7.1, respectively, were used for enzymatic and physico-chemical tests. Both fractions were completely resistant to RNase, confirming once more the blocking action on RNase of 3N-methylation.

If we examine the effect of substitution in the pyrimidine ring on the action of RNase, we find that substitution of the 5-position by $-CH_3$ [11] or —Br is without influence, as is reduction of the 5, 6 double bond

[1] Such a procedure has been used to prepare N-methyluridylic acid (A. M. Michelson, private communication).

[12]; but 3N-methylation blocks the enzyme. It has been suggested that the number 3N position is a point of attachment for the enzyme [7] but this may be an oversimplification. It should be recalled that 5-ribosyluracil -2′ : 3′-phosphate [2] [22] and poly-(5-ribosyluracil) [2] are likewise substrates for RNase. It would therefore be of interest to see whether N-methylation would render these substrates resistant to the enzyme. The question of RNase specificity is further complicated by the fact that the enzyme will slowly degrade poly-ribosephosphate [35] and, in high concentrations, poly-A [4].

Snake venom phosphodiesterase *very slowly* hydrolyzes VI to mononucleotides. The apparent resistance of VI is in this instance probably due to two factors: (a) the presence in the oligonucleotide of a mixture of 2′, 5′ and 3′, 5′ internucleotide bonds, and (b) the fact that we are dealing with short chains terminated by 3′ (2′)-phosphate [24].

Kidney alkaline phosphatase readily dephosphorylates all the above compounds and, following extended incubation, hydrolyzes the internucleotide linkages of VI, indicating the presence in this enzyme preparation of a non-specific phosphodiesterase [cf. 31]. On the other hand, prostate phosphomonoesterase removes only the end phosphate groups in VI, at acid pH, and was consequently suitable for determinations of chain length.

As mentioned in the introduction, it had been hoped to synthesize high molecular weight polymers of BrU and MeBrU. To this end 5-bromouridine-5′-pyrophosphate (BrUDP, VII) and MeBrUDP (VIII) were prepared as substrates for polynucleotide phosphorylase, by bromination of the tri-*n*-octylamine salts with N-bromosuccinimide at room temperature, as above. Anhydrous conditions were found to be of considerable importance in this case, even traces of water resulting in partial hydrolysis of the products to the 5′-monophosphates. Both VII and VIII were quantitatively transformed by snake venom to the corresponding nucleosides.

Attempts to obtain polymers from VII and VIII with polynucleotide phosphorylase were unsuccessful [cf. 23] and, even when a mixture of VII with UDP (1 : 1) was used as a substrate, only poly-U was formed; spectral observations and hydrolysis followed by chromatography demonstrated the total absence of bromouridylic acid in the poly-U. Compound VII was, however, found to appreciably inhibit the formation of poly-U,

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[2] This compound, named "pseudouridylic acid" by Cohn [6] and Michelson [22], is perhaps not the most fortunate choice since there may also exist a 6-ribosyluracil derivative, as well as a 3-β-ribosyluracil [25].

indicating some reaction with the enzyme[3]. It is consequently of interest to note that while MeUDP [32] and 2-thio-UDP [14] will undergo polymerization, as will likewise 5-methyl-UDP [11], 5-Br-UDP will not. Furthermore 5,6-dihydro-UDP is not polymerized by polynucleotide phosphorylase [23, 32].

Although it is at the moment rather difficult to draw any particular conclusions with regard to the specificity of polynucleotide phosphorylase (bearing in mind the fact that it will likewise polymerize purine nucleoside pyrophosphates), it would appear that enzyme specificity is not in this instance related to any ability of the polymer to form internal hydrogen bonds as is the case for deoxynucleotide polymerase [13]. It is, of course, to be borne in mind that the mechanism of polymer formation is not quite the same for the two enzymes: polynucleotide phosphorylase does not polymerize *via* a "matrix" [13] but rather through a „primer"

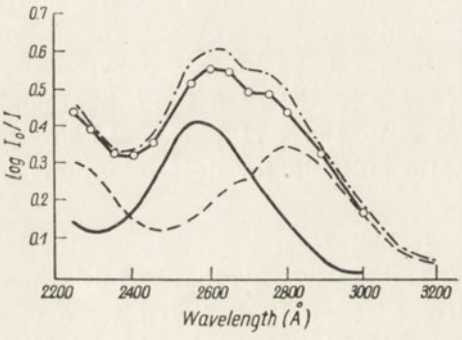

Fig. 1. Spectral evidence for complex formation between poly-A and oligo-BrU: ——poly-A; - - - oligo-BrU; —.—, additive absorption of poly-A and oligo-BrU; O, observed absorption of poly-A and oligo-BrU. molar ratio 1 : 1, at 9° under standard conditions in 0.15 M-NaCl + 0.015 M-sodium citrate, pH 6.7

[29]; nonetheless the foregoing suggestion retains some validity in view of the undoubted existence in RNA of secondary structure. Finally, from the point of view of specificity, it would be of interest to synthesize and examine the behaviour of 5-ribosyl-UDP as a potential substrate.

In view of the negative results with BrUDP as substrate, additional experiments were limited to the use of oligo-BrU and oligo-MeBrU. Both oligonucleotides, with mean chain lengths of 7 or more, were quantitatively hydrolyzed in 1 N-HCl overnight to mononucleotides. The resulting hyperchromicity was 11.5% for the former and 13.3% for the latter. The small difference between these two once more supports the previous suggestion [19, 31] that the hyperchromicity of such oligonucleotides is not

[3] Since completion of the above work, a report has appeared by Barker *et al.* [3], in which it was also found that BrUDP is not polymerized by polynucleotide phosphorylase. The authors report, however, that an exchange of phosphorus with the substrate does occur, in agreement with our observation of the inhibitory effect of BrUDP on polymerization of UDP.

due to internal hydrogen bonding but is rather the result of mutual interaction between adjacent pairs of residues in the chains. This is further supported by the fact that, in contrast to poly-U [15, 32], neither oligonucleotide exhibited any evidence of secondary structure in the presence of 0.05 M-$MgCl_2$ at temperatures down to 3°.

Oligo-MeBrU (VI) showed no evidence of formation of a twin strand complex with poly-A either under standard conditions [34, 8] or upon increase in ionic strength of the solution, or on reduction of the temperature to 5°. This again confirms the previous suggestion [32] that it is sufficient to block one of the two hydrogen bonds between the base pair U-A to prevent twin-strand complex formation.

On the other hand, control experiments demonstrated that oligo-BrU will form complexes with poly-A. The maximum hyperchromicity of such a complex, at 2650 Å, was 13% at a 1 : 1 ratio of A : BrU (Fig. 1), and is therefore quite similar to that for complex formation between oligo-U and poly-A [32]. This confirms once more that 2′, 5′ internucleotide linkages do not hinder complex formation and, more interesting still, that such complex formation is practically unaffected by substitution of bromine in the 5-position of the uracil ring. It seems reasonable to conclude that in more highly polymerized chains BrU may replace U without any marked change in secondary structure of the chain.

EXPERIMENTAL

5-bromouridine-2′ (3′)-phosphate (I) and 5-bromouridine-2′ : 3′-phosphate (II). Compound I was obtained in 80% yield by the action of N-bromosuccinimide at room temperature on the anhydrous tri-*n*-octylamine salt of uridine-2′ (3′)-phosphate in dry dioxane, as described for the 5′ isomer [18, 30].

To 50 mg. of I, dissolved in 2 ml. dimethylformamide, was added 180 mg. DCC; following 1 hour paper chromatography demonstrated the absence of I and appearance of the cyclic phosphate [28]. Solvent was removed under reduced pressure (at a temperature not exceeding 30°), 3 ml. water added to the residue, followed by filtration and washing of the precipitated dicyclohexylurea. The filtrate was concentrated to 1 ml., carefully brought to pH 8.5 with ammonia, extracted twice with 3 ml. ether and an excess of ethanolic $CaCl_2$ and two volumes ethanol were then added. The precipitated calcium salt of II was collected by centrifugation and washed with ethanol and ether; it was chromatographically homogeneous in solvents A and B.

N-methyl-5-bromouridine-2′ (3′)-phosphate (V) : 400 mg. (1 mM) of I, dried over P_2O_5, was dissolved in 10 ml. dry dimethylformamide and

1.5 gm. (7.3 mM) DCC added. After 1 hour at room temperature, crystals of dicyclohexylurea appear and chromatography shows the absence of I and appearance of II. The reaction vessel was then immersed in ice-water and the reaction mixture saturated with gaseous diazomethane (an ether solution of CH_2N_2 from 5.15 gms. N-methylnitrosourea [2], warmed under reflux and the resulting gas dried over granulated NaOH prior to passing into the reaction mixture). The course of the reaction was followed by removing aliquots at 10 - 15 minute intervals and measuring optical densities at 2750 Å, at pH 7 and 12. The reaction is complete after about 40 mins. Chromatography in solvents A and B show the absence of II and quantitative formation of N-methyl-5-bromouridine-2′ : 3′-methylphosphate (III). About 10 ml. water was then added and the precipitated dicyclohexylurea filtered off and washed with water. The filtrate was evaporated to dryness under reduced pressure (temp. 30°) and the residue dissolved in 20 ml. 0.1 N-HCl and left 8 hrs. at room temperature. Chromatography in solvents A and B now showed that over 90% of III underwent decyclization to N-methyl-5-bromouridine-2′ (3′)-methylphosphate (IV). The solution was cooled to 0°, acidified to pH 0.5 with 5 N-HCl and left overnight at 37°, following which chromatography in solvents A and B demonstrated the disappearance of IV and the formation of N-methyl-5-bromouridine-2′ (3′)-phosphate (V). The solution was then brought to pH 3.5 at 0° by careful addition of 1 N-NaOH, concentrated under reduced pressure to about 5 ml., and water then removed by azeotropic distillation with benzene and ethanol. The precipitated crystals of NaCl were washed with ethanol, the filtrates concentrated to 5 ml., following which was added 25 µl. triethylamine and then an excess of alcoholic $CaCl_2$. The resulting precipitate was collected by centrifugation, washed with ethanol and ether, again dissolved in the minimum volume of water (with addition of a drop of 1 N-HCl to assist in complete dissolution) and precipitated with two volumes ethanol. The resulting calcium salt of V was washed with aqueous ethanol (1 : 1), ethanol and ether and dried at 110° under reduced pressure. Yield 280 mg. (61.2% theoretical).

Calculated for $C_{10}H_{12}O_9N_2PBrCa$: P,6.81%; N, 6.15%
Determined: P, 6.5%; N, 5.82%

$$\lambda_{max} - 2775\ Å; \frac{\varepsilon_{2800}}{\varepsilon_{2600}} = 1.51; \frac{\varepsilon_{2600}}{\varepsilon_{2500}} = 1.65.$$

A product identical with V was also obtained by the direct action of diazomethane on a dry dioxane solution of I and hydrolysis of the resulting N-methyl-5-bromouridine-2′ (3′)-dimethylphosphate in 1 N-HCl for 60 hrs. at 37°.

Behaviour of V towards acid and alkali: In 1 N-HCl for 12 hrs. at 60°, V is stable. In 0.5 N-KOH it is completely decomposed in 30 mins. at room temperature and this is not reversible by acidification. The half-time for decomposition at pH 12.5, measured by the rate of decrease of the absorption maximum at 2775 Å, is 60 mins. The products of decomposition in alkali exhibit no absorption in the ultraviolet.

Oligonucleotides of N-methyl-5-bromouridine-2′ (3′)-phosphate (oligo--MeBrU, VI): 110 mg. (0.25 mM) of the calcium salt of V was dissolved in 6 ml. water and calcium removed by passing the solution through a column of Amberlite IR-120 (H^+ form, 1.5 × 5 cm.); the column was washed with two volumes of water and the combined filtrates concentrated under reduced pressure, following which water was removed by azeotropic distillation with ethanol and benzene. To the solution was added 100 µl. (0.25 mM) tri-*n*-octylamine, following which it was dried by several distillations with dry benzene and toluene and the residue stored over P_2O_5 overnight. It was then dissolved in 1.5 ml. dry dioxane, followed by the addition of 80 µl. diphenylphosphorochloridate (DPPC) and 150 µl tri-*n*-butylamine. After 5 hrs. at room temperature chromatography in solvents A and B showed that the main product (about 90% by visual observation) was the cyclic phosphate. An additional 80 µl. DPPC and 150 µl. tri-*n*-octylamine were added, and the solution left overnight. Chromatography then showed that polymerization had taken place. A 100 µl. aliquot was then acidified to 0.1 N-HCl and left at room temperature for 6 hrs., following which chromatography exhibited the presence of oligonucleotides with R_F values below that for V (from 0 to 0.22), as well as some unreacted V. Solvent was then removed under reduced pressure, the residue washed twice with ether by decantation and then dissolved in 2.5 ml. water. The aqueous solution was brought to pH 8.5 with 1 N-ammonia and extracted three times with 10 ml. ether. Two volumes of ethanol were then added, followed by the dropwise addition, at 0°, of saturated ethanolic $CaCl_2$. Centrifugation and washing produced 40 mg. of oligonucleotide, VI contaminated with 8% V, as determined by elution from paper chromatograms.

Dialysis of oligonucleotides (VI) and chain lengths of individual fractions. About 3 mg. of VI was dissolved in 0.6 ml. water at pH 5.5 and dialyzed against 20 ml. water for 48 hrs., then against 2 M-NaCl at 0°, and finally against water to remove salt. Mean chain lengths were estimated from terminal phosphate liberated by prostate phosphomonoesterase. The contents of the various fractions and mean chain lengths are presented in Table 1. No attempt was made to measure the chain length of the H_2O dialyzate since it contained also monomers.

Table 1

Mean chain lengths of fraction of oligo-MeBrU

	Dialyzate against water	Dialyzate against 2 M-NaCl	Dialysis residue
Oligonucleotide content	37.2%	56.5%	6.3%
Mean chain length (no. of residues)	—	3.2	7.1

Acid hydrolysis of VI in 1 N-HCl at 37° overnight resulted in quantitative conversion to mononucleotides.

Acid hyperchromicity of VI: A sample of oligonucleotide was dissolved in 1 N-HCl and the optical density at 2775 Å measured immediately. After 16 hrs. at 37° the increase in extinction, for a sample with a mean chain length of 7.1, was 11.5%.

In an analogous experiment the hyperchromicity of a sample of oligo--BrU (kindly supplied by Dr. A. M. Michelson) was found to be 13.3%.

Complex formation of oligo-BrU, and oligo-MeBrU (VI), with poly-A: It was first established that neither oligonucleotide exhibited secondary structure by showing that they exhibited no change in absorption at temperatures down to 3°, even when the solutions were made 0.05 M in $MgCl_2$ [10, 15].

Under standard conditions, as described by Warner [34] and Doty *et al.* [8], poly-A was titrated with oligo-BrU at 10° in 0.15 M-NaCl + + 0.015 M-sodium citrate at pH 6.7. The complex obtained exhibited maximum hyperchromicity of 13% at a molar ratio of BrU to A of 1 : 1 (Fig. 1) and dissociated over the temperature range 14 - 39° with a T_m of 24°. A repetition of the foregoing, using oligo-MeBrU (mean chain length 7.1 residues) in place of oligo-BrU gave a mixture, the extinction of which was always the arithmetic sum of poly-A and oligo-MeBrU, even when the salt concentration was raised to 0.5 M.

5-bromouridine-5'-pyrophosphate (BrUDP, VII): 112 mg. (0.25 mM) of the calcium salt of UDP was dissolved in water, calcium removed by passing through a column of Amberlite IR-120 (H^+ form) which was then washed with water and the combined percolates concentrated under reduced pressure and water removed by azeotropic distillation with ethanol and benzene. To the ethanolic solution was added 176 mg. (0.5 mM) tri-*n*-octylamine and drying was then carried out by successive distillation with dry benzene, toluene and dioxane. The resultant salt was dried overnight over P_2O_5 and dissolved in 10 ml. dry dioxane, to which was added 176 mg. (1 mM) N-bromosuccinimide. The reaction mixture was left for 5 days at room temperature, following which chromatography

in solvent B showed that the main reaction product was VII, accompanied by traces of the starting compound and about 10% of BrUMP. Solvent was removed under reduced pressure, the residue dissolved in 5 ml. aqueous ethanol (1 : 2) and triethylamine added to bring the pH to 5. The calcium salt was then precipitated by addition of ethanolic $CaCl_2$ and two volumes ethanol. The crude calcium salt, 134 mg. after washing and drying, was dissolved in 3 ml. water, acidified to pH 2.5 with 1 N-HCl and 7 ml. ethanol then added. The precipitated acid salt of BrUDP was collected by centrifugation, washed and dried over P_2O_5. Yield 72 mg. (54.5% theor.), chromatographically homogeneous in solvent B and in propan-2-ol-1% $(NH_4)_2SO_4$ (60 : 40, v/v) [1].

Calculated for $C_9H_{11}O_{12}N_2P_2BrCa$: N, 5.36%; P, 11.81%,
Determined: N, 5.57%; P, 11.6%.

N-methyl-5-bromouridine-5'-pyrophosphate (MeBrUDP, VIII): The procedure was identical to that for VII described above. From 120 mg. (0.25 mM) MeUDP [32] the yield of VIII was 85 mg. (60.7% theor.) and was chromatographically homogeneous in solvent B and in propan-2-ol - 1% $(NH_4)_2SO_4$ (60 : 40, v/v) [1].

Calculated for $C_{10}H_{13}O_{12}N_2P_2BrCa$: N, 5.25%; P, 11.6%;
Determined: N, 5.32%; P, 11.4%.

Attempts to polymerize BrUDP: 12 mg. of the calcium salt of VII in 0.5 ml. water was passed through an IR-120 (H^+ form) column to remove calcium. The solution was brought to pH 7.6 with NaOH and reduced to 0.1 ml., followed by the addition of 0.1 ml. 0.2 M-tris buffer, pH 8.1, 2 µl. M-$MgCl_2$ and 20 µl. polynucleotide phosphorylase (the enzyme was a gift from Dr. S. Ochoa). No polymer formation could be observed even after 18 hrs. incubation.

In another experiment a 1 : 1 mixture of UDP and BrUDP was submitted to the action of the enzyme. The polymer obtained was exhaustively dialyzed against 0.005 M-NaCl and then hydrolyzed in 1 N-HCl for 16 hrs. at 37°. Paper chromatography of the hydrolyzate demonstrated the presence only of uridylic acid; no bromouridylic acid could be detected. However, the yield of poly-U was only 20% of that to be expected in the absence of BrUDP under analogous conditions, indicating that BrUDP does compete with the enzyme for its natural substrate, UDP.

Enzymatic trials

Substrates were prepared at a concentration of 10 mg/ml. in appropriate 0.1 M-buffers and enzyme added to a concentration of 1 mg./ml. except where otherwise indicated. Incubation was at 37° and samples

Table 2

R_F values for various compounds

Paper chromatography: The following solvent systems were used in ascending chromatography with Whatman paper No. 1:

A — propan-2-ol - NH_4OH (d - 0.88) - H_2O (70 : 1 : 30, v/v/v)
B — ethanol - 1 M-ammonium acetate - (5 : 2, v/v)
C — *n*-butanol - glacial acetic acid - H_2O (5 : 2 : 3, v/v/v).

Compound	R_F in solvent		
	A	B	C
Uridine	0.58	0.75	0.54
Uridine-2′(3′)-phosphate	0.22	0.25	0.26
Uridine-5′-pyrophosphate	—	0.10	—
5-bromouridine	0.69	0.82	0.59
5-bromouridine-2′(3′)-phosphate (I)	0.24	0.28	0.35
5-bromouridine-2′ : 3′-phosphate (II)	0.42	0.62	—
N-methyl-5-bromouridine-2′ : 3′-methyl phosphate (III)	0.68	0.88	—
N-methyl-5-bromouridine-2′(3′) - methyl phosphate (IV)	0.47	0.58	—
N-methyl-5-bromouridine-2′(3′) - phosphate (V)	0.34	0.38	0.42
Oligo BrU	0.0	0.0	—
Oligo-MeBrU			
Dialyzate vs. 2 M-NaCl	0.1—0.15	0.12—0.16	—
Dialysis residue	0.0	0.0	—
5-bromouridine-5′-pyrophosphate (VII)	—	0.14	—
N-methyl-5-bromouridine-5′-pyrophosphate (VIII)	—	0.18	—

withdrawn at various time intervals for chromatographic analysis. Insofar as oligo-BrU is concerned, samples used had a mean chain length of 3.1 residues.

(*a*) *Pancreatic ribonuclease* (Armour). Incubation at pH 7.5, in acetate buffer. N-methyl-5-bromouridine-2′ : 3′-phosphate (III), the methyl esters of III and IV, and oligo-MeBrU (VI) were all resistant to the enzyme after 40 hrs. incubation. On the other hand, 5-bromouridine-2′ : 3′-phosphate (II) was quantitatively converted to the mononucleotide (I) in 2 hrs.; this reaction could be followed not only chromatographically but also spectrally since λ_{max} I is at 2750 Å while λ_{max} II is at 2775 Å.

(*b*) *Snake venom* (*Crotalus adamanteus*). Incubation at pH 9.2 in borate buffer in presence of $MgCl_2$. Both VII and VIII were quantitatively converted to the corresponding nucleosides in 1 hr.

(*c*) *Snake venom phosphodiesterase* (prepared in this laboratory by Mrs. F. Rzendowska by fractionation on a cellulose column). Incubation

at pH 8.8 in presence of magnesium. Oligo-MeBrU (VI) was slowly hydrolyzed, mononucleotides making their appearance after 20 hrs. incubation.

(*d*) *Alkaline phosphatase* (Worthington). Incubation at pH 8.7 in borate buffer; 5-bromouridine-2′(3′)-phosphate (I) and the N-methylated derivative (V) were transformed to the corresponding nucleosides in 1 hr. Incubation of the oligonucleotide VI led to the appearance of smaller fragments, not containing end phosphate groups, after about 2 hrs.; after 6 hrs. there were traces of N-methyl-5-bromouridine, and this latter was the sole product after 20 hrs. incubation.

(*e*) *Prostate phosphomonoesterase* (prepared according to Loring *et al.* [17]). Incubation in acetate buffer at pH 5. Compounds I and V underwent dephosphorylation to the corresponding nucleosides in 1 hr. The oligonucleotide VI was slowly hydrolyzed to corresponding fragments without end phosphate groups. No N-methyl-5-bromouridine could be detected even after 30 hrs. incubation.

SUMMARY

1. Several 5-brominated derivatives of uridine phosphates have been prepared.

2. 5-bromo-uridine-2′ : 3′-phosphate is a substrate for ribonuclease but its N-methylated analogue is not. Oligo-BrU is also attacked by ribonuclease but oligo-MeBrU is resistant. The latter polymer is, however, slowly hydrolyzed by snake venom phosphodiesterase.

3. Oligo-BrU is capable of forming a twin-stranded complex with poly-A, but N-methylation of the brominated polymer prevents the formation of such complexes.

4. The hyperchromicities of oligo-BrU and oligo-MeBrU are approximately equal, testifying to the fact that such hyperchromicity is due, not to hydrogen bonding, but to interaction between adjacent rings in the chain.

5. Neither 5-BrUDP nor N-methyl-5-BrUDP are substrates for polynucleotide phosphorylase but 5-BrUDP partially inhibits the enzyme.

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SYNTEZA ORAZ WŁAŚCIWOŚCI FIZYKO-CHEMICZNE I ENZYMATYCZNE 5-BROMOPOCHODNYCH KWASU URYDYLOWEGO I JEGO POLIMERÓW

Streszczenie

1. Otrzymano pochodne fosforanów urydyny, bromowane w pozycji 5 pierścienia uracylu.

2. 2′ : 3′-fosforan (cykliczny) 5-bromourydyny stanowi substrat dla rybonukleazy z trzustki, a jego N-metylowy analog jest odporny na działanie rybonukleazy. Oligo-BrU ulega również hydrolizie pod wpływem rybonukleazy, natomiast oligo-MeBrU jest odporny na jej działanie, ale jest powoli hydrolizowany przez fosfodwuesterazę jadu węża.

3. Oligo-BrU tworzy podwójny kompleks z poli-A, natomiast N-metylowanie bromowanego polimeru uniemożliwia utworzenie takiego kompleksu.

4. Efekty hiperchromowe oligo-BrU i oligo-MeBrU są jednakowego rzędu. Potwierdza to wniosek, że tego rodzaju hiperchromazja spowodowana jest współdziałaniem sąsiednich pierścieni zasad, bez wytworzenia pomiędzy nimi wiązań wodorowych.

5. Ani 5-BrUDP, ani N-metylo-5Br-UDP nie są substratami dla fosforylazy polinukleotydowej; 5-BrUDP hamuje częściowo działanie enzymu.

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METABOLISM OF ^{14}C-L-CARBAMYLASPARTIC ACID TO PYRIMIDINE DERIVATIVES IN EXCISED WHEAT BLADES

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Recent investigations into the biosynthesis of pyrimidine nucleotides in animal organs and microorganisms have established the enzymatic system as well as the main intermediate compounds that participate in this process [18, 3]. On plant material however only rudimentary work has been carried out thus far [19, 4]. Preliminary results indicate that on the whole the path of pyrimidine nucleotide synthesis in the plant is similar to that in other organisms, yet certain distinct differences were also established. It was previously observed that plants fed with CA[1] accumulate large amounts of OA and some uridine [19]. Feeding plants with OA leads to the synthesis of 5′-UMP, 5′-CMP, uracil and uridine [4]. These last two mentioned substances do not appear on the main path of pyrimidine nucleotide synthesis in animal organs and microorganisms.

An attempt has been made to establish the biosynthetic sequence in which the particular pyrimidine derivatives may arise from a common aliphatic precursor in higher plants. With this in view plants were fed with ^{14}C-CA labelled in the carbamyl group and samples were withdrawn for analysis in a dynamic experiment after 1, 2, 4 and 8 hours incubation.

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[1] Following abbreviations were used throughout: CA, L-carbamylaspartic acid; OA, orotic acid; OMP, orotidine-5′-phosphate; 5′-UMP, uridine-5′-phosphate; 2′(3′)-UMP, uridine-2′(3′)-phosphate; 5′-CMP, cytidine-5′-phosphate; 2′(3′)-CMP, cytidine-2′(3′)-phosphate.

MATERIALS AND METHODS

Reagents

CA was synthesised from L-aspartic acid and potassium cyanate, using the method of Nyc & Mitchell [17].

Radioactive CA (specific activity: 1000 counts/sec./μmol.) labelled in the carbamyl group was synthesised as above, using $Na^{14}CNO$ in place KCNO. $Na^{14}CNO$ was made from $Na^{14}CN$ by oxidation [16].

All other reagents were of commercial origin.

Introduction of CA into the plant

Feeding experiments were carried out on 6 days old wheat blades (ca 7 cm. in length), variety Dańkowska 40.

In the first experiment 2 g. of excised blades were immersed with the cut off ends in 1 ml. of a 33 mM solution of CA with specific activity of 1000 counts/sec./μmol. After 1, 2, 4 and 8 hr. the blades were treated as described previously [19].

In the second experiment four samples were fed with ^{14}C-CA as above. After 4 hr. of incubation, the four samples were treated as follows: the first sample without further treatment was prepared for analysis, the second one was continuously fed for 4 more hr. with active CA, the third was immersed for 4 hr. in 1 ml. of inactive CA (33 mM), and the fourth sample was immersed for 4 hr. in 1 ml. of distilled water prior to their preparation for analysis.

Isolation of radioactive compounds

Acid-soluble fraction. The preparation of material for analysis, the extraction with cold 0.6 N-$HClO_4$, the determination of absorbed and metabolized CA were carried out as described previously [19]. The elution with ethanol of pyrimidine compounds adsorbed on active charcoal [4] was slightly modified, using hot ethanol for the final elution, which guaranteed quantitative recoveries of adsorbed compounds. The combined charcoal eluates were evaporated under reduced pressure in a dessicator over NaOH at room temperature. The dry residue was dissolved in about 10 ml. of distilled water and put through a Dowex-1-Cl^- column (1,5 × × 50 cm., 50 - 100 mesh). The non-adsorbable substances were quantitatively washed out with distilled water and the adsorbed compounds were eluted with dilute HCl (speed of elution about 0.8 ml./min.). 400 fractions of 5 ml. each were collected on the collector, using 0.002 N-HCl for

fractions 1 - 50, 0.004 N-HCl for fractions 51 - 200, and 0.015 N-HCl for fractions 201 - 400.

All 400 fractions were investigated for radioactivity and their optical density was measured at 270 mμ in order to ascertain which fractions should be used for the chromatographic isolation of radioactive compounds absorbing ultraviolet light. Thus uracil and uridine which are not adsorbed on the Dowex-1-Cl^- column were recovered from water washings, 5′-CMP from the combined eluates of fractions 12 - 30, 5′-UMP from the combined eluates of fractions 241 - 294 and OA from fractions 301 - 360. The chromatographic isolation of pure pyrimidine derivatives, their identification and quantitative spectrophotometric determination were carried out as previously described [4, 19].

Fractions 76 - 110 and 128 - 190 contained at least two substances with considerable radioactivity but which did not show any absorption in ultraviolet light. However it was proved that these compounds were not products of enzymatic reactions. When namely to inactive $HClO_4$ extract ^{14}C-CA was added, and the mixture then partitioned as above, then no radioactivity in the pyrimidines could be observed. Yet fractions 76 - 110 and 128 - 190 have shown the same radioactivity as in the normal feeding experiment.

Acid-insoluble fraction. The residual plant material, from which all $HClO_4$-soluble compounds have been removed, was hydrolyzed with 1 N-HCl at 100° for period of 1 hr. [13]. After cooling, the supernatant was separated by centrifugation and evaporated in a dessicator under reduced pressure over NaOH at room temperature. The dry residue was dissolved in about 10 ml. H_2O, put through a Dowex-1-Cl^- column and the pyrimidine derivatives isolated by means of paper chromatography as above. Fractions 17 - 43 contained 2′ (3′)-CMP, and 2′ (3′)-UMP were recovered from fractions 255 - 318. During hydrolysis about 10% of mononucleotides was decomposed to corresponding nucleosides and these losses were accounted for by isolation of cytidine and uridine from the water solution following the adsorption of the nucleotides on the Dowex-1-Cl^- column.

Measurement of radioactivity

Radioactivity was measured with a window counter (1.4 mg./cm^2.), type BAT 25/1 for varying periods of time so that the standard error did not exceed 5%. Whenever this requirement was not met on counting for 3000 sec., then the equation according to Verchovskaja [20] was employed for the control of accuracy of measurements.

Table 1

Amounts and specific activity of pyrimidine derivatives in plants fed with radioactive CA

Amounts expressed in μmoles/2 g. fresh weight

No.	Time of feeding (hr.)	CA			Found in acid-soluble fraction										Found in acid--insoluble fraction			
		absorbed		metabolized	uracil		uridine		5′-UMP		5′-CMP		OA		2′(3′)-UMP		2′(3′)-CMP	
		μmoles	counts/sec./μmol.	μmoles	μmoles	counts/sec./μmol.	μmoles	counts/sec./μmol.	μmoles	counts/sec./μmol.	μmoles	counts/sec./μmol.	μmoles	counts/sec./μmol.	μmoles	counts/sec./μmol.	μmoles	counts/sec./μmol.
1	1	1.72	1000	0.58	0.12	55	0.20	67	0.23	218	0.06	40	0.00	—	1.30	2.3	1.60	2.3
2	2	3.33	1000	1.26	0.32	234	0.66	163	0.36	700	0.08	75	0.00	—	1.35	4.6	1.72	4.5
3	4	7.60	1000	2.97	0.46	379	0.48	308	0.45	729	0.12	255	0.01	800	1.33	11.0	1.88	10.2
4	8	16.82	1000	8.17	1.84	479	0.53	313	1.13	722	1.32	469	2.93	785	1.25	23.3	2.00	18.0

RESULTS

After feeding ^{14}C-CA for 1 hr., 5′-UMP contained most of the radioactivity accounted for in plant material (Table 1). 5′-CMP, uridine and uracil have shown considerable less activity. 2′ (3′)-UMP and 2′ (3′)-CMP obtained after hydrolysis of the polynucleotides have shown distinct but very small specific activity. Contrary to 5′-CMP and 5′-UMP from the acid-soluble fraction, with considerably different specific activities, the 2′ (3′)-mononucleotides have shown identical radioactivity.

Eluates containing purine derivatives have not been radioactive at all, which is in agreement with data obtained by other workers [1, 9].

Extention of feeding time beyond 1 hr. caused further considerable increases of radioactivities as well as increases in the absolute amounts of recovered pyrimidine derivatives in the plant. Again 5′-UMP showed highest specific activity, followed by uracil, uridine and 5′-CMP. Already after 2 hr. the specific activity of 5′-UMP amounted to 70% of the specific activity of CA employed in the experiment. The activity of pyrimidines obtained from the hydrolysis of the polynucleotides continues to rise slowly throughout the experiment up to 8 hr. of feeding, but without any increases in the absolute quantities recovered.

Entirely different observations were made as regards OA. Up to 2 hr. of feeding no OA could be found in the plant. Only after 4 hr. traces of OA could be detected. Yet its specific activity was extremely high from the start and amounted to 80% of the initial activity of CA used in the experiment. All the other pyrimidine derivatives of the acid-soluble fraction continued to increase in quantity as well as in specific activity, with the exception of 5′-UMP which has apparently reached the limits of saturation already after 2 hr. of feeding with CA.

Further interesting observations as regards quantities and activities were made after 8 hr. of feeding with radioactive CA. The concentration and activity of uridine and 5′-UMP underwent no further changes. On the other hand quantities of uracil and 5′-CMP increased fivefold and elevenfold respectively, with considerable increases of specific activity. A rapid accumulation of OA was noticeable, which exceeded in quantity all the other pyrimidine derivatives (Table 1).

The absence of OA up to 4 hr. of feeding the plant with CA and its subsequent emergence with extremely high specific activity lead to the assumption that OA may have an additional role to fulfill in the process of biosynthesis of pyrimidine nucleotides in the plant. Therefore an experiment was carried out in which wheat blades were fed for 4 hours with labelled CA. As already mentioned above, this is the shortest time

Table 2

Amounts and specific activity of OA in plants fed with CA

Amounts expressed in μmoles/2 g. of fresh weight. Radioactivity of ^{14}C-CA 1000 counts/sec./μmol.

No.	Feeding	Found (μmoles)	Specific activity (counts/sec./ /μmol.)
1	4 hr. ^{14}C-CA	0.02	800
2	4 hr. ^{14}C-CA + 4 hr. ^{14}C-CA	2.55	809
3	4 hr. ^{14}C-CA + 4 hr. CA	2.94	138
4	4 hr. ^{14}C-CA + 4 hr. H_2O	0.00	—

required for the appearance of OA in plants fed with CA. If after 4 hr. of above treatment the plant was continuously fed with active CA, then rapid accumulation of OA with very high specific activity was noticeable. If however the treatment with active CA was interrupted after 4 hours and blades transferred onto a solution containing inactive CA, then OA still accumulated rapidly as above, but the specific activity was falling considerably. Finally, if blades treated for 4 hr. with active CA were immersed for further 4 hours in H_2O, then no OA accumulated and the small quantities arising after the first 4 hr. of feeding with CA disappeared entirely from the plant material (Table 2).

DISCUSSION

It is essential to state that no unequivocal answer has been obtained concerning the biosynthetic sequence of emergence of various pyrimidine derivatives from CA, their common precursor. According to literature [18,3] the first pyrimidine which arises from CA in animal organs and microorganisms is OA. In plant material however OA appears only after 4 hr. of CA feeding, while the appearance of other pyrimidines and their derivatives could be observed already after 1 hr. On the other hand OA is characterized from the start by extremely high specific activity, which suggests that it actually is the first pyrimidine compound arising from CA, but that it undergoes rapid metabolic changes to other pyrimidines. This view is supported by direct as well as indirect evidence: (1) Feeding plants with OA, we were able to show synthesis of 5′-UMP, 5′-CMP, uridine and uracil [4]. (2) The specific activity of six isolated pyrimidine derivatives rises gradually with the extention of time of feeding, whereas OA shows greatest activity throughout. (3) Specific activity of OA decreases

rapidly on transfer of the plant material onto inactive CA, suggesting strongly that OA is a very active metabolite supplying the pyrimidine ring structure to the other derivatives (Table 2, No 3).

The intense accumulation of OA after 8 hr. of CA feeding (Table 1, No 4 and Table 2, No 2), as well as its rapid metabolism (Table 2, No 3) indicate that OA is not only a very active intermediate on the path of pyrimidine nucleotide synthesis, but it is also the substance in which the plant may temporarily accumulate the preformed cyclic pyrimidine structure.

This interpretation unites to a certain extent Mitchell's view [14] that OA is a by-product of pyrimidine nucleotide synthesis with the thesis of Reichard [18] that OA must be considered a true intermediate in this process. According to our interpretation it may be assumed that all radioactive products that appear already after 1 hr. arise from ^{14}C-CA by way of OA as true intermediate.

5′-UMP shows highest specific activity after short time feeding, which indicates that higher plants contain an enzyme system similar to animals and microorganisms, catalizing ribotidation of OA [12, 2] and decarboxylation of OMP [12, 10].

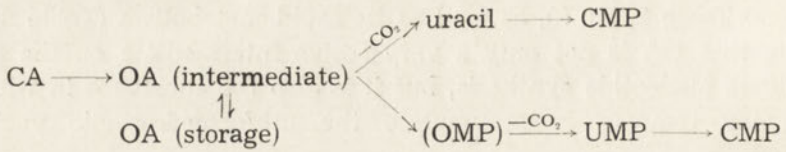
After 1 hr. of CA feeding uracil and uridine show distinct radioactivity which rises sharply on extention of the time of feeding. This is in agreement with the observation of uracil and uridine synthesis in plants fed with OA [4]. It would therefore appear that higher plants possess an OA decarboxylase thus placing uracil on the main path of further anabolic reactions. It has been recently reported that animals [5, 15] and microorganisms [8, 7] also use uracil in similar processes although the path of uracil synthesis is quite different and results from the products of reversible reactions of reductive degradation (β-alanine, carbamyl-β-alanine and dihydrouracil) and not from decarboxylation of OA.

The striking increases in the quantities of uracil and 5′-CMP after 4 hr. of feeding, with corresponding increases of specific activity may perhaps suggest the amination of uracil to cytosine prior to the synthesis of nucleotides. However this observation may be coincidental, as it is known that only uridine triphosphate undergoes amination [11, 6] and further detailed experimental evidence is obviously required.

No differences appeared in the specific activity of both pyrimidine nucleotides, obtained on hydrolysis of the polynucleotide fraction. This would suggest that their precursors should have activities of the same magnitude, yet radioactivity of 5′-UMP and 5′-CMP in the acid-soluble fraction was distinctly different. The answer to this question cannot be

expected without additional information concerning the di- and triphosphonucleosides, the immediate precursors of polynucleotides.

Tentatively the following scheme of pyrimidine mononucleotide synthesis may be suggested:



SUMMARY

Excised wheat blades were fed for 1, 2, 4, and 8 hr. with CA labelled with ^{14}C in the carbamyl group. Already after 1 hr. considerable radioactivity was noticed in 5′-UMP, uracil, uridine, 5′-CMP as well as in 2′ (3′)-UMP and 2′ (3′)-CMP of the polynucleotide fraction.

OA appears only after 4 hr. of feeding, showing from the start extremely high radioactivity amounting to 80% of the specific activity of CA employed.

The double role of OA as a true intermediate in the synthesis of pyrimidine nucleotides as well as the store of pyrimidine ring structure has been discussed.

The existence of OA decarboxylase in higher plants and possible role of uracil in pyrimidine metabolism has been suggested.

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PRZEMIANA KWASU ^{14}C-L-KARBAMYLOASPARAGINOWEGO DO POCHODNYCH PIRYMIDYNOWYCH W ODCIĘTYCH ŹDŹBŁACH PSZENICY

Streszczenie

Odcięte źdźbła pszenicy dokarmiano kwasem L-karbamyloasparaginowym znakowanym ^{14}C w grupie karbamylowej przez okres 1, 2, 4 i 8 godz. Już po godzinie stwierdzono włączanie ^{14}C do 5′-UMP, uracylu, urydyny i 5′-CMP, oraz 2′(3′)-UMP i 2′(3′)-CMP frakcji polinukleotydowej.

Kwas orotowy pojawia się w roślinie dopiero po 4 godz. dokarmiania. Posiada on jednak odrazu wyjątkowo wysoką radioaktywność, wynoszącą 80% aktywności właściwej użytego CA.

W dyskusji wysunięto przypuszczenie, że OA odgrywa podwójną rolę w biosyntezie pirymidyn, a mianowicie jako aktywny produkt pośredni i jako magazyn przejściowego nadmiaru prekursora pirymidyn kwasów nukleinowych. Ponadto wskazano na możliwość występowania dekarboksylazy kwasu orotowego w roślinie i udziału uracylu w syntezie mononukleotydów.

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ИССЛЕДОВАНИЯ НАД СИНТЕЗОМ БЕЛКОВ В КЛЕТОЧНЫХ ЯДРАХ

Резюме

Исследовалось включение ^{14}C-аминокислот *in vitro* и *in vivo* в рибонуклеопротеидовые частицы (RNP) и растворимую фракцию, полученные путем центрифугирования дезинтегрированных ядер печени морской свинки при 40 000 × г.

Синтез белков в выделенных частицах RNP протекал в присутствии растворимой фракции из ядер и глюкозы, а также в присутствии цитоплазматической растворимой фракции и системы регенерирующей ATP. Сама надосадочная жидкость ядер интенсивно включала аминокислоты.

В клеточных ядрах найдены нуклеопептиды, похожие на описанные ранее [19] нуклеопептиды из цитоплазмы печени морской свинки.

В. ДРАБИКОВСКИ

СВЯЗЫВАНИЕ ATP СЫВОРОТОЧНЫМ АЛЬБУМИНОМ В РАСТВОРЕ

Резюме

При применении ультрафильтрации и компенсационного диализа установлено возникновение соединений ATP с сывороточным альбумином в растворах. Способность связывания ATP уменьшается с увеличением pH и возрастает с увеличением концентрации ATP, так же как и в случае связывания нуклеотидов осажденными белками.

Спектрофотометрические исследования смеси ATP и альбумина сыворотки показали, что присутствие белка не влияет на ультрафиолетовый спектр ATP, что подтверждает предположение о том, что

кольцо аденина не принимает участия в образовании исследуемых соединений.

При pH, меньшем изоэлектрической точки, наблюдалось заметное влияние ATP на элоктрофоретическую подвижность альбумина на фильтровальной бумаге.

ГАННА СТШЕЛЕЦКА-ГОЛАШЕВСКА

НЕКОТОРЫЕ СВОЙСТВА СОЕДИНЕНИЙ АДЕНИН-НУКЛЕОТИДОВ С МЫШЕЧНЫМИ БЕЛКАМИ

Резюме

Исследовалось влияние pH и некоторых неорганических солей (NaCl, KCl, $CaCl_2$ и $MgCl_2$) на соединения мышечных белков содержащимися в мышце нуклеотидами. Опыты проводились на ацетоновых порошках из мышцы лягушки. Установлено наличие заметной зависимости количества связываемых нуклеотидов от pH.

Больше всего нуклеотидов (до 90 - 100% общего количества в материале) остается в связанной форме при pH 2,2 - 2,8. Добавление неорганических солей в зависимости от их концентрации и от pH среды, вызывало снижение или увеличение количества связываемых нуклеотидов или же не оказывало никакого влияния. На основании полученных результатов обсуждается возможность наличия между белками и нуклеотидами различных связей в разных условиях.

БРОНИСЛАВА МОРАВЕЦКА

ИЗМЕНЕНИЯ В БЕЛКАХ ЖЕЛТОГО ЛЮПИНА (*LUPINUS LUTEUS* L.) ПРИ ПРОРАСТАНИИ

Резюме

Белки боратных вытяжек из семян и проростков желтого люпина разделяли путем бумажного электрофореза на пять фракций. Исследовались изменения отдельных фракций в I, III и V фазе развития семян. При проростании общий белок уменьшается с 40% сухого веса семян до 1,3%. Между I и III фазой развития резко снижается количество канглютина β, а в V фазе не удалось уже обнаружить его присутствия. В V фазе усчезает также альбумино-глобулиновая фракция.

ПАУЛИНА ВЛОДАВЕР

ВКЛЮЧЕНИЕ ^{32}P В ФОСФОРНЫЕ СОЕДИНЕНИЯ *GALLERIA MELLONELLA*

Резюме

1. При помощи ортофосфата, меченого по ^{32}P, исследовался обмен фосфорных соединений у голодающих и потребляющих пищу гусениц *Galleria mellonella*.

2. Содержание ^{32}P в кислоторастворимой фракции непосредственно после прекращения потребления изотопа, остается высоким и значительно уменьшается при переходе на „нормальное" потребление пищи. Одновременно с уменьшением специфической активности в кислоторастворимой фракции наблюдается увеличение специфической активности фосфолипидов и нуклеиновых кислот. Специфическая активность фосфолипидов достигает максимума через 24 часа после начала нормального потребления пищи и превышает активность кислорастворимых фосфорных соединений.

3. Выделяемый ^{32}P содержится почти исключительно во фракции полифосфатов.

4. При голодании гусениц наблюдается меньшее падение удельной активности кислоторастворимой фракции, чем при кормлении гусениц, а изменения удельной активности фосфолипидов и нуклеиновых кислот незначительны.

5. В кислоторастворимых фосфорных соединениях изотопное равновесие устанавливается уже в течение двух дней голодания, тогда как при кормлении это явление не наблюдается.

ЦЕЛИНА ЯНИОН и Д. ШУГАР

ТИМИДИН-ФОСФОРИЛАЗА И ДРУГИЕ ФЕРМЕНТЫ РЕГЕНЕРИРУЮЩЕЙ ПЕЧЕНИ КРЫСЫ

Резюме

Для выяснения роли тимидин-фосфорилазы исследовались изменения этого фермента при синтезе DNA в регенерирующей печени крысы. Одновременно определялись 5′-нуклеотидаза и кислая фосфатаза, содержание DNA и белка, а также увеличение веса печени. В период наиболее интенсивного синтеза DNA активность тимидин-фосфо-

рилазы по отношению к общему белку уменьшается; в следующих этапах регенерации ее активность возрастает, не отличаясь на активности других ферментов.

Полученные результаты указывают на то, что этото фермент принимает участие скорее в деградации, чем в синтезе DNA.

В. ОСТРОВСКИ и З. ВАЛЬЧАК

НУКЛЕОЛИТИЧЕСКИЕ ФЕРМЕНТЫ *TH. THIOPARUS*. ОЧИЩЕНИЕ И СВОЙСТВА РИБОНУКЛЕАЗЫ

Резюме

В экстрактах клеток *Th. thioparus* найдено, по крайней мере, три рибонуклеазы, вызывающие гидролиз RNA олигонуклеотидов. Путем фракционирования экстракта сульфатом протамина, сульфатом аммония, хроматографии на СМ-целлюлозе и на Амберлите IRC-50 была достигнута значительная степень очищения (более чем 300 раз) одной из рибонуклеаз, устойчивой к нагреванию и к действию низкого pH. Оптимум pH фермента равняется 8,5, оптимум температуры гидролиза RNA приблизительно 45°. Фермент активируется ионами Fe^{2+}. Фермент вызывает гидролиз дрожжевой RNA, RNA выделенной из тех же клеток и медленнее „сердцевину" из дрожжевой RNA. Приблизительно в 25 раз медленне по сравнению с RNA разлагается этим ферментом синтетическая полиадениловая кислота.

Выделенная рибонуклеаза не разлагает дезоксирибополинуклеотидов. Предварительные исследования над специфичностью фермента указывают, что легче гидролизуются связи между пиримидиновыми и пурино-пиримидиновыми нуклеотидами, чем между пуриновыми нуклеотидами. Основными продуктами гидролиза RNA являются олигонуклеотиды, состоящие из двух или трех нуклеотидов.

В. ШЭР и Д. ШУГАР

СИНТЕЗ И ФИЗИКО-ХИМИЧЕСКИЕ И ЭНЗИМАТИЧЕСКИЕ СВОЙСТВА 5-БРОМОПРОИЗВОДНЫХ УРИДИЛОВОЙ КИСЛОТЫ И ЕЕ ПОЛИМЕРОВ

Резюме

1. Получены производные уридин-фосфата, бромированные в позиции 5 кольца урацила.

2. 2′ : 3′-фосфат (циклический) 5-бромуридина является субстратом панкреатической рибонуклеазы, а его N-метил аналог устойчив к дей-

ствию рибонуклеазы. Олиго-BrU также подвергается гидролизу под влиянием рибонуклеазы, тогда как олиго-MeBrU устойчив к ее действию, но медленно гидролизуется под влиянием фосфодиэстеразы змеиного яда.

3. Олиго-BrU образует двойной комплекс с поли-A, тогда как N-метилированный бромированный полимер не может образовать этого комплекса.

4. Гиперхромные эффекты олиго-BrU и олиго-MeBrU того же порядка. Это подтверждает вывод, что такая гиперхромазия вызвана взаимодействием соседних колец оснований без образования между ними водородных связей.

5. Ни 5-BrUDP, ни N-метил-5 BrUDP не могут быть субстратами для полинуклеотид-фосфорилазы; 5-BrUDP частично тормозит активность этого фермента.

Е. БУХОВИЧ, И. РЕЙФЕР и Е. МАКОВСКИЙ

ПРЕВРАЩЕНИЕ ^{14}C-L-КАРБАМИЛАСПАРАГИНОВОЙ КИСЛОТЫ В В ПИРИМИДИНОВЫЕ ПРОИЗВОДНЫЕ В ОБОСОБЛЕННЫХ СТЕБЛЯХ ПШЕНИЦЫ

Резюме

Обособленные стебли пшеницы инкубировали с *L*-карбамиласпарагиновой кислотой, меченой по ^{14}C в карбамильной группе, в течение 1, 2, 4 и 8 часов. Установлено, что уже через 1 час инкубации происходит включение ^{14}C в 5′-UMP, урацил, уридин и 5′-CMP, а также в 2′ (3′)-UMP и 2′ (3′)-CMP полинуклеотидной фракции.

Оротовая кислота появляется в стеблях пшеницы только после 4-х часов инкубации. Отмечено, что образующаяся оротовая кислота с самого начала обладает исключительно высокой радиоактивностью, составляющей около 80% удельной активности *L*-карбамиласпарагиновой кислоты, взятой для инкубации.

В дискуссии высказано предположение, что оротовая кислота играет двойную роль в биосинтезе пиримидинов. Она может быть активным промежуточным продуктом синтеза и с другой стороны — специальной формой аккумуляции временного избытка предшественника пиримидинов, входящих в состав нуклеиновых кислот. Рассмотрена возможность присутствия в высших растениях декарбоксилазы оротовой кислоты и участия урацила в синтезе мононуклеотидов.