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STUDIES ON δ-AMINOLAEVULIC ACID TRANSAMINATION

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δ-Aminolaevulic acid (ALA) is derived from glycine and "active" succinate under the influence of an unidentified enzyme system. The next stage of porphyrin biosynthesis is the conversion of ALA into porphobilinogen catalyzed by ALA dehydrase. However, there exists still another pathway of ALA metabolism. According to Shemin [8], ALA gives up the amino group, loses one atom of carbon to give succinate, closing a cycle called by Shemin the succinate-glycine cycle. This second pathway of ALA conversion starts with deamination — and as further studies of Nemeth, Russel and Shemin show [5] — is connected with the utilization of a carbon atom for the synthesis of the purine ureido group. This pathway is as important as the pathway leading to porphyrin biosynthesis.

The knowledge of conditions enabling ALA transformation by one of the possible metabolic route may be useful to clear up the porphyrin and purine cell metabolism and its disturbances in disease.

We pointed out in a previous paper [4] that the ALA deamination postulated by Shemin is an enzymatic transamination. We called the enzyme which catalyzes this process δ-aminolaevulic acid transaminase (TrALA). In this paper there will be presented further studies on ALA transamination [1]).

MATERIALS

Solutions of 0.1 M-α-oxoglutaric acid (GLA), sodium pyruvate (PA), alanine (AL), aspartic acid (ASPA) and ALA in 0.06 M-phosphate buffer at pH 7.6 were used. All reagents except ALA were La Roche or B. D. H. ALA

[1]) The results were presented in part at the 4th International Congress of Biochemistry in Vienna and at the 7th Congress of the International Society of Hematology in Rome, on September 1958.

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was synthesized, according to Neuberger and Scott [6] [2]). Before use ALA was twice recrystallized from a mixture of ethyl acetate and methyl alcohol. The melting point of the used ALA hydrochlorid was 149—151°. The remaining reagents used were analytical grade, made in Poland.

Homogenates of rat tissues were prepared immediately after killing the animal. The animals were anaesthetized with ether and killed by bleeding after incision of jugular veins. 7 g. of wet tissue were homogenized with 30 ml. water in a M. S. E. blendor, unless it is otherwise stated. The homogenates were used immediately after preparation.

METHODS

In all experiments the activity of ASPA transaminase (TrASPA) was assayed to compare the known transaminase with the ALA transamination. The incubated mixtures, according to Karmen, Wróblewski and La Due [2] consisted of: 0.5 ml. of homogenate, 0.5 ml. of the donor amino acid (ALA or ASPA), 0.5 ml. of the acceptor amino acids (0.1 M-GLA and PA mixed in equal quantities) and 1.5 ml. of 0.06 M-phosphate buffer (pH 7.6), and they were incubated in a water bath at 37° for 2.5—3.0 hrs. unless it is otherwise stated.

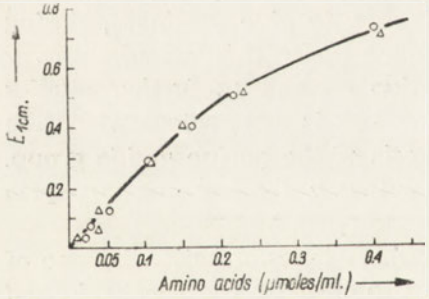

Fig. 1. Absorption of ninhydrine complexes of glutamic acid △, and alanine ○, extracted from chromatograms of standard solutions

After incubation 10 ml. absolute alcohol was added and centrifuged for 5 minutes at 4000 r.p.m. The precipitated protein was washed with 70% alcohol (v/v). The combined alcohol extracts were evaporated in water bath until dry. The sediments were kept overnight at 0° then redissolved in 0.5 ml phosphate buffer. 10 μl. of this solution (twice 5 μl.) was chromatographed. The amino acid chromatography was carried out according to Kay, Harris and Entenman [3] on Whatman No. 1 paper, developed in phenol-water at room temperature for 20—23 hrs. Chromatograms were dried for 40—44 hrs. and then sprayed with ninhydrine solution (2% in ethyl alcohol containing 0.2% NaOH), dried for 0.5 to 1 hr. and developed at 65 to 70° for 5 minutes. Spots corresponding to the determined amino acids were cut out and ground in 15 ml. centrifuge tubes containing 3.5 ml. absolute alcohol. The paper pulp was spun by centrifugation and the absorption was measured at 475 mμ. The amounts of

[2]) The synthesis was carried out by Mr. M. Wolff, M. Sc., Bydgoska Wytwórnia Chemiczna, whom we are very indebted.

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amino acids were established by means of standard curve (Fig. 1) which is identical for both glutamic acid and alanine, the concentration of these amino acids being expressed in μmoles.

Table 1

Incubation mixtures for determination of δ-aminolaevulic acid transaminase

	ml.	Simultaneous incubation					
		I	II	III	IV	V	VI
δ-Aminolaevulic acid 0.1 M	0.5	+	+	—	—	—	—
Aspartic acid 0.1 M	0.5	—	—	+	+	—	—
α-Oxoglutaric acid 0.1 M	0.25	+	—	+	—	+	—
Pyruvic acid 0.1 M	0.25	+	—	+	—	+	—
Homogenate (0.7 g. of tissue per 3 ml. H_2O)	0.5	+	+	+	+	+	+
Buffer pH 7.6 to	3.0	+	+	+	+	+	+

The enzymatic activity of the homogenates was expressed as net increments of the appropriate amino acids per gram of wet tissue or mg. of protein nitrogen estimated by Kjeldahl method. An incubation mixture

Table 2

Glutamic acid and alanine at "0 time" and after 3 hours of incubation of a mixture of equal parts of rat liver and kidney homogenates with and without substrates (δ-aminolaevulic acid (ALA), oxoglutaric acid (GLA) and pyruvic acid (PA))

All values are μmoles/g. wet tissue

Incubation time (hours)	Homogenate without substrates		Homogenate +ALA+GLA+PA		Net increase	
	Glutamic acid	Alanine	Glutamic acid	Alanine	Glutamic acid	Alanine
0	0.05	0.05	0.06	0.05	—	—
	0.07	0.08	0.08	0.10		
	0.07	0.08	0.07	0.10		
3	0.16	0.15	11.4	9.8	11.24	9.65
	0.20	0.23	16.7	10.2	16.50	9.97
	0.19	0.22	12.3	11.1	12.11	10.98

without the amino group donor (ALA or ASPA) was used as control. Other controls consisted of incubation systems in which homogenates or amino group donors or amino group acceptors were omitted, respectively. All incubation mixtures are shown in Table 1. Table 2 gives values of

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glutamic acid and alanine found in one typical experiment at "0 time" and after 3 hr. of incubation of homogenate alone or of homogenate with ALA and amino group acceptor.

RESULTS

1. The results obtained when the mixture of rat liver and kidney homogenates was heated for 10 minutes at 100° before incubation point

Table 3

Effect of heating (10 minutes at 100°) on the transamination activity of a mixture of equal parts of rat liver and kidney homogenates

Incubation time 2.5 hours. The activity expressed as increase of amino acids in μmoles per mg. protein nitrogen

Substrates	δ-Aminolaevulic acid		Aspartic acid	
Products	Glutamic acid	Alanine	Glutamic acid	Alanine
Unheated homogenate	0.42	0.70	5.51	11.00
Heated homogenate	0.14	0.19	1.10	0.55

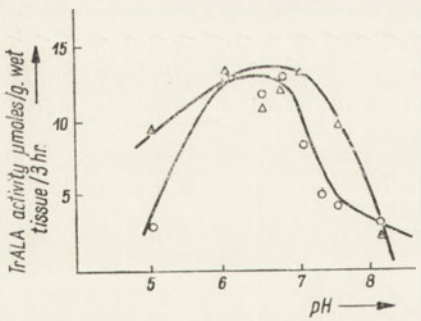

Fig. 2. Effect of pH on the activity of δ-aminolaevulic acid transaminase in a mixture of equal parts of rat liver and kidney homogenate. Glutamic acid △, and alanine ○, formed

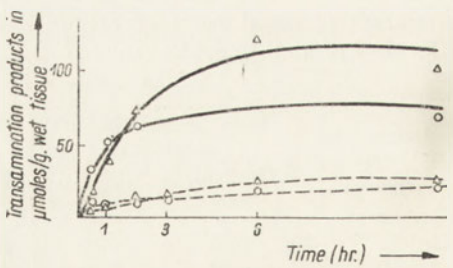

Fig. 3. Effect of incubation time on δ-aminolaevulic acid – – –, and aspartic acid ———, transamination in mixture of equal parts of rat liver and kidney homogenates. Glutamic acid △, and alanine ○, formed

to an enzymatic nature of ALA transamination. As can be seen from Table 3 both TrALA and TrASPA show considerably lower values in the heated homogenates as compared with unheated.

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2. The optimum pH of TrALA activity is 6.5 to 7.0 (Fig. 2). The samples tested were adjusted to the desired pH with a solution of 0.1 N-NaOH or HCl before incubation.

3. Figure 3 shows the relation between the incubation time and the efficiency of transamination in mixed rat liver and kidney homogenates. After 3 hrs. an equilibrium is reached; there is only a slight increase of amino acids.

4. Figure 4 shows the effect of substrate concentration upon the transamination. The efficiency is directly proportional to the concentration of substrate. In one series of experiments the enzyme concentration and in another the substrate concentration were changed. The results of both series of experiments expressed in μmoles per mg. of protein nitrogen of formed glutamic acid or alanine, respectively, are presented in Fig. 4.

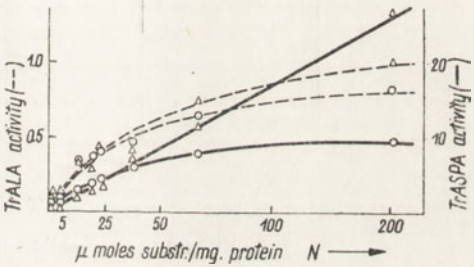

Fig. 4. Effect of substrate concentrations on δ-aminolaevulic acid — — —, and aspartic acid ———, transamination in a mixture of equal parts of rat liver and kidney homogenates. The activity is expressed as glutamic acid △, and alanine ○, formed in μmoles/mg. of protein N/2.5 hr.

5. In order to state whether ALA transaminase is a specific enzyme or its activity is a part of the known transaminases only, the activity of transamination from ALA, ASPA and AL to α-oxoglutaric acid in heart, liver and kidney of rat were followed. As can be seen from data presented in Table 4 there is no apparent correlation between the activities of ALA and ASPA transamination. However, comparison of ALA and AL employed as substrates shows changes of the same kind.

6. Attempts have been made to purify the enzyme by the ammonium sulphate fractionation. The homogenate of fresh ox kidney was used, all operations were carried out at 0°. After each stage of fractionation both TrALA and TrASPA were determined. The results shown in Table 5 indicate that it was possible to obtain preparations with TrALA activity 18-fold increased, while the increase of TrASPA activity was 4.5-fold only. The different degree of purification may suggest TrALA specificity.

Using acetone for fractionation, we were not able to obtain any increase of enzyme concentration.

7. To get some indication of a biological role of TrALA, the enzyme was studied in various tissues. It can be seen from the Table 6 that kidney and small intestine contain considerably greater amounts of TrALA than brain, red corpuscules or striated muscle. This may suggest some re-

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Table 4

Activities of different transaminases in rat liver, kidney and heart muscle

Transamination expressed as increase of glutamic acid in μmoles per g. of wet tissue

	Substrates for transamination		
	δ-Aminolaevulic acid	Aspartic acid	Alanine
Heart muscle	1.39	385.7	102.8
	1.56	300.0	71.2
	2.12	278.0	85.7
Liver	5.56	145.7	300.0
	3.51	167.0	180.0
	2.73	113.0	120.0
Kidney	—	300.0	12.80
	—	180.0	5.56
	2.34	137.0	7.22

Table 5

Enzyme purification with ammonium sulphate

Fraction	Protein mg. N/ml.	Specific activity*				Degree of purification*			
		a	b	c	d	a	b	c	d
Ox kidney homogenate	5.56	0.29	0.15	7.48	0.54	—	—	—	—
Supernatant	2.80	0.68	0.50	10.40	0.84	2.3	3.2	1.4	1.6
I Fraction (0.4 amm. sulph. sat.)	0.38	3.32	2.86	16.10	2.38	11.5	18.6	2.1	4.5
II Fraction (0.55 amm. sulph. sat.)	0.60	1.92	1.20	4.8	1.62	6.6	7.8	0.6	3.0
III Fraction (0.7 amm. sulph. sat.)	0.93	0.97	1.04	4.48	0.16	3.3	6.8	0.6	2.2

* a = δ-aminolaevulic acid-glutamic acid transaminase
b = δ-aminolaevulic acid-alanine transaminase
c = Aspartic acid-glutamic acid transaminase
d = Aspartic acid-alanine transaminase

lationship between ALA transamination and the nucleic acid metabolism because the organs with a higher TrALA content possess a high nucleic acid metabolism.

8. To test the relationship between TrALA and erythropoietic activity we studied the transaminase activity in nucleated erythroblasts of rabbit bone marrow, and compared it with the activity in erythrocytes of the peripheral blood of the same animal. The marrow of heparinized animals

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Table 6

The average values of δ-aminolaevulic acid transaminase activity in organs of the rat

Net increase of alanine and glutamic acid in μmoles/g. wet tissue per 3 hr. incubation. The mean values of 10 assays are given, ± S.D.

Tissue	Alanine	Glutamic acid
Kidney	18.37 ± 13.0	29.3 ± 18.5
Small intestine	11.88 ± 8.6	14.79 ± 11.2
Spleen	9.64 ± 7.77	17.15 ± 9.3
Liver	6.86 ± 3.26	11.06 ± 9.9
Heart	7.33 ± 5.27	7.35 ± 6.1
Lung	5.41 ± 3.03	7.67 ± 4.02
Bone marrow	4.38 ± 1.75	10.3 ± 5.6
Brain	4.0 ± 1.48	8.3 ± 2.03
Plasma	5.33 ± 3.5	2.59 ± 1.8
Erythrocytes	3.44 ± 0.34	3.24 ± 1.55
Muscle	1.94 ± 0.6	4.78 ± 2.3

Table 7

Activity of δ-aminolaevulic acid transaminase in rabbit erythrocytes and erythroblastes

Net increase of amino acids in μmoles/mg. protein nitrogen/3 hr.

Animal	Erythrocytes of peripheral blood		Bone-marrow erythroblastes	
	Alanine	Glutamic acid	Alanine	Glutamic acid
1	0.01	0.02	0.83	0.29
2	0.03	0.02	0.27	0.18
3	0.015	0.01	0.81	0.60
4	0.025	0.015	0.77	0.19

was suspended in saline and ejected through syringe needles of decreasing diameter, thereafter centrifuged for 10 minutes at 2000 r.p.m. The bottom layer with the highest concentration of erythroblasts was washed with saline, centrifuged and then used. The results (Table 7) expressed as activity per mg. of protein nitrogen indicate that the transamination in the nucleated bone marrow cells is much higher than that of erythrocytes of peripheral blood which is negligible. The transamination in nucleated rabbit bone marrow cells reaches values similar to the transaminase activity found in rat liver or kidney homogenates. Nucleated hen erythrocytes show a negligible TrALA content like non-nucleated rat and rabbit erythrocytes.

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DISCUSSION

In the above study it could be shown that the transamination in biological material of ALA to GLA and to PA is of enzymatic nature. This conclusion may be drawn from the comparison of TrALA activity in heated and unheated homogenates as well as from the physico-chemical features of the reaction which are enzymatic in character (dependence of the enzyme activity on substrates concentration, pH and incubation time).

The question of the existence of a transaminase as an enzyme with group specificity is not definitely explained. It is not clear whether any individual transaminase exists for each amino acid or there is only one enzyme for all amino acids. It is known that many amino acids undergo transamination, but the obtained preparations of purified enzyme as yet were able to catalyze only a few or one reaction of this type. Therefore it seems more probable that the transamination of each amino acid is catalyzed by a specific enzyme.

The comparison of the various organs of transaminase activity with ALA, ASPA and AL, as well as the results obtained during the process of enzyme purification seem suggest that TrALA activity is not a partial function of the known transaminases (TrASPA and TrAL) but is a specific enzyme.

Roberts [7] showed that not only α-amino acids undergo transamination but also β- and γ-amino acids. The transamination of δ- or γ-amino acids (δ-aminoornithine, γ-aminobutyric acid) stated by Roberts, was supported in our experiments with δ-aminolaevulic acid. Like the γ-aminobutyric acid transamination the efficiency of ALA transamination is rather low as compared to the α-amino acid transamination.

ALA after transamination seems to be changed into α-oxoglutaric semialdehyde which takes part in the succinate-glycine cycle as shown by Shemin [8]:

$$\underset{\text{aminolaevulic acid}}{HOOC\cdot CH_2\cdot CH_2\cdot CO\cdot CH_2\cdot NH_2} + CH_3\cdot CO\cdot COOH \rightleftarrows$$

$$CH_3\cdot CH(NH_2)COOH + \underset{\text{oxoglutaric semialdehyde}}{HOOC\cdot CH_2\cdot CH_2\cdot CO\cdot CHO}$$

We could not demonstrate the presence of oxoglutaric semialdehyde in our incubation mixture because of the lack of adequate analytical tests. But Nemeth, Russel and Shemin [5], using oxoglutaric semialdehyde labelled with ^{14}C, showed that this compound is a precursor of the purine ring in nucleic acid biosynthesis.

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It is possible that the relationship between TrALA and ALA dehydrase activities in tissues determines the fate of ALA in cell metabolism and the rate of its utilisation in heme or purine biosynthesis. If so, cells with a pronounced nucleic acid metabolism should show a higher transaminase activity than cells with poor nucleic acid metabolism do. The reported results seem to support this supposition, since homogenates of organs with high nucleic acid metabolism show a higher TrALA content. The ripening of erythroblasts which is connected with a decrease of nucleic acid metabolism and an increase of haemoglobin content is characterized by a low TrALA activity. The found activity of birds (hen) nucleated erythrocytes which have no nucleic acid metabolism [1] did not reveal a higher TrALA content, is consistent with this hypothesis.

The skilled technical assistance of Mrs. D. Rzepniewska, Miss Z. Długoborska, Mr. T. Turkiewicz and Mr. K. Radziszewski is gratefully acknowledged.

SUMMARY

The enzymatic nature of the transamination of δ-aminolaevulic acid--α-oxoglutaric acid and δ-aminolaevulic acid-pyruvic acid was proved. The effect of pH, substrate concentration and incubation time has been examined. 18-fold increase of the δ-aminolaevulic acid transaminase activity could be obtained by fractionation with ammonium sulphate.

Organs with high nucleic acid metabolism as well as erythroblasts of bone marrow showed high δ-aminolaevulic acid transaminase activity, mature erythrocytes showed only negligible activity. The role of this transaminase has been discussed.

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BADANIA NAD TRANSAMINACJĄ KWASU δ-AMINOLEWULINOWEGO

Streszczenie

Potwierdzono enzymatyczny charakter transaminacji: kwas δ-aminolewulinowy-kwas α-ketoglutarowy i kwas δ-aminolewulinowy-kwas pirogronowy. Zbadano wpływ pH, stężenia substratów i czasu inkubacji. Stosując frakcjonowanie siarczanem amonu uzyskano preparaty wykazujące 18-krotne zwiększenie aktywności transaminazy kwasu δ-aminolewulinowego (TrALA).

Narządy odznaczające się żywą przemianą nukleinową i erytroblasty szpiku wykazały wysoką aktywność TrALA. Dojrzałe erytrocyty posiadają jedynie nieznaczną aktywność. Przedyskutowano rolę tej transaminazy.

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WPŁYW PROMIENIOWANIA RENTGENOWSKIEGO NA TRANSAMINACJĘ KWASU δ-AMINOLEWULINOWEGO I ASPARAGINOWEGO W NIEKTÓRYCH NARZĄDACH SZCZURÓW

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Wyniki badań nad wpływem promieniowania jonizującego na transaminację [1, 2, 3, 5, 7, 8] prowadzonych na różnych obiektach, różnymi metodami i dotyczących różnych transaminaz trudno jest porównywać ze sobą. Tonhazy, White i Umbreit [8] nie stwierdzili istotnych zmian czynności transaminazowej w sercu i wątrobie napromieniowanych zwierząt. Milch i Albaum [7] wykazali, że aktywność transaminazy glutaminowo-szczawiooctowej w osoczu krwi królików i szczurów wzrasta już w kilka godzin po napromieniowaniu. Autorom tym nie udało się jednakże stwierdzić zależności pomiędzy aktywnością transaminazy a długością czasu przeżycia napromienionych zwierząt. Brin i McKee [2] stwierdzili zwiększenie aktywności transaminazy glutaminowo-szczawiooctowej i glutaminowo-pirogronowej w wątrobie a zmniejszenie w jelitach napromienianych szczurów. Największe zmiany autorzy stwierdzili około 4 dnia od napromieniania, a około 10 dnia aktywność transaminazy powracała do wartości normalnej. Brent i współprac. [1] badając poziom transaminazy osoczowej królików i szczurów w różnych odstępach czasu od napromienienia wykazali, że wzrost aktywności jest wyraźny 24 godziny po napromienieniu. Autorzy ci stwierdzają jednak, że wobec dużych różnic indywidualnych właściwa interpretacja wyników możliwa jest jedynie wówczas, kiedy znany jest poziom transaminazy przed napromienieniem badanego osobnika.

Celem niniejszej pracy, będącej dalszym ciągiem studiów nad transaminacją kwasu δ-aminolewulinowego (ALA) było poznanie wpływu pro-

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mieniowania rentgenowskiego na transaminację ALA w niektórych narządach szczura. W celach porównawczych badano również transaminację kwasu asparaginowego (ASPA).

MATERIAŁY I METODY

Do badań użyto białe szczury rasy Wistar wagi 150—250 g, płci męskiej, karmione standardowym pokarmem.

Aktywność transaminacji oznaczano w homogenatach wątroby, nerki i śledziony wg metody opisanej szczegółowo w poprzednim doniesieniu [6]. Miarą aktywności były przyrosty netto kwasu glutaminowego i alaniny po 2,5-godzinnej inkubacji badanego układu. Przyrosty aminokwasów

Tablica 1

Czynność transaminazy kwasu δ-aminolewulinowego w śledzionie, nerce i wątrobie szczura napromienionego na całe ciało różnymi dawkami promieniowania rentgenowskiego

Aktywność transaminazy mierzona jako przyrost kwasu glutaminowego (Gl) i alaniny (Al) w μmolach/g mokrej tkanki. ± Średnie odchylenie

Czas od napromieniania	Liczba zwierząt n	Transaminaza kwasu aminolewulinowego					
		śledziona		nerka		wątroba	
		Al	Gl	Al	Gl	Al	Gl
		0 rentgenów					
—	10	9,6 ± 7,7	17,5 ± 9,3	18,4 ± 13,0	29,3 ± 18,5	6,9 ± 3,3	11,1 ± 9,9
		50 rentgenów					
1 d	6	4,8 ± 0,6	— —	13,1 ± 2,7	19,3 ± 5,7	14,8 ± 3,3	6,1 ± 1,0
3 d	6	8,5 ± 1,9	4,6 ± 0,3	6,8 ± 1,6	16,3 ± 4,5	5,8 ± 1,8	5,3 ± 1,1
5 d	6	3,9 ± 0,9	3,6 ± 0,4	5,4 ± 1,4	9,1 ± 3,5	5,0 ± 0,6	4,5 ± 1,8
10 d	6	3,7 ± 0,7	1,7 ± 0,1	9,3 ± 2,2	19,1 ± 6,5	9,1 ± 3,7	6,0 ± 1,2
		400 rentgenów					
1 d	4	5,6 ± 0,2	4,3 ± 0,1	6,0 ± 1,3	12,1 ± 7,1	4,5 ± 0,5	2,3 ± 0,2
3 d	6	9,0 ± 5,1	2,3 ± 1,0	4,1 ± 2,2	5,5 ± 4,3	13,2 ± 3,2	5,9 ± 1,9
18 d	6	4,3 ± 1,2	1,4 ± 0,6	6,5 ± 0,9	9,9 ± 3,0	8,3 ± 0,4	2,3 ± 1,1
21 d	6	3,1 ± 1,0	1,5 ± 1,0	7,2 ± 0,9	5,4 ± 2,5	4,8 ± 0,9	3,8 ± 0,7
		650 rentgenów					
3 g	5	3,4 ± 3,5	2,1 ± 1,8	4,2 ± 2,0	9,8 ± 6,5	10,0 ± 5,3	7,0 ± 3,2
6 g	3	9,3 ± 3,0	5,9 ± 2,8	2,6 ± 0,2	12,6 ± 3,1	9,8 ± 2,5	11,7 ± 3,5
12 g	3	7,9 ± 1,7	1,7 ± 0,3	7,2 ± 1,5	13,0 ± 0,5	4,6 ± 0,6	10,6 ± 1,9
1 d	6	7,2 ± 2,4	4,8 ± 1,6	4,4 ± 2,2	12,7 ± 0,7	14,0 ± 7,1	7,0 ± 1,2
3 d	2	16,2 ; 0,0	5,1 ; 11,2	3,3 ; 12,9	24,3 ; 23,8	9,0 ; 1,0	6,3 ; 6,2
7 d	2	6,9 ; 23,2	5,6 ; 27,8	3,2 ; 5,1	12,2 ; 11,8	7,5 ; 11,7	2,8 ; 14,0
10 d	2	1,8 ; 11,3	16,7 ; 0,0	6,4 ; 10,2	17,6 ; 11,7	6,3 ; 8,8	5,7 ; 13,8

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obliczano w μmolach na gram mokrej tkanki, a w niektórych próbkach również na mg azotu białkowego oznaczonego metodą Kjeldahla.

Do napromieniania stosowano aparat rentgenowski typu „Stabilipan" z lampą TR-220 d; napięcie 160 kV, prąd 15 mA, filtr 0,5 Cu. Dawkę promieniowania ustalono pomiarem w powietrzu za pomocą dawkomierza Siemens Universal Dosismesser. Zwierzęta umieszczano w tekturowych pudełkach o wielkości odpowiadającej wielkości zwierząt. Odstęp od ogniska lampy do środka pudełka wynosił 93 cm. Dawka mierzona wewnątrz pudełka w tych warunkach 9,5 r/min. Wielkość żądanej dawki globalnej uzyskano regulując długość czasu napromieniania. Napromieniano równocześnie 2—6 zwierząt.

Dla celów kontrolnych wykonano oznaczenie średnich aktywności transaminazy kwasu δ-aminolewulinowego (TrALA) i transaminazy kwasu

Tablica 2

Czynność transaminazy kwasu asparaginowego w śledzionie, nerce i wątrobie szczura napromienionego na całe ciało różnymi dawkami promieniowania rentgenowskiego

Aktywność transaminazy mierzona jako przyrost kwasu glutaminowego (Gl) i alaniny (Al) w μmolach/g mokrej tkanki ± średnie odchylenie

Czas od napromienienia	Liczba zwierząt n	Transaminaza kwasu asparaginowego					
		śledziona		nerka		wątroba	
		Al	Gl	Al	Gl	Al	Gl
		0 rentgenów					
—	10	2,8± 1,4	74,3± 20,9	11,4± 2,1	92,0± 13,5	75,4± 15,0	47,8± 6,8
		50 rentgenów					
1 d	6	1,0± 0,9	60,4± 17,5	19,8± 9,7	97, ± 59,0	97,4± 16,4	44,1± 8,3
3 d	6	4,4± 0,1	115, ± 3,8	8,7± 3,8	120,0± 19,0	90,7± 23,4	51,8±28,2
5 d	6	3,7± 0,4	76,1± 8,2	12,8± 4,9	72,3± 15,4	73,2± 3,0	39,5± 1,5
10 d	6	0,2± 0,3	123, ± 19,7	8,8± 1,7	158,0± 31,5	77,7± 3,8	63,0± 5,0
		400 rentgenów					
1 d	4	3,7± 0,4	66,3± 1,4	17,8± 1,6	76,7± 4,6	79,4± 14,1	43,2± 7,4
3 d	6	2,9± 0,9	73,4± 18,0	13,1± 2,9	114,5± 54,5	70,5± 13,7	43,1±11,9
18 d	6	2,3± 0,7	86,3± 23,0	14,4± 4,5	119,2± 51,0	117,1± 27,5	50,4± 9,6
21 d	6	3,7± 1,2	67,3± 10,8	15,7± 7,0	94,3± 12,2	84,7± 19,4	50,3± 9,3
		650 rentgenów					
3 g	5	—	86,8± 20,7	4,4± 2,7	96,6± 7,1	48,3± 16,8	40,1±11,6
6 g	3	5,0± 0,2	103,3± 46,0	9,6± 3,8	76,9± 8,4	73,2± 29,5	47,2±20,5
12 g	3	2,1± 1,4	146,0± 63,5	10,1± 4,1	123,5± 5,0	94,0± 17,6	106,0±17,5
1 d	6	2,4± 1,5	191,0± 23,8	7,0± 0,3	200,0± 25,0	126,0± 37,3	129,8±30,8
3 d	2	6,6 ; 13,0	120,0 ; 220,0	8,6 ; 22,6	75,0 ; 106,0	60,0 ; 95,5	45,3 ; 44,6
7 d	2	8,0 ; 22,1	135,0 ; 185,0	10,5 ; 12,8	79,2 ; 102,0	59,3 ; 51,1	57,5 ; 51,9
10 d	2	6,9 ; 7,7	140,0 ; 240,0	16,8 ; 15,9	78,5 ; 113,0	85,6 ; 117,0	69,2 ; 95,8

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asparaginowego (TrASPA) w narządach zwierząt nie napromienianych (10 szczurów). Zwierzęta napromieniane badano po upływie 3, 6, 12 godz. i 1, 3, 7 i 10 dni od napromieniania dawką 650 r; 1, 3, 18, 21 dni od napromieniania dawką 450 r oraz 1, 3, 5 i 10 dni od chwili napromieniania dawką 50 r.

WYNIKI

Wyniki wykonanych oznaczeń TrALA i TrASPA, w przeliczeniu na gram mokrej tkanki przedstawiono na tablicy 1 i 2.

Uzyskane dane wskazują, że TrALA ulega pod wpływem promieniowania na ogół nieznacznym zmianom. Leżą one w obszarze wartości normalnych (średnia $\pm 3\sigma$). W śledzionie i nerce wskazują one na zmniejszanie się aktywności TrALA pod wpływem promieniowania. W wątrobie zmiany aktywności TrALA po napromienieniu w niektórych przypadkach mają raczej tendencję wzrostową. Dotyczy to zarówno transaminacji ALA — kwas α-ketoglutarowy, jak i ALA — kwas pirogronowy. W żadnym narządzie nie stwierdzono wyraźnej zależności zmian aktywności TrALA od okresu choroby popromiennej.

W przeciwieństwie do TrALA aktywność TrASPA w przypadku kwasu α-ketoglutarowego jako biorcy grupy aminowej wykazuje tendencję zwyżkową; chociaż i w tym przypadku wszystkie uzyskane wartości pokrywają się z obszarem wartości normalnych (średnia $\pm 3\sigma$).

Transaminaza ASPA — kwas pirogronowy występuje w ilościach rzędu TrASPA — kwas α-ketoglutarowy jedynie w wątrobie. I w tym też narządzie zachowuje się pod wpływem promieniowania podobnie jak TrASPA — kwas α-ketoglutarowy. W niektórych przypadkach aktywność jej ulega nieznacznemu zwiększeniu.

W śledzionie i nerce wartości TrASPA są rzędu TrALA. W niektórych przypadkach aktywność TrASPA w tych narządach również wykazuje nieznaczne zwiększenie po napromieniowaniu zwierząt. Aktywności TrALA i TrASPA po przeliczeniu na mg azotu białkowego wykonywane w części materiału również pokrywają się z obszarem wartości normalnych, nie wykazując statycznie pewnej zależności czynności tych enzymów od napromieniowania zwierząt.

DYSKUSJA

W przeciwieństwie do innych enzymów, u których łatwo stwierdzić można wpływ promieniowania, transaminazy są enzymami o stosunkowo małej promienioczułości [7, 8] i trudno jest stwierdzić istotne zmiany ich aktywności po napromieniowaniu. Wahania zawartości enzymu w narzą-

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dach zwierząt zdrowych są tak wielkie, że rozrzuty wartości uzyskanych u różnych osobników są przeważnie większe aniżeli ewentualne zmiany po napromienieniu [1]. Podobne trudności nasunęły się również przy interpretacji naszych wyników.

Oznaczenia TrALA wykonane w analogicznych warunkach przebiegu choroby popromiennej u szczurów jak w naszej pracy na temat wpływu promieniowania na czynność dehydrazy ALA [4] nie wykazują zwiększenia aktywności enzymu, jak w przypadku dehydrazy ALA, a nawet sugerują, że promieniowanie zmniejsza aktywność TrALA w śledzionie, a w niektórych przypadkach i w nerce. W wątrobie natomiast w niektórych przypadkach obserwowano tendencję wzrostową aktywności TrALA. Statystycznie różniących się wartości aktywności TrALA w obu grupach zwierząt (kontrolnych i napromienianych) nie uzyskano. Różnic takich nie wykazują również badania innych autorów [1, 8].

Również w przypadku TrASPA aktywności oznaczone w narządach zwierząt napromienianych leżą w obszarze wartości normalnych (średnia $\pm 3\sigma$). W przeciwieństwie do TrALA jednak TrASPA — kwas α-ketoglutarowy wykazuje tendencję wzrostową po napromieniowaniu. Zachowanie się tej transaminazy, wybranej dla porównawczego oznaczania, jest w zasadzie zgodne z obserwowanymi przez Brina i McKee [2] zmianami transaminazy w wątrobie po napromienieniu zwierzęcia.

Zachowanie się TrALA pod wpływem napromienienia zwierząt różni się od zachowania się TrASPA, nawet jeśli wyniki przeliczone zostaną na zawartość azotu białkowego. Fakt ten popiera w pewnym stopniu postulowaną poprzednio [6] specyficzność TrALA.

Poprzednie nasze badania wykazały, że enzym katalizujący przemianę kwasu δ-aminolewulinowego w porfobilinogen i skierowujący go na drogę biosyntezy porfiryn i hemu — dehydraza kwasu δ-aminolewulinowego ulega w śledzionie i szpiku kostnym napromienionych zwierząt zwiększeniu w pierwszych dniach po napromienieniu dawką 50—100 r i w drugim, regeneracyjnym okresie choroby popromiennej (18—21 dni po dawce 400 r). Zakładając, w wyniku prowadzonych badań nad transaminacją ALA, że proces ten jest konkurencyjnym procesem przemiany ALA w stosunku do syntezy porfobilinogenu, można było oczekiwać mniejszej aktywności TrALA w warunkach zwiększonej syntezy hemu, tzn. w warunkach zwiększenia funkcji dehydrazy ALA. Duże rozrzuty aktywności TrALA nie pozwalają jednak na wyciągnięcie jednoznacznych wniosków i tym samym na bezwzględne stwierdzenie przeciwstawnego w stosunku do dehydrazy ALA zachowania się transaminazy ALA w narządach napromienianych zwierząt. Brak wyraźnego zwiększenia aktywności tego enzymu po napromienieniu szczurów, przy tendencji wzrostowej TrASPA stwier-

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dzonej w tych warunkach, upoważnia nas jednakże do przypuszczenia, że konkurencyjna rola TrALA w stosunku do dehydrazy ALA w przemianie kwasu δ-aminolewulinowego nie może być wyłączona.

Asystentom technicznym, D. Rzepniewskiej, Z. Długoborskiej, W. Trojanowskiej i K. Radziszewskiemu składamy podziękowanie za pomoc w seryjnych analizach wykonywanych w toku niniejszej pracy.

STRESZCZENIE

Aktywność transaminacji kwasu δ-aminolewulinowego i kwasu asparaginowego badana w homogenatach wątroby, nerki i śledziony szczurów napromienionych dawką 50, 400 lub 650 r różni się jedynie nieznacznie od wartości oznaczonych w tych narządach zwierząt nie napromienionych. Wartości TrALA są przy tym nieco niższe, a TrASPA nieco wyższe od normalnych.

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THE INFLUENCE OF X-IRRADIATION ON δ-AMINOLAEVULIC AND ASPARTIC ACID TRANSAMINATION IN SOME ORGANS OF THE RAT

Summary

δ-Aminolaevulic and aspartic acid transamination has been examined in homogenates of liver, kidney and spleen of rats after whole body irradiation with doses of 50, 400 or 650 r. It could be stated only insignificant differences between those values and corresponding activities in organs of non-irradiated animals. TrALA activity after irradiation has tendency to decrease and TrASPA activity to increase.

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ARGINASE IN *CELERIO EUPHORBIAE*

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The work of Garcia, Tixier and Roche [6] pointed to the presence of the ornithine cycle components and to the enzymatic degradation of arginine to ornithine in insects such as *Bombyx mori, Apis mellificia* and *Locusta migratoria.* They failed, however to demonstrate the accompanying urea formation. Kilby and Neville [9] found in 1957 a rather high arginase activity in *Locusta,* resulting in production of urea and ornithine. Desai and Kilby did not find any arginase activity in *Calliphora* larvae [4].

The present work aimed at researches of arginase in *Celerio euphorbiae* and at a study of its activity in various tissues during different stages of the insect's growth.

EXPERIMENTAL AND RESULTS

Feeding caterpillars, "running" caterpillars, "spindle-forms", two months old pupae and the adult moths of *Celerio euphorbiae* were used. Whole insects or isolated tissues were homogenized in a glass homogenizer with 2—10 volumes of distilled water, according to the expected activity.

Urea contents was determined by (I) the Fosse's xanthydrol method as modified by Engel and Engel [5]. Xanthydrol was prepared from xanton, according to the Werner's method described by Greenberg [7]. It was then dissolved in methanol at some few days intervals and stored at 4°. (II) with urease, ammonium being estimated by Conway's method [2]. Soluble urease (E. R. Squibb and Sons, New York) and arginine (Chemapol, Prague, Czechoslovakia) were used.

The preliminary experiments were carried out on homogenates of caterpillars in the last instar and of adult moths. The formation of urea was assumed to be an evidence for the arginase activity.

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The incubation mixture consisted of 0.5 ml. of the 0.85 M-arginine solution (425 μmoles) adjusted to pH 9.5 and 1 ml. of the homogenate, containing 100 to 300 mg. of tissue, according to its enzymatic activity. The mixture was incubated for 10 min. at 25°. Two control tests were incubated at the same time. One of them contained arginine solution at pH 9.5, the other homogenate only. The reaction was stopped by the addition of 2 ml. 87% acetic acid. The precipitated protein was removed by centrifugation, and urea estimated by means of xanthydrol in the supernatant. No urea was found in either control test, it was found every time when arginine was incubated with the whole insect homogenates.

Table 1

Estimation of urea by xanthydrol and by urease method after the incubation of fat-body and muscle homogenate of C. euphorbiae with arginine

0.5 ml. 0.85 M-arginine, 1 ml. homogenate; pH 9.5, incubation 2-hr.

Experiment No.	μmoles of urea formed	
	xanthydrol method	urease method
1	5.3	5.4
2	4.0	4.2

To confirm the urea formation and to check the values obtained by xanthydrol, the urease method was also applied. 1 ml. of the homogenized fat bodies and muscles of adult moth was incubated with 0.5 ml. of arginine solution at pH 9.5 for 2 hours. Protein being removed, the filtrate was divided into two parts. Urea was estimated by xanthydrol method in one of them, while the other was adjusted to pH 7.0. Then, the 0.5 ml. samples were placed in Conway's diffusion dish, added with 0.2 ml. phosphate buffer pH 7.00 and 0.3 ml. of the urease solution. The incubation was carried out for 3 hours at 25°. 1 ml. of the saturated K_2CO_3 solution was added, and the whole allowed to stand overnight. Nessler reagent being added, the ammonia contents was estimated colorimetrically. The values found for ammonia in control tests were accounted for, and the amount of urea was calculated. Table 1 shows the urea contents as measured by either xanthydrol or urease methods. It can be seen, that the results obtained by both methods are in accordance.

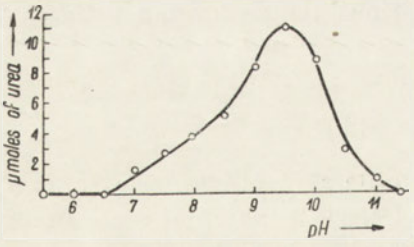

Fig. 1. The effect of pH upon the arginase activity in fat-body of feeding caterpillars *C. euphorbiae*. Homogenate 0.5 ml; 0.85 M-arginine solution, 0.5 ml; 0.2 M-phosphate or glycine buffer 0.5 ml.

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To follow the effect of pH upon the arginase activity the homogenized fat bodies of feeding caterpillars were used. Phosphate buffers were applied for pH 5.0 to 7.0, whereas glycine buffers for pH 7 to 11.5. Results (Fig. 1) indicated pH 9.5 as an optimum for arginase activity, according with the data referred to for arginase from other sources.

The distribution of the arginase activity in various tissues of *Celerio euphorbiae* was studied during different stages of its growth. Results listed in Table 2 show, that the arginase activity has been found in all examined stages of growth. It has been present in fat-body and muscles but not in haemolymph. The highest activity has been found in the fat--body of feeding caterpillars and of adult moths.

Table 2

Arginase activity in various tissues of C. euphorbiae in different stages of its growth

10 ml. of homogenate, 0.5 ml. 0.85 M-arginine solution, pH 9.5, incubation 10 min.

Age	Experiment No.	Urea formed (μmoles/g. of tissue)		
		fat-body	muscles	haemolymph
Feeding caterpillars, last instar	1	10.8	6.0	0.0
	2	11.1	5.3	0.0
	3	12.2	3.8	0.0
Running caterpillars	1	5.8	3.9	0.0
	2	4.4	4.0	0.0
	3	6.3	4.5	0.0
Spindle forms	1	5.8	3.7	0.0
	2	4.4	4.0	0.0
	3	4.7	—	—
Pupae*	1	3.3		0.0
	2	2.5		0.0
Adult moths	1	9.7	4.2	—
	2	10.5	3.2	—

* The homogenate was preparated from fat-body and muscles altogether.

DISCUSSION

The enzymatic degradation of arginine to urea is rather an unexpected process in insects, since uric acid is known to be the end product of their protein metabolism.

The contribution of urea to the uric acid synthesis as referred to by Leifert [10] has not been supported by other authors. The investigations of Desai and Kilby [3] as well as those of Anderson and Patton [1] did not show any increase of uric acid in the presence of urea nor any other

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simple compound. On the other hand, Heller and Jeżewska [8] have shown in this Laboratory that the uric acid synthesis in the Oak feeding Silkworm follows the pattern given by Levenberg, Helnick and Buchanan [11] and generally accepted for higher animals.

The papers quoted above [6, 9] point to the presence of arginase in some insects during their all life, while in certain stages only in others. Some elucidation of the problem may be expected only when more experimental data were gathered and confronted with the other known information dealing with the metabolism of the given insect and stage.

As yet it is possible only to speculate that there may exist some relationship between the arginase activity and the metabolic pathway leading from ornithine to orotic acid and pyrimidine bases. In favour of such a suggestion it may be quoted that Lieberman and Kornberg [12] have shown that orotic acid may be degradated to dihydroorotic acid and ureido-succinic acid.

SUMMARY

The arginase activity has been found in fat-body and in muscles of the *Celerio euphorbiae* at various stages of its life. No activity has been found in the haemolymph.

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ARGINAZA U MOTYLA *CELERIO EUPHORBIAE*

Streszczenie

Stwierdzono aktywność arginazy w ciele tłuszczowym i mięśniu u motyla *Celerio euphorbiae* w różnych stadiach rozwojowych. Nie znaleziono jej natomiast w hemolimfie owada.

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J. FRENDO, A. KOJ i J. M. ZGLICZYŃSKI

PRZEMIANY ZWIĄZKÓW SIARKOWYCH W PŁYTKACH KRWI LUDZKIEJ. TWORZENIE TAURYNY

Zakład Chemii Fizjologicznej A. M. w Krakowie
Kierownik Zakładu: prof. dr B. Skarżyński

Płytki krwi ludzkiej są komórkami niezbyt dokładnie poznanymi zarówno pod względem składu chemicznego jak i przemian enzymatycznych. Dotychczasowe badania nad zawartością i rolą rozmaitych organicznych związków siarki w płytkach mają charakter fragmentaryczny. Pierwszy Chargaff [7], a później Numata [14] zwrócili uwagę na związki siarkowe w trombocytach. Następnie Szirmai [16] oraz Koppel i Olwin [11] donieśli o wybitnej aktywacji dehydrogenaz i metabolizmu gazowego płytek pod wpływem cysteiny i glutationu. Dopiero jednak chromatograficzna analiza cytolizatów i hydrolizatów płytkowych dokonana przez Morita i Asada [13] rzuciła więcej światła na to zagadnienie. Według tych autorów płytki zawierają m. in. wolną cystynę, kwas cysteinowy, metioninę, glutation oraz pokaźne ilości wolnej tauryny. Zawartość tauryny jest tak znaczna, że badacze japońscy uznali ją za substancję wyróżniającą trombocyty od innych elementów morfotycznych krwi. Fakt ten pozwala na postawienie pytania, jakie jest pochodzenie tauryny w płytkach, a więc czy jest ona tylko transportowana, czy też wytwarzana czynnie na drodze odpowiednich przemian enzymatycznych. Doświadczenia Truhaut i Clanet [18] ze znakowaną tauryną wstrzykiwaną dożylnie szczurom wskazują na możliwość wybiórczego gromadzenia się tauryny w płytkach. Natomiast zawartość innych połączeń siarkowych, mogących być prekursorami tauryny wskazuje raczej na endogenne pochodzenie tego związku.

W pracy naszej pragnęliśmy określić ilościowo zawartość tauryny w płytkach krwi ludzkiej, rozstrzygnąć czy tauryna może być w płytkach wytwarzana, a jeśli istotnie powstaje, to na jakiej drodze i jakie związki są dla niej substancjami macierzystymi. Mechanizm powstawania tauryny w organizmie zwierzęcym jest ciągle dyskutowany. Opierając się na doświadczeniach Bernheim [5], Virtue [19], Blaschko [6] oraz Medes

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i Floyd [12], wielu autorów uważa, że tauryna powstaje przez utlenienie cysteiny do kwasu cysteinowego i późniejszą jego dekarboksylację. Natomiast badania wykonane w ostatnich latach przez Bergeret, Chatagner i Fromageot [3, 4] oraz Awapara i Wingo [1, 2], wskazują na centralną rolę kwasu cysteinosulfinowego. Kwas ten ma ulegać dekarboksylacji do hypotauryny, a ta dalej utleniać się do tauryny.

Zdolność tworzenia tauryny przez tkanki można badać dwojako: albo metodami manometrycznymi śledzić procesy dekarboksylacji kwasu cysteinowego i cysteinosulfinowego, lub też w inny sposób oznaczać ilościowo przyrost tauryny. W naszych badaniach z trombocytami stosowaliśmy oba sposoby postępowania.

METODY I PRZEBIEG DOŚWIADCZEŃ

Wyosabnianie płytek

Zawiesinę płytek użytą w naszych doświadczeniach otrzymywano z 40 ml świeżej krwi ludzi zdrowych. Krew była mieszana z 5% wodnym roztworem versenu [8] w stosunku 9 : 1 i wirowana dla otrzymania osocza bogatego w płytki. Osocze zbierano cienką pipetką i przez dalsze wirowanie otrzymywano osad płytkowy, który przemywano dwukrotnie izotonicznym roztworem NaCl. W przebiegu doświadczeń płytki pozostawały zawieszone w roztworze soli fizjologicznej z dodatkiem (3 : 1) buforu fosforanowego o pH 7,3. W dwóch eksperymentach zamiast roztworu NaCl użyto płynu Krebsa-Ringera fosforanowego. Zawiesina płytek o końcowej objętości około 1 ml zawierała zwykle 2—3 miliony płytek w mm^3 liczonych metodą Reesa-Eckera [17]. Zanieczyszczenie leukocytami i erytrocytami nie przekraczało w sumie 0,1% ogólnej ilości komórek. W części doświadczeń aparatura szklana była powlekana silikonem; nie stwierdzono jednak różnic w otrzymywaniu płytek ani w wynikach. Płytki otrzymane w opisany sposób wykazywały przemianę gazową przez okres ponad 10 godzin gdy badano ich oddychanie w nurkach Kartezjusza.

Metody manometryczne

Dekarboksylację kwasu cysteinowego i cysteinosulfinowego badano gazometrycznie jako ilość wytworzonego CO_2, inkubując przez kilka godzin płytki z substratem w nurkach Kartezjusza. Używano serii 6 nurków o stałych od 11—13 μl. Objętość kropli dennej z zawiesiną płytkową i substratem wynosiła 1,2 μl. Dla uzyskania warunków beztlenowych nurki przedmuchiwano pod wodą przez 10 min. atmosferą azotu. Pozostałe szczegóły techniki nurków nie odbiegały od zasad podanych w podręczniku Glicka [9].

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Ilościowe oznaczanie tauryny

Oznaczanie zawartości tauryny w płytkach. Próbkę zawiesiny płytkowej o objętości 0,3 ml cytolizowano przez zamrożenie i szybkie ogrzanie oraz następną inkubację w ciągu 20 min. z kroplą toluenu [11]. Cytolizat denaturowano przez zagotowanie z kwasem octowym i przepuszczano przez kolumnę Zeo-karbu 225 w postaci wodorowej. W tych warunkach żywica zatrzymuje sole i aminokwasy, a tauryna i kwas cysteinowy przechodzą ilościowo do przesączu [15]. Przesącz zagęszczano do sucha w próżni, a następnie rozpuszczano w 0,2 ml redestylowanej wody. Próbkę tego roztworu o objętości 15 μl nakładano brzeżnie na krążek bibuły Whatman nr 1 i rozprowadzano przez 15 godz. w komorze chromatograficznej w układzie: woda-butanol-kwas octowy (5:4:1) lub w układzie fenol-woda (7:3) z oksyizochinoliną i w parach amoniaku. Plamy na chromatogramie wykrywano przez barwienie ninhydryną [10]. Ilościowe oznaczenie tauryny, a także kwasu cysteinowego i cysteinosulfinowego, przeprowadzano przez pomiar ekstynkcji alkoholowych eluatów plam z bibuły używając spektrofotometru fotoelektrycznego marki Coleman lub Hilger. Błąd metody oznaczony na trzech równolegle badanych próbkach płytek z krwi jednego osobnika wyniósł $\pm$ 3,3%.

Badanie tworzenia tauryny przez płytki

W tym celu 0,3 ml zawiesiny płytek inkubowano z dodatkiem 0,02 M-roztworu cysteiny albo kwasu cysteinowego lub kwasu cysteinosulfinowego w ciągu 15 godz. w powietrzu lub w atmosferze azotu w temp. 37°. Roztwory substratów przyrządzano każdorazowo świeże przez rozpuszczenie substancji w buforze fosforanowym o pH 7,3. Używana w naszych doświadczeniach cysteina pochodziła z firmy La Roche, kwas cysteinowy z firmy Light, a cysteinosulfinowy łaskawie użyczył prof. Blaschko z Oxfordu. W kilku doświadczeniach czas inkuacji skrócono do 2 godzin. Po okresie inkubacji próbki były cytolizowane i oznaczano taurynę w sposób podany wyżej. W każdym doświadczeniu ilość tauryny w płytkach inkubowanych porównywano z zawartością tego składnika w płytkach bez inkubacji (ślepa).

Metody izotopowe

Zdolność tworzenia tauryny przez płytki staraliśmy się potwierdzić używając w dwóch doświadczeniach cysteiny znakowanej ^{35}S (preparat otrzymany z Instytutu Badań Jądrowych w Warszawie) dodanej do 0,02 M-roztworu cysteiny zwykłej w buforze fosforanowym o pH 7,3. Aktywność właściwa naszego preparatu była niewielka i wynosiła około

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$1{,}0 \times 10^5$ imp./mg/min. Płytki z dodatkiem tego substratu były inkubowane przez 15 godzin, a ich cytolizat przygotowywano do oznaczeń chromatograficznych w sposób opisany wyżej. Na chromatogramie oznaczano rozmieszczenie połączeń radioaktywnej siarki umieszczając odpowiednie fragmenty bibuły pod okienkiem licznika G. M. Jednocześnie sporządzano autoradiogram przez dwutygodniową ekspozycję filmu rentgenowskiego na działanie promieniowania β wysyłanego przez radioaktywną siarkę zlokalizowaną w plamach chromatogramu.

WYNIKI DOŚWIADCZEŃ

Wykonaliśmy 27 oznaczeń zawartości wolnej tauryny w płytkach krwi 24 zdrowych ludzi, znajdując, że średni poziom tej substancji w przeliczeniu na 1 płytkę wynosi $7{,}8 \times 10^{-8}$ μg, przy najniższej stwierdzonej wartości $5{,}0 \times 10^{-8}$ μg i najwyższej 11×10^{-8} μg oraz standardowym odchyleniu $\pm 1{,}5 \times 10^{-8}$ μg. W przeliczeniu na wagę komórek daje to około 1,8 mg tauryny na 1 g mokrej masy płytek. Oznaczono również stosunek tauryny do azotu płytkowego; okazuje się, że azot tauryny stanowi w przybliżeniu 1% azotu ogólnego oraz 15% azotu pozabiałkowego.

W 4 doświadczeniach badano przy pomocy nurków Kartezjusza dekarboksylację kwasu cysteinowego i cysteinosulfinowego przez płytki w atmo-

Tablica 1

Zmiany zawartości tauryny w płytkach po 15 godz. inkubacji z kwasem cysteinowym

Warunki doświadczeń podane w tekście

Nr dośw.	Przyrost (+) lub ubytek (—) tauryny w %	Uwagi
1	+ 20	
2	+ 10	
3	0	
4	— 10	
5	0	Po 2 godz. inkubacji
6	+10	Po 2 godz. inkubacji
7	+ 20	
8	— 15	Inkubacja w płynie Krebsa-Ringera-fosf.
9	+ 20	
10	— 10	
11	+ 10	Inkubacja w atmosferze $N_2 + CO_2$

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sferze powietrza i azotu. Nie stwierdzono wyraźnego wytwarzania CO_2 przy inkubacji z żadnym z tych substratów. Zatem za pomocą nurków nie udało się wykazać wytwarzania tauryny przez płytki.

W 11 doświadczeniach oznaczano poziom tauryny w płytkach po inkubacji z kwasem cysteinowym i w 6 przypadkach uzyskano przyrost tauryny o 5—20% w porównaniu ze ślepą. W pozostałych doświadczeniach ilość tauryny po inkubacji była równa lub nawet mniejsza niż w ślepej. (Tabl. 1). Natomiast w toku doświadczenia zaznaczał się prawie zawsze wyraźny spadek ilości dodanego kwasu cysteinowego.

Cysteinę jako substrat użyto w 12 eksperymentach i w 9 przypadkach uzyskano po inkubacji przyrost zawartości tauryny o około 20—40%. (Tabl. 2).

Tablica 2

Zmiany zawartości tauryny w płytkach po 15 godz. inkubacji z cysteiną

Warunki doświadczeń podane w tekście

Nr dośw.	Przyrost (+) lub ubytek (—) tauryny w %	Uwagi
1	+ 20	Inkubacja w atmosferze $N_2 + CO_2$
2	+ 10	
3	+ 30	
4	0	
5	+ 33	
6	+ 15	Po 2 godz. inkubacji
7	0	
8	+ 40	
9	0	Inkubacja w płynie Krebsa-Ringera-fosf.
10	+ 35	
11	+ 20	Inkubacja w płynie Krebsa-Ringera-fosf.
12	+ 40	

Jeśli inkubowano równolegle dwie próbki płytek, jedną z kwasem cysteinowym a drugą z cysteiną, znajdowano większy przyrost ilości tauryny w próbce z cysteiną. Przykład typowego doświadczenia przedstawiono w tablicy 3.

Wytwarzanie tauryny przez płytki potwierdzono używając cysteiny znakowanej ^{35}S; po inkubacji plama tauryny wykazywała promieniowanie rzędu 100 imp./min. Jest rzeczą ciekawą, że na chromatogramie znaleziono także inne, bliżej nieokreślone substancje radioaktywne, nie dające reakcji z ninhydryną i wędrujące szybciej niż tauryna w układzie butanolowym.

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Dane uzyskane za pomocą licznika G. M. potwierdziła analiza autoradiogramu. Wykazano na tej drodze oprócz prążka tauryny powstawanie przynajmniej dwóch radioaktywnych substancji w toku inkubacji z cysteiną znakowaną.

Tablica 3

Tworzenie tauryny przez płytki w doświadczeniu z dnia 11.XI.58 r.

Zawiesina płytek	Sposób przygotowania próbki	Wyniki w μg na 54×10^6 płytek	
		tauryna	kw. cysteinowy
Krew grupy O 2 600 000 płytek/mm^3 1 100 erytr./mm^3 700 leuk./mm^3	Ślepa Płytki cytolizowane i denaturowane + kw. cysteinowy i cysteina	4 3	68
	Próbka I Płytki inkubowane z cysteiną w ciągu 15 godz. w powietrzu	6.3	ślad
	Próbka II Płytki inkubowane z kw. cysteinowym w ciągu 15 godz. w powietrzu	5,0	60

Tablica 4

Tworzenie tauryny przez płytki po 15 godz. inkubacji z kwasem cysteinosulfinowym

Nr dośw.	Ilość płytek w 0,3 ml zawiesiny	Zawartość tauryny w próbce ślepej (μg)	Zawartość tauryny w próbce inkub. (μg)	Przyrost tauryny w (%)
1	72×10^7	50,4	74,9	50
2	81×10^7	63,2	81,8	30

Wreszcie przeprowadzono dwa oznaczenia, używając jako substratu kwasu L-cysteinosulfinowego. Mała ilość doświadczeń z tym substratem wynikła z faktu, że dysponowaliśmy ograniczoną ilością tego związku. W doświadczeniach tych uzyskano po inkubacji przyrost ilości tauryny o około 40% w stosunku do ślepej (Tabl. 4); cyfrę tę trudno uznać za pewną z uwagi na zbyt małą ilość oznaczeń. Zestawienie użyteczności rozmaitych substratów dla produkcji tauryny przedstawia rys. 1.

Przebadano również wpływ środowiska inkubacyjnego na tworzenie tauryny. Użycie płynu Krebsa-Ringera fosforanowego lub dodatek osocza,

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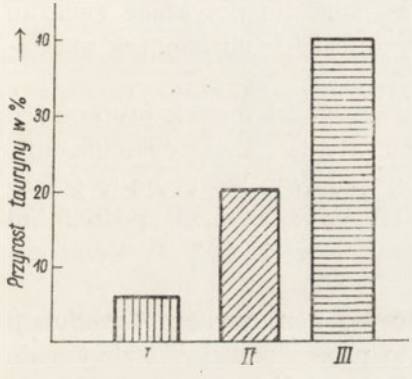
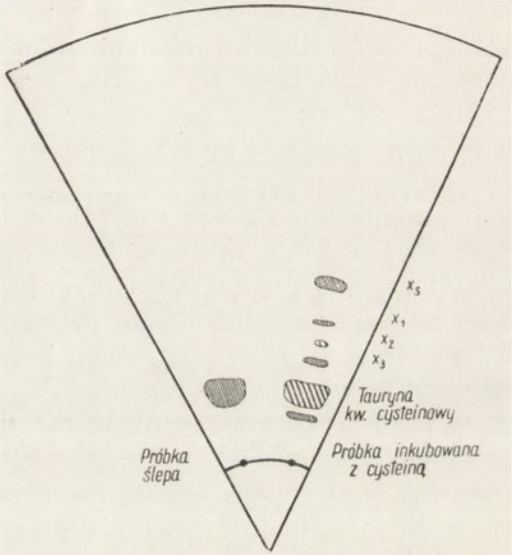
a także rodzaj atmosfery gazowej w toku inkubacji nie wpłynął w sposób widoczny na wynik doświadczeń.

Skrócenie okresu inkubacji do 2 godzin zastosowane w trzech doświadczeniach dało mniejszy przyrost tauryny. Zatem wytwarzanie tauryny przez płytki wydaje się być procesem przebiegającym stopniowo i powoli.

Na większości chromatogramów cytolizatów płytek czystych przepuszczonych przez kolumnę Zeo-karbu oprócz plamy tauryny zaobserwowano ślad kwasu cysteinowego oraz 4 prążki substancji barwiących się



Rys. 1. Średni przyrost tauryny po inkubacji: *I* — z kwasem cysteinowym; *II* — z cysteiną; *III* — z kwasem cysteinosulfinowym



Rys. 2. Wycinek typowego chromatogramu przedstawiony schematycznie

ninhydryną, których rozmieszczenie na chromatogramie przypomina niezidentyfikowane związki z autoradiogramu. Prążki te oznaczono na rys. 2 jako X_1, X_2, X_3, X_S.

Ustalono R_F tych substancji w układzie butanolowym na: $X_1 = 0{,}32$, $X_2 = 0{,}28$, $X_3 = 0{,}22$ oraz $X_S = 0{,}4$ (R_F tauryny $= 0{,}16$). Przechodzenie tych związków przez Zeo-karb przemawia za ich kwaśnym charakterem, a reakcja z ninhydryną za obecnością grup aminowych. Substancje *X* nie dają dodatniego odczynu Pauliego, charakterystycznego dla większości polipeptydów, ani nie wykazują fluorescencji lub absorpcji w świetle ultrafioletowym. Po potraktowaniu 5% roztworem H_2O_2 nałożonej próbki na chromatogramie przed rozprowadzeniem w butanolu, substancje X_1 i X_2 ulegają przemieszczeniu i znajdują się wtedy jako jeden prążek poniżej tauryny. Wykonane dodatkowo doświadczenie pozwala przypuszczać, że prążek X_3 odpowiada zredukowanemu glutationowi. Najwyraźniejsze

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prążki substancji *X* otrzymano przy inkubacji płytek z cysteiną w atmosferze powietrza, ale znaleźć można je było również na chromatogramach płytek nieinkubowanych. Choć nie udało się zidentyfikować wszystkich tych związków, zmienność ich występowania wydaje się wskazywać na powiązanie ze stanem funkcjonalnym płytek.

DYSKUSJA

Stwierdzenie stałego poziomu wolnej tauryny w płytkach krwi ludzkiej, jej wysoka zawartość w przeliczeniu na masę płytek i zawartość azotu oraz możliwości jej powstawania w tych elementach morfotycznych przemawiają za istotną rolą tego związku w metabolizmie płytek. Jednakże za wcześnie jest wysuwać jakieś sugestie dotyczące związku tauryny z zasadniczą funkcją trombocytów, to jest ich udziałem w mechanizmie hemostazy.

Doświadczenia dowiodły wyraźnie, że dla całych komórek płytkowych najlepszym substratem dla wytwarzania tauryny jest kwas cysteinosulfinowy i cysteina a mniej przydatnym kwas cysteinowy. Wskazuje to jednocześnie przypuszczalną drogę tworzenia tauryny: etapami pośrednimi byłyby więc, zgodnie z poglądami innych autorów [1, 2, 3, 4], kwas cysteinosulfinowy i hypotauryna.

Metodami manometrycznymi nie udało się nam wykazać produkcji tauryny, prawdopodobnie dlatego, że jest to proces powolny i zbyt mało wpływający na zmiany fazy gazowej nurka, aby stał się uchwytny. Przyjmując średnią zdolność tworzenia tauryny przez płytki na 30% pierwotnej zawartości, uzyskujemy około 2×10^{-2} μl CO_2 wyzwolonego przy dekarboksylacji. Wartość ta po uwzględnieniu stałych nurków odpowiada około 20 mm słupa płynu Brodiego w manometrze. Pamiętając o powolnym procesie tworzenia tauryny, zmiana ta może się pojawić po kilkunastu godzinach doświadczenia, gdy dyfuzja gazów i zmiany termobarometryczne utrudniają wyciągnięcie wniosków.

Stwierdzenie w toku inkubacji wyraźnego ubytku kwasu cysteinowego i cysteinosulfinowego, ubytku większego niżby to odpowiadało przyrostowi tauryny, wskazuje na inne drogi przemiany tych związków w płytkach. W kilku doświadczeniach z kwasem cysteinowym zaobserwowano spadek ilości tauryny w płytkach po inkubacji. Może to sugerować, że tauryna nie jest ostatecznym produktem przemiany związków siarkowych. Fakty te oraz dostrzeżone przez nas niezidentyfikowane substancje *X* skłaniają do przypuszczenia, że płytki dysponują wielotorowym i bogato zróżnicowanym metabolizmem. Sprawa udziału związków siarkowych w tych przemianach oraz powiązanie wytwarzania tauryny z funkcją płytek wymaga dalszych badań.

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STRESZCZENIE

Badano zawartość i tworzenie tauryny w płytkach krwi ludzkiej. Ustalono, że poziom wolnej tauryny w płytkach jest stały i wynosi średnio $7{,}8 \times 10^{-8}$ μg/płytkę. Tauryna może być w płytkach wytwarzana, przy czym substancją macierzystą dla niej jest cysteina, a droga powstawania wiedzie prawdopodobnie przez kwas cysteinosulfinowy.

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METABOLISM OF SULPHUR COMPOUNDS IN HUMAN THROMBOCYTES. TAURINE FORMATION

Summary

The concentration and the formation of taurine in human thrombocytes has been followed. It has been found that the level of free taurine in thrombocytes is rather stable and amounts 7.8×10^{-8} μg per 1 thrombocyte on an average. It is possible that taurine may arise in thrombocytes and that cysteine is the precursor while cysteinosulphinic acid is probably the intermediary step in the process.

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J. BRAHMS and CZESŁAWA RŻYSKO

PHOSPHORYLATION OF H-MEROMYOSIN IN THE COURSE OF ATP SPLITTING

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Kierownik: prof. dr W. Niemierko

The presence of phosphorus compounds in myosin and actomyosin was established by Smith [2], Bailey [1], Buchtal *et al.* [7, 8], Lajtha [12], Mihalyi, Laki, Knoller [14]. Buchtal *et al.* [7, 8] found that one of the phosphorus fractions of myosin and actomyosin is a loosely bound orthophosphate; during incubation of myosin with ATP this orthophosphate fraction considerably increases.

In our previous investigation [4, 5] it was found that the amount of protein-bound orthophosphate increases at the beginning of incubation of myosin with ATP and diminishes towards the end of the enzymic process. These facts suggested the possibility of the formation of a phosphorylated protein as an intermediate. It seemed to be interesting to investigate the mechanism of ATP splitting by H-meromyosin which, being a fragment of the myosin molecule, retains the ATP-ase activity. The purpose of the present investigation was to find out whether a phosphorylated H-meromyosin may also be formed. A preliminary account of the work has already appeared [6].

METHODS

Actin free L-myosin three times precipitated was preparated from rabbit muscle according to Perry [16]. The absence of actin was controlled by the viscosimetric test (absence of changes of viscosity after addition of ATP). H-meromyosin was prepared according to the method of Szent-Györgyi [20] with a slight modification. The procedure was as follows.

The following abbreviations will be used in this paper: ATP = adenosinetriphosphate, ADP = adenosinediphosphate, p = inorganic phosphate, DFP = diisopropyl fluorophosphate, tris = tris(hydroxymethyl)aminomethane, DNP=2,4-dinitrophenol, PCMB = = p-chloromercuribenzoate.

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Myosin was incubated with trypsin and the digestion was stopped by the addition of DFP. The solution obtained after trypsin digestion was dialyzed against 0.0067 M-tris buffer, pH 7.0 overnight in the cold. The H-meromyosin (crude product) was separated from L-meromyosin by a short centrifugation and purified by ammonium sulfate fractionation. The solution was brought to 42% ammonium sulfate saturation; the slight precipitate was removed by centrifugation. Ammonium sulfate was added to the solution to bring the saturation to 55%; the precipitate formed was collected by centrifugation, redissolved in 0.0067 M neutral tris buffer and dialyzed against the same buffer. The solution obtained contained the purified H-meromyosin.

ATP was from Light and Co. ltd. or from Instytut Farmaceutyczny (Warszawa); both preparations contained about 50%—60% ATP; Glutathione was from Fluka.

H-meromyosin solution (about 15 mg. in 3 ml. final volume) was incubated with 0.1 M-KCl-glycine buffer, pH 9.0 and 0.002 M-ATP at 20°. The enzymic process was stopped by precipitating H-meromyosin by addition of $(NH_4)_2SO_4$ to 55% saturation and cooling to 0°. The precipitated H-meromyosin was separated by centrifugation at 17.000× g and washed twice with $(NH_4)_2SO_4$ 55% saturation.

"Free" orthophosphate was estimated in the supernatant directly, and the protein-bound orthophosphate was determined in the precipitated H-meromyosin after treatment with cold 10% trichloracetic acid. The inorganic phosphate was determined by the Berenblum and Chain method as modified by Martin and Doty [13]. The ATP-ase activity was determined according to Perry and expressed in Q_P units [16].

RESULTS

Fig. 1 shows that during enzymic splitting of ATP by H-meromyosin binding of orthophosphate to protein occurs. The amount of orthophosphate bound to protein is high during the initial period of incubation with ATP but diminishes towards the end of the enzymic process.

The following results of our experiments seem to indicate that the binding of orthophosphate by H-meromyosin is not a simple adsorption:

1. At the end of ATP splitting a decrease in the content of orthophosphate bound to protein could be observed although the solution contained at this moment the highest concentration of orthophosphate.

2. H-meromyosin preparations with a very low ATP-ase activity did not bind the orthophosphate which was always present in a small quantity

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in the added ATP solution (Fig. 2). The same results were observed with H-meromyosin after its inactivation by treatment with 0.0005 M p-chloromercuribenzoate (Fig. 4).

3. When ATP was substituted by ortophosphate in equimolar concentration the precipitated H-meromyosin contained only a very small amount of orthophosphate (Fig. 1) which did not change during incubation.

The question arises whether the observed binding of orthophosphate to protein is due only to H-meromyosin or is a result of the presence of

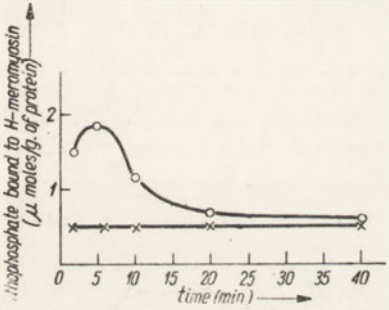

Fig. 1. Binding of orthophosphate to H-meromyosin during incubation with ATP (o) or with orthophosphate (x) (ATP-ase activity: $Q_p = 2100$). The mixture consisted of: 2.3 ml. of protein solution (0.5 — 1%); 0.2 ml. glutathione (3 mg/ml.); 0.1 ml. 10^{-1} M-$MgCl_2$; 0.1 ml. of 0.1M-KCl--glycine buffer pH 9.0; 0.3 ml. $2 \cdot 10^{-2}$M-ATP or -orthophosphate. The solutions of protein, gluthatione and ATP were adjusted to pH 9.0

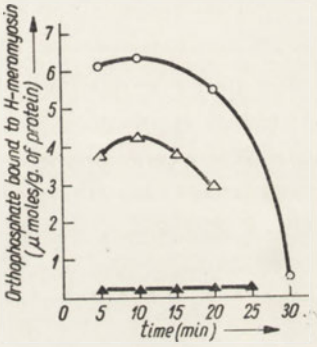

Fig. 2. Binding of orthophosphate to H-meromyosin preparations of different activity

○ $Q_p = 6320$;

△ $Q_p = 4000$;

▲ $Q_p = 0$

some other protein which may contaminate the H-meromyosin preparations. Using different H-meromyosin preparations it was observed that the maximum amount of orthophosphate bound to protein depends upon, and is to a certain extent proportional to the ATP-ase activity of the preparation (Fig. 2 and 3).

In the presence of 0.005 M-glutathione an increase of the ATP-ase activity was observed as well as an increase of the maximum amount of orthophosphate bound to protein (Table 1). *p*-Chloromercuribenzoate ($5 \cdot 10^{-4}$ M) inhibited completely both the ATP-ase activity and the binding of orthophosphate to H-meromyosin (Fig. 4). Magnesium ions 0.005 M, which decrease the rate dephosphorylation of ATP [15, 3, 10], activated the binding of orthophosphate to H-meromyosin (Fig. 4). Addition of 2,4-dinitrophenol ($5 \cdot 10^{-3}$ M) increased the dephosphorylation of ATP twofold. This is in agreement with the results of Greville and Needham

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Table 1

Influence of glutathione on ATP-ase activity and the binding of orthophosphate to H-meromyosin

	ATP-ase activity (Q_P units)	Maximum amount of orthophosphate bound by protein (μmoles/g. of protein)
Without GSH	3.000	2.8
With GSH 0.005 M	5.700	5.6
Without GSH	1.800	1.9
With GSH 0.005 M	2.400	2.4

[11], and Perry and Cheppel [17] obtained on myosin ATP-ase. At the same time DNP diminished the amount of orthophosphate bound to H-meromyosin (Fig. 4).

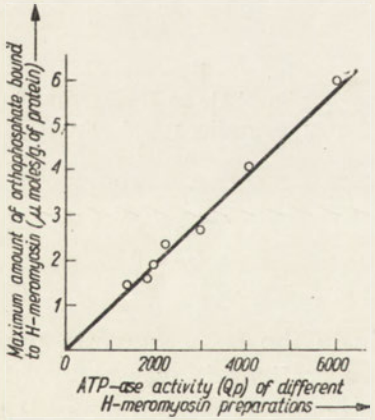

Fig. 3. Maximum content of orthophosphate in H-meromyosin in relation to its enzymic activity

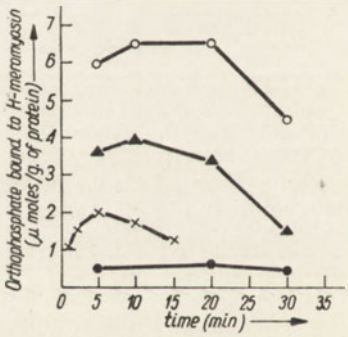

Fig. 4. Effect of Mg^{2+}, DNP and PCMB upon amount of orthophosphate bound to H-meromyosin during incubation with ATP

▲ H-meromyosin + ATP
○ H-meromyosin + ATP + Mg^{2+}
× H-meromyosin + ATP + DNP
● H-meromyosin + ATP + PCMB

DISCUSSION

The results presented above seem to indicate that during the splitting of ATP by H-meromyosin the formation of a phosphorylated protein occurs. The enzymic cleavage of ATP may be represented by the following scheme:

$$E + ATP \rightleftarrows E \cdot ATP$$
$$E \cdot ATP \rightleftarrows E \cdot P + ADP$$
$$E \cdot P \longrightarrow E + P$$

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This means that in the course of ATP splitting, after formation of the enzyme-substrate complex (E·ATP), phosphorylation of protein takes place followed by dephosphorylation.

The experiments performed show that the maximum amount of orthophosphate bound to H-meromyosin depends upon the ATP-ase activity of the different H-meromyosin preparations (Fig. 3). This may be interpreted as an indication that the rate of ATP splitting is proportional to the number of sites in the protein molecule to which orthophosphate is bound. Further evidence of a correlation between the rate of splitting of ATP and binding of orthophosphate was obtained when the binding of orthophosphate by H-meromyosin was compared with the changes in the rate of ATP hydrolysis during the enzymic process (Fig. 5). Simulta-

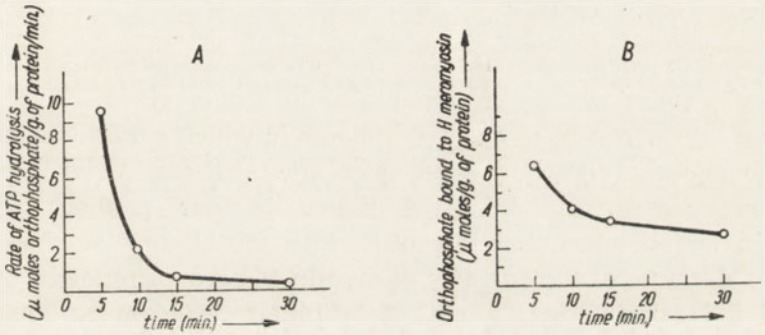

Fig. 5. Binding of orthophosphate to H-meromyosin during incubation with ATP (Figure B) compared with the rate of ATP hydrolysis (Figure A)

Conditions: 0.005 M-$MgCl_2$, 0.001 M-ATP, 0.0005 M-glutathione, 0.1 M-glycine-KCl buffer, pH 9.0, + 20°

The enzymic reaction was interrupted, by ammonium sulfate (Fig. B), and by precipitation of H-meromyosin with 10% TCA (Fig. A)

neously with the decrease in the rate of ATP hydrolysis a decrease in the content of protein-bound orthophosphate was also observed.

The opposite effect of $MgCl_2$ on the phosphorylation of protein and on the ATP-ase activity seems to indicate that the process of phosphorylation of the protein is stimulated by Mg^{2+}, whereas the process of dephosphorylation is inhibited.

In contrast to the inhibitory effect of Mg^{2+} on the ATP-ase activity, DNP acts as a stimulator of ATP splitting, but the amount of phosphate bound to the protein in the latter case is diminished as compared with that in the former. One can suppose therefore that DNP accelerates the dephosphorylation of the protein. Levy and Koshland [12a] investigated

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ATP splitting by muscle proteins in the presence of H_2O^{18} and Mg^{2+}. Their experiments gave some evidence of the existence of a phosphorylated intermediate. As has been shown the results of the present investigation, although obtained with an entirely different method, lead to the same conclusion. Some recent experiments of Cheesman and Keller [9] on the mechanical rigidities of films of myosin and actomyosin are also in good agreement with the hypothesis of the formation of a phosphorylated protein.

One can suppose that in the interaction between ATP and H-meromyosin at least two kinds of sites in the protein molecule may be involved. One of them is occupied by ATP during the formation of the enzyme-substrate complex, whereas the other is occupied by orthophosphate derived from ATP.

It seems to be possible that in the process of phosphorylation the sulfhydryl groups of the protein molecule are involved. This view is supported by the results of our experiments in which glutathione activated both the phosphorylation process and the ATP-ase activity, whereas p-chloromercuribenzoate inhibited them. In our previous work on L-myosin we observed simultaneously with the binding of phosphate to protein a decrease of the quantity of myosin-SH groups followed by a subsequent liberation of -SH groups [5]. Similar results were obtained by Pogłazov *et. al.* [18]. Snellman [19] isolated peptides containing sulfhydryl groups from a myosin hydrolysate and suggested that the sulfhydryl groups may from the active center of ATP-ase.

SUMMARY

During ATP splitting by H-meromyosin formation of a phosphorylated H-meromyosin, as a possible intermediate, was observed.

Magnesium ions ($5 \cdot 10^{-3}$ M) depress the enzymic splitting of ATP but activate the phosphorylation of H-meromyosin. DNP ($5 \cdot 10^{-3}$M) has an opposite effect: the enzymic activity of H-meromyosin is increased while phosphorylation is decreased. Glutathione activates both ATP splitting and protein phosphorylation, whereas PCMB ($5 \cdot 10^{-4}$ M) inhibits these processes.

The authors wish to thank Professor W. Niemierko for his interest and criticism.

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FOSFORYLACJA H-MEROMIOZYNU PODCZAS ROZKŁADU ATP

Streszczenie

Podczas rozkładu ATP przez H-meromiozyn obserwowano tworzenie się ufosforylowanego białka jako możliwego pośredniego produktu reakcji enzymatycznej. Jony magnezu ($5 \cdot 10^{-3}$ M) hamują enzymatyczny rozpad ATP, lecz aktywują sam proces fosforylacji białka. DNP ($5 \cdot 10^{-3}$ M) wywołuje odwrotny efekt: podwyższa enzymatyczną aktywność H-meromiozynu, natomiast obniża stopień ufosforylowania białka.

Glutation aktywuje zarówno rozpad ATP, jak i fosforylację H-meromiozynu, podczas gdy *p*-chlorortęciobenzoesan ($5 \cdot 10^{-4}$ M) jest inhibitorem obu tych procesów.

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AMINOACYDURIA WYWOŁANA KWASEM MALEINOWYM

II. WPŁYW DIETY*

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Kierownik: prof. dr Wł. Mozołowski

Przemiana kwasu maleinowego i jego działanie w ustroju zwierzęcym są mało dotychczas poznane. Doświadczenia przeprowadzone *in vivo* zdają się wskazywać, że w wyniku zatrucia kwasem maleinowym istotnym zaburzeniom ulega praca nerki [2]. Harrison i Harrison [7] w badaniach na szczurach krzywiczych stwierdzili aminoacydurię jako jeden z objawów zatrucia kwasem maleinowym. W poprzednich naszych badaniach [1] używaliśmy do doświadczeń szczury zdrowe, żywione pokarmem, który pod względem jakościowym i kalorycznym zaspokajał potrzeby dorosłych szczurów; uważaliśmy ten pokarm za dietę „fizjologiczną", która bez szkody dla zdrowia zwierzęcia zastępowała dietę naturalną. Na tej podstawie wynik zatrucia kwasem maleinowym traktowaliśmy jako wyraz działania kwasu maleinowego na zdrowe, prawidłowo odżywiane szczury.

Przez podanie kwasu maleinowego, w dawce 400 mg na 1 kg wagi ciała, dootrzewnowo wywołaliśmy aminoacydurię znacznego stopnia. Dobowa ilość wydalonego w moczu azotu α-aminowego osiągnęła, w drugim dniu po wstrzyknięciu, wartości 10-krotnie wyższe, w porównaniu ze stanem przed zatruciem. Dominującą cechą moczu zatrutych szczurów była aminoacyduria. Uzyskane wyniki stwarzały podstawę do tego, żeby w dalszych badaniach traktować aminoacydurię jako istotny wskaźnik, miernik działania kwasu maleinowego na ustrój szczura.

Ponieważ doświadczenia miały być wykonywane na większej liczbie zwierząt, a niektóre składniki mieszanki witaminów były drogie lub trudno dostępne (inozytol), próbowaliśmy zastąpić mieszankę witaminów

* Część I ukazała się w *Acta Biochimica Polonica* 5, 431, 1958.

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przez suszone drożdże piekarskie. Uzyskane wyniki skłoniły nas do wykonania dodatkowych doświadczeń, w których odtłuszczone suszone mleko zastąpiliśmy innymi składnikami. Wyniki przedstawione w tej pracy obrazują wpływ diety na aminoacydurię wywołaną przez parenteralne podanie szczurom kwasu maleinowego.

METODY

Do poszczególnych grup doświadczeń używano szczury jednej płci, o zbliżonej wadze. Szczury pochodziły częściowo z PZH w Warszawie, częściowo z I Kliniki Położn. AM w Gdańsku, w większości z własnej hodowli. Zwierzęta trzymano pojedynczo, w klatkach metabolicznych, pozwalających na ilościowe zbieranie moczu bez domieszek kału i pokarmu. Pożywienie oraz woda były udostępnione zwierzętom do woli. Zobojętniony kwas maleinowy firmy Schuchardt podawano w jednej porcji, jako jednomolowy roztwór wodny. Mocz do czasu analizy (zwyczajnie w tym samym lub następnym dniu) przechowywano z dodatkiem tymolu w lodówce. Gdy mocz zatrutych szczurów dawał dodatni odczyn z kwasem sulfosalicylowym białko usuwano alkoholem etylowym. Azot α-aminowy oznaczano w moczu metodą Yemma i Cockinga [14].

Mocz uwalniano od amoniaku w następujący sposób: Do kolbki na 25 ml odmierzano 0,5 ml wody, 0,25 ml lub 0,1 ml moczu i 0,5 ml 1 N-NaOH. Kolbki umieszczano w eksykatorze próżniowym nad stężonym H_2SO_4 na przeciąg około 60 min. Po zobojętnieniu i dopełnieniu kolbek wodą wolną od amoniaku, odmierzano do probówek po 1 ml badanego roztworu, 0,5 ml buforu cytrynianowego o pH 5,0, 0,2 ml 5% roztworu ninhydryny w metyloglikolu oraz 1 ml roztworu 0,5 mM-KCN w metyloglikolu. Probówki umieszczano we wrzącej łaźni wodnej na przeciąg 15 min. Po oziębieniu (przez 5 min.) dodawano po 10 ml 60% etanolu i kolorymetrowano przy długości fali 570 mμ. Do prób kontrolnych oraz do sporządzania odczynników używano wodę wolną od amoniaku (każdorazowo wytrząsaną przez 20 min. z permutytem). Krzywe standardowe wykreślono dla standardu glicyny.

ODŻYWIANIE SZCZURÓW

Używane w doświadczeniach diety zawierały następujące składniki: odtłuszczone suszone mleko (Farmers Co-op. Dairy Mass. USA), kazeina podpuszczkowa cz. (Spółdzielnia Chemiczna „Pokój" w Krakowie), skrobia pszenna (apteczna), sacharoza (handlowa), laktoza (przepakowana w „Cefarm" w Katowicach), olej rzepakowy (handlowy, jadalny), mieszanka soli, sporządzona według Wessona [13].

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Na 1000 g diety dodawano 1336 mg mieszanki witaminów o następującym składzie: 5,0 mg tiaminy, 9,6 mg ryboflawiny, 60,0 mg niacyny, 4,8 mg pirydoksyny, 30,0 mg pantotenianu wapnia, 100,0 mg kwasu para-aminobenzoesowego, 1,2 mg kwasu folowego, 0,4 mg biotyny, 120,0 mg inozytolu, 1000 mg choliny HCl, 5 mg menadionu (w roztworze). Oprócz tego dodawano 150 mg witaminu E, 25.000 j. m. witaminu A i 2,500 j. m. witaminu D.

Tablica 1

Skład używanych diet

Składniki	Dieta				
	mleczna			laktozowa witaminowa	bezlaktozowa witaminowa
	witaminowa	drożdżowa + wit. E	drożdżowa bez wit. E		
	I	II	III	IV	V
Odtłuszczone suszone mleko	480 g	480 g	480 g	—	—
Kazein	—	—	—	170 g	170 g
Skrobia pszenna	270 g	270 g	270 g	270 g	480 g
Sacharoza	130 g	130 g	130 g	130 g	230 g
Laktoza	—	—	—	310 g	—
Olej rzepakowy	88 g	88 g	88 g	88 g	88 g
Mieszanka soli	32 g	32 g	32 g	32 g	32 g
Razem	1000 g	1000 g	1000 g	1000 g	1000 g
Mieszanka witaminowa	1336 mg	—	—	1336 mg	1336 mg
Witamin A	25 000 j.m	25 000 j.m	25 000 j.m	25 000 j.m	25 000 j.m
Witamin D	2 500 j.m	2 500 j.m	2 500 j.m	2 500 j.m	2 500 j.m
Witamin E	150 mg	150 mg	—	150 mg	150 mg
Suszone drożdże piekarskie	—	50 g	50 g	—	—

Skład używanych diet podaje tablica I. Skład stosowanych diet opierał się na diecie I Chorążego [4], nazywanej w tej pracy dietą mleczną witaminową. Dieta ta zawiera dostateczne ilości białka, węglowodanów i tłuszczów, w odpowiednim wzajemnym stosunku (w dietach dla szczurów, stosowanych przez różnych badaczy, stosunek ten jest podobny i wynosi 2:7:1) [8, 11, 12]. Dobór jakościowy i ilościowy mieszanki soli oraz mieszanki witaminów w pełni pokrywa zapotrzebowanie dorosłych szczurów. Specjalną cechą tej diety jest duża zawartość laktozy (30% pokarmu i przeszło 3/7 zawartych w nim węglowodanów).

W części doświadczeń mieszanka witaminów została zastąpiona przez suszone drożdże piekarskie (50 g na 1 kg diety). Dodawano wówczas tylko witaminy A, D i E. W części doświadczeń nie dodawano witaminu E.

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W jednym doświadczeniu odtłuszczone suszone mleko zastąpiono przez mieszaninę kazeinu i laktozy (przybliżony skład mleka w proszku przyjęto wg Chorążego [4]). W 1 kg diety, jako równoważnik 480 g mleka było 170 g kazeinu i 310 g laktozy.

W części doświadczeń odtłuszczone mleko zastąpiono przez mieszaninę kazeinu, skrobi i sacharozy, eliminując laktozę. W 1 kg diety jako równoważnik 480 g mleka było 170 g kazeinu, 210 g skrobi i 100 g sacharozy.

Zastąpienie mleka w proszku przez mieszaninę ściśle określonych substancji chemicznych pozwala eliminować wpływ wszystkich dodatkowych nieznanych lub bliżej nieokreślonych składników, jakie znajdują się w mleku sproszkowanym.

WYNIKI

Dootrzewnowe podanie maleinianu w dawce 400 mg na 1 kg wagi, szczurom karmionym dietą mleczną zawierającą komplet witaminów, powoduje aminoacydurię znacznego stopnia (Tabl. 2). Maksimum wydalania azotu α-aminowego przypada zwykle na 2—3 dzień po zatruciu. Wartości wydalanego na dobę azotu α-aminowego wzrastają 5 do 10 razy w porównaniu ze stanem przed zatruciem. Po tygodniu ilości wydalanego azotu α-aminowego zbliżają się do wartości normalnych.

Tablica 2

Wpływ diety mlecznej witaminowej (I) na aminoacydurię wywołaną przez dootrzewnowe podanie kwasu maleinowego

Czas stosowania diety do wstrzyknięcia kwasu maleinowego: 14 dni. Dawka kwasu maleinowego: 400 mg na 1 kg wagi, dootrzewnowo. Szczury: samice, wagi 210—225 g.

Szczur nr	Azot-α-aminowy, mg w dobowym moczu										
	dni przed zatruciem				dni po zatruciu						
	—4	—3	—2	—1	+1	+2	+3	+4	+5	+6	+7
1	5,7	4,4	4,0	4,5	9,3	12,1	27,5	12,2	6,3	8,4	8,6
2	2,0	3,3	2,8	2,8	9,0	15,3	23,0	6,5	2,6	6,0	9,4
3	1,6	4,2	2,2	2,4	5,0	5,4	11,5	8,7	6,0	10,8	9,9
4	3,5	3,1	3,0	3,0	7,7	6,6	21,5	11,7	9,5	6,0	5,9
5	3,2	3,0	4,4	3,4	6,6	15,0	19,0	8,6	8,2	7,6	7,5

W wyniku podania dootrzewnowego przynajmniej część kwasu maleinowego zostaje skierowana wprost do wątroby. Można przypuszczać, że ulega on w wątrobie przemianom powodującym osłabienie, lub zniesienie jego toksycznych własności. Podanie podskórne zapewnia przedostanie się kwasu maleinowego wprost do ogólnego krążenia. Okazało się bowiem, że podanie podskórne maleinianu w dawce obniżonej do 300 mg na 1 kg

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Tablica 3

Wpływ diety mlecznej witaminowej (I) na aminoacydurię wywołaną przez podskórne podanie kwasu maleinowego

Czas stosowania diety do wstrzyknięcia kwasu maleinowego: 5 dni. Dawka kwasu maleinowego: 300 mg na 1 kg wagi, podskórnie. Szczury: samce, wagi 265—315 g

Szczur nr	Azot-α-aminowy, mg w dobowym moczu							
	dni przed zatruciem		dni po zatruciu					
	— 2	— 1	+ 1	+ 2	+ 3	+ 4	+ 5	+ 6
6	1,8	1,5	11,0	20,4	15,6	10,3	6,6	2,2
7	1,4	1,6	2,5	3,2	14,4	5,5	3,0	1,6
8	2,5	0,8	10,3	6,4	16,8	15,7	3,1	2,3
9	1,5	1,3	4,4	7,2	11,9	22,6	5,7	1,2

wagi powoduje aminoacydurię tego samego rzędu co dootrzewnowe wstrzyknięcie 400 mg na 1 kg wagi (Tabl. 3).

Jeżeli szczury są karmione dietą bezlaktozową zawierającą komplet witaminów wówczas dootrzewnowe podanie maleinianu w dawce 400 mg na 1 kg wagi nie powoduje aminoacydurii (Tabl. 4). Wartości azotu α-ami-

Tablica 4

Wpływ laktozy w diecie na zawartość azotu-α-aminowego w moczu szczurów po zatruciu kwasem maleinowym

Czas stosowania diety do pierwszego wstrzyknięcia kwasu maleinowego 8 dni; do drugiego wstrzyknięcia 7 dni, tj. w 2 tygodnie później od pierwszego zatrucia. Szczury: samce, wagi 270—290 g.

Szczur nr	Azot-α-aminowy, mg w dobowym moczu													
	Dieta bezlaktozowa witaminowa (V) Dawka kwasu maleinowego 400 mg na 1 kg wagi, dootrzewnowo							Dieta mleczna witaminowa (I) Dawka kwasu maleinowego 300 mg na 1 kg wagi, podskórnie						
	dni przed zatruciem	dni po zatruciu						dni przed zatruciem	dni po zatruciu					
	—1	+1	+2	+3	+4	+5	+6	—1	+1	+2	+3	+4	+5	+6
10	2,5	3,5	1,7	3,1	3,8	8,7	8,3	5,5	8,0	11,0	9,6	10,0	6,9	27,5
11	5,0	3,5	1,1	2,4	3,1	4,0	6,3	3,2	6,5	14,4	12,5	13,5	15,0	9,0
12	4,4	3,4	1,7	2,6	3,3	5,5	6,8	1,8	7,0	2,1	4,5	3,5	9,2	6,2
13	7,5	4 4	2,2	7,7	5,4	6,3	1,4	1,5	8,0	9 4	10,4	13,0	14,3	24,2
14	5,7	5,8	1,7	1,1	12,0	9,4	5,4	3,4	6,7	3,2	7,7	14,0	19,0	13,4

nowego wydalanego w ciągu 6 dni po zatruciu są rzędu wartości wydalanych przed zatruciem. W 7 dniu doświadczenia zmieniono u tych samych szczurów dietę bezlaktozową na dietę mleczną witaminową i po tygodniu (2 tygodnie od poprzedniego zatrucia) podano szczurom maleinian w dawce 300 mg na 1 kg wagi podskórnie. Wystąpiła wyraźna aminoacyduria (u niektórych szczurów opóźniona).

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Tablica 5

Wpływ diety laktozowej witaminowej (IV) na aminoacydurię wywołaną kwasem maleinowym

Czas stosowania diety do wstrzyknięcia kwasu maleinowego: 9 dni. Dawka kwasu maleinowego: 400 mg na 1 kg wagi dootrzewnowo. Szczury: samce, wagi 120—180 g.

Szczur nr	Azot-α-aminowy, mg w moczu dobowym					
	dni przed zatruciem	dni po zatruciu				
	—1	+1	+2	+3	+4	+5
37	4,0	31,6	15,0	28,0	25,0	8,6
38	8,1	25,4	22,0	57,0	17,3	7,0
39	6,0	14,8	15,0	48,0	30,5	15,9
40	7,0	18,4	20,0	16,0	28,5	15,5

Okazało się, że wywołanie aminoacydurii kwasem maleinowym uzależnione jest od diety. Daje się wywołać aminoacydurię u szczurów karmionych dietą mleczną (zawierającą laktozę), natomiast nie udaje się to u szczurów karmionych dietą bezlaktozową. Potwierdzeniem tego, że wywołanie aminoacydurii parenteralnym podaniem kwasu maleinowego wymaga poprzedniej diety zawierającej znaczne ilości laktozy, jest wynik doświadczenia podanego w tablicy 5.

Tablica 6

Wpływ diety mlecznej drożdżowej bez witaminu E (III) na aminoacydurię wywołaną kwasem maleinowym w dawce 400 mg na 1 kg wagi

Czas stosowania diety do wstrzyknięcia dootrzewnowo kwasu maleinowego: 14 dni. Szczury: samice, wagi 215—235 g.

Szczur nr	Azot-α-aminowy, mg w dobowym moczu										
	dni przed zatruciem				dni po zatruciu						
	—4	—3	—2	—1	+1	+2	+3	+4	+5	+6	+7
15	2,9	2,4	2,6	2,2	5,8	4,2	7,8	5,9	7,0	9,0*	
16	6,7	3,9	4,0	3,0	5,9	1,8	3,7	3,6*			
17	3,4	3,2	2,9	4,0	4,8	4,7	8,4	8,1	9,6	17,7	*
18	3,3	3,0	3,0	3,1	7,1	1,7	3,2	5,0	10,7	3,1	
19	4,2	3,4	3,8	3,3	3,1	2,2	3,2	3,6	9,4	6,9	11,4
20	4,2	5,9	3,8	2,5	7,2	17,6	21,5	19,3	7,4	5,7	7,0

* W dniu oznaczonym * szczur padł.

Zastąpienie mieszanki witaminów przez suszone drożdże piekarskie wywiera szczególny wpływ na aminoacydurię. Jeżeli szczury karmić dietą mleczną (zawierającą laktozę), w której mieszankę witaminów zastąpiono całkowicie drożdżami (z wyjątkiem witaminów A i D dodawanych osobno), wówczas stwierdza się, że 400 mg maleinianu na 1 kg wagi, dawka dobrze

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Tablica 7

Wpływ diety mlecznej drożdżowej bez witaminu E (III) na aminoacydurię wywołaną kwasem maleinowym w dawce 250 mg na 1 kg wagi

Czas stosowania diety do wstrzyknięcia dootrzewnowo kwasu maleinowego: 10 dni. Szczury samce, wagi 145—245 g.

Szczur nr	Azot-*a*-aminowy, mg w dobowym moczu					
	dni przed zatruciem			dni po zatruciu		
	—3	—2	—1	+1	+2	+3
21	8,6	5,9	5,7	46,0	59,0	36,0
22	3,9	2,8	3,2	33,4	47,0	29,0
23	6,9	4,0	2,0	33,6	64,5	26,0
24	4,8	3,4	4,5	86,0	80,0	52,0
25	—	2,4	2,2	39,0	28,0	28,0
26	4,0	—	2,8	50,0	36,5	13,5

znoszona przez szczury karmione dietą zawierającą komplet witaminów, działa bardzo silnie toksycznie. Zachowanie się szczurów wskazywało na znaczny stopień zatrucia. Z sześciu szczurów użytych do doświadczenia trzy padły, jeden zareagował, wykazując znaczną aminoacydurię, u dwóch pozostałych wystąpiła nikła, opóźniona reakcja (blisko 3-krotny wzrost wartości wydalanego azotu *a*-aminowego). Wynik tego doświadczenia po-

Tablica 8

Wpływ diety mlecznej drożdżowej z dodatkiem witaminu E (II) na aminoacydurię wywołaną kwasem maleinowym w dawce 400 mg na 1 kg wagi

Czas stosowania diety do wstrzyknięcia dootrzewnowo kwasu maleinowego: 28 dni. Szczury: samce, wagi 220—230 g.

Szczur nr	Azot-*a*-aminowy, mg w dobowym moczu								
	dni przed zatruciem			dni po zatruciu					
	—3	—2	—1	+1	+2	+3	+4	+5	+6
27	7,8	5,8	6,1	2,5	5,0	1,8	*		
28	8,6	6,7	6,6	7,7	2,7	9,1	4,4	13,9	12,9
28	5,9	4,7	6,7	9,4	26,5	25,0	19,0	19,0	14,6
30	10,5	7,5	6,2	18,5	47,0	28,0	15,0	10,0	10,8

* Szczur padł.

danego w tablicy 6 zdaje się wskazywać, że zastąpienie mieszanki witaminów całkowicie przez drożdże piekarskie w jakiś sposób uwrażliwia ustrój szczura na toksyczne działanie kwasu maleinowego, wyrażające się znaczną śmiertelnością. Potwierdzeniem tego może być fakt, że przy użyciu tej diety, można przez podanie maleinianu w dawce prawie o połowę

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Tablica 9

Wpływ diety mlecznej drożdżowej z dodatkiem witaminu E (II) na aminoacydurię wywołaną kwasem maleinowym w dawce 250 mg na 1 kg wagi

Czas stosowania diety do wstrzyknięcia dootrzewnowo kwasu maleinowego: 20 dni. Szczury: samce, wagi 280—345 g.

Szczur nr	Azot-α-aminowy, mg w dobowym moczu					
	dni przed zatruciem		dni po zatruciu			
	—2	—1	+1	+2	+3	+4
31	2,0	5,0	10,6	8,6	6,2	8,7
32	2,0	4,0	2,7	0,6	3,0	2,4
33	2,6	2,2	15,6	5,8	4,5	3,2
34	1,2	4,0	9,8	3,9	2,3	2,8
35	1,5	7,0	11,3	5,5	6,3	4,3
36	1,6	3,2	6,7	2,2	5,2	1,3

mniejszej (250 mg na 1 kg wagi) wywołać znaczną aminoacydurię (Tabl. 7).

Maksymalne wartości wydalanego azotu α-aminowego (w pierwszych dniach po zatruciu) były 10—15 razy wyższe w porównaniu ze stanem przed zatruciem. Wszystkie szczury użyte do doświadczenia przeżyły nie wykazując oznak silnego zatrucia. Większa wrażliwość szczurów na zatrucie kwasem maleinowym po zastąpieniu w diecie mieszanki witaminów przez drożdże piekarskie może mieć przyczynę w jakimś działaniu uczulającym przez substancje zawarte w drożdżach, lub w tym, że drożdże nie zawierają dostatecznych ilości potrzebnych witaminów. Jeżeli do diety mlecznej, zawierającej drożdże zamiast mieszanki witaminów, dodano witamin E, wówczas szczury lepiej znosiły dawkę 400 mg kwasu maleinowego na 1 kg wagi, mimo pewnych oznak silnego zatrucia (Tabl. 8). Z 4 szczurów użytych do doświadczenia padł jeden, dwa zareagowały wyraźnie, wykazując w 2 dniu po zatruciu 5-krotny wzrost wydalania azotu α-aminowego, czwarty wykazał nikłą opóźnioną reakcję. Przy użyciu tej diety dawka 250 mg kwasu maleinowego na 1 kg wagi okazała się niewystarczająca do wywołania aminoacydurii (Tabl. 9), w pierwszym dniu wystąpił niewielki wzrost wydalania azotu α-aminowego.

DYSKUSJA

Szkodliwy wpływ laktozy na szczury jest znany od dawna; dane piśmiennictwa do 1944 r. zbierają Ershoff i Deuel [5]; ich doświadczenia stwierdzają szczególną wrażliwość młodych szczurów, które na diecie zawierającej 73% laktozy zdychają po około 5 dniach. Handler badał na szczurach wpływ diet o różnej zawartości laktozy [6] i stwierdził jej

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szkodliwość, gdy stanowi więcej niż połowę wagi diety; mocz takich zatrutych szczurów cechuje (prócz znacznej zawartości galaktozy) zwiększenie objętości, podwyższenie zawartości wapnia i amoniaku przy podwyższonej kwasowości aktualnej i potencjalnej. Careddu i Cabassa [3] podają, że szczury na diecie zawierającej galaktozę w ilości około 25% wykazują po 15 dniach do 2 miesięcy aminoacydurię znacznego stopnia. Z tymi wynikami nie zgadzają się doświadczenia Humana, Middletona i Geigera [10], którzy nie stwierdzają aminoacydurii u szczurów trzymanych na diecie zawierającej 52 lub 73% galaktozy. Natomiast galaktozemii u ludzi towarzyszy, jak wiadomo, aminoacyduria znacznego stopnia (dane piśmiennictwa [9]).

Dla interpretacji naszych doświadczeń zdają się mieć znaczenie wyniki pracy Harrisonów [7], którym udało się przez podanie kwasu maleinowego wywołać u szczurów krzywiczych zespół objawów zbliżony do zespołu Fanconiego u ludzi.

W naszych doświadczeniach wykazano, że szczury trzymane przez tydzień lub dłużej na diecie zawierającej około 30% laktozy nie wykazują aminoacydurii, ale po parenteralnym podaniu kwasu maleinowego wydalają znaczne ilości aminokwasów w moczu. Natomiast szczury, w których diecie zastąpiono laktozę przez sacharozę nie wykazują tych objawów. Ten fakt, ilustrowany bardzo wyraźnie przez porównanie ze sobą tablic 4 i 5 uważamy za najważniejszy wynik naszych doświadczeń.

Dieta zawierająca laktozę okazała się dogodna w badaniu wpływu działania kwasu maleinowego na ustrój szczura. Czynnikiem wywołującym aminoacydurię jest kwas maleinowy, natomiast laktoza zdaje się pełnić rolę czynnika warunkującego wystąpienie aminoacydurii po zatruciu kwasem maleinowym. Jednotygodniowy okres odżywiania pokarmem zawierającym laktozę dostatecznie przygotowuje szczury na działanie kwasu maleinowego, wywołujące aminoacydurię.

Stwierdziliśmy też, że istotny wpływ na działanie kwasu maleinowego na szczury ma dieta, w której mieszaninę witaminów zastąpiono przez drożdże. Suszone drożdże piekarskie, jako zastępcze źródło witaminów, uwrażliwiają ustrój szczura na działanie kwasu maleinowego. Wyraża się to śmiertelnością przy dawce dobrze znoszonej na diecie witaminowej i znaczną aminoacydurią przy dawce prawie o połowę mniejszej. Takie działanie drożdży można starać się wytłumaczyć dodatkowym wpływem uczulającym na ustrój szczura, lub inaczej, że drożdże jako zastępcze źródło witaminów nie zawierają dostatecznych ilości potrzebnych witaminów. Przemawia za tym fakt, że dodanie obok drożdży witaminu E zmniejsza wyraźnie ich działanie uwrażliwiające. Dawka 250 mg na 1 kg wagi jest wówczas niewystarczająca do wywołania aminoacydurii; zmniejsza się też śmiertelność przy dawce 400 mg na 1 kg wagi.

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STRESZCZENIE

1. Jednorazowe parenteralne podanie szczurom kwasu maleinowego w dawkach 300—400 mg na 1 kg wagi wywołuje aminoacydurię znacznego stopnia u szczurów odżywianych pokarmem zawierającym laktozę.

2. W stosowanych dietach laktoza (w postaci czystej, lub jako składnik mleka sproszkowanego) stanowiła 30% pokarmu.

3. U szczurów odżywianych dietą, z której wyeliminowano laktozę, zastępując ją mieszaniną sacharozy i skrobi, te same dawki kwasu maleinowego nie wywołują aminoacydurii.

4. Jednotygodniowy okres odżywiania pokarmem zawierającym laktozę wystarczająco przygotowuje szczury na działanie kwasu maleinowego wywołujące aminoacydurię.

5. Zastąpienie w diecie zawierającej laktozę mieszanki witaminów przez suszone drożdże piekarskie powoduje znaczną śmiertelność, przy dawce kwasu maleinowego dobrze znoszonej na diecie witaminowej i znaczną aminoacydurię, przy dawce prawie o połowę mniejszej. Dodanie obok drożdży witaminu E wyraźnie zmniejsza to działanie.

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AMINOACIDURIA CAUSED BY MALEIC ACID

II. THE EFFECT OF DIET

Summary

1. A single parenteral administration of maleic acid in doses ranging from 300 to 400 mg./kg. body weight results in a considerable aminoaciduria in rats fed with the lactose containing diet.

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2. The diet applied contained 30% of lactose (as pure lactose added or as a component of powdered skim milk).

3. The same doses of maleic acid did not induce any aminoaciduria in rats fed with diet containing a mixture of sucrose and starch instead of lactose.

4. One week of the lactose containing diet appears to be sufficient to render rats sensible to the aminoaciduria inducing effect of maleic acid.

5. The vitamin mixture was replaced by dried baker's yeasts in the lactose containing diet. Rats fed with such a diet showed a considerable mortality when maleic acid was administered to them in doses well tolerated by rats on vitamin diet. A half of those doses of maleic acid administered to rats fed with so modified diet resulted in a considerable aminoaciduria. The addition of vitamin E to the diet abolished this effect.

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TREHALOSE IN *CELERIO EUPHORBIAE*

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Wyatt and Kalf [5] have found trehalose to be the main sugar in haemolymph of eight insect species. Howden and Kilby [3] found also this sugar in *Schistocerca gregaria*. It is now generally accepted that trehalose is a typical blood sugar of the majority of insects especially in larval stage. However, Evans and Dethier [1] could not find any trehalose chromatographically in the blood of *Phormia regina* larvae although it is the main sugar in adult insect.

In our studies on *Celerio euphorbiae* we have stated the presence of trehalose in haemolymph and tissues of pupae, while we have failed to show it in the caterpillars haemolymph. Thus, we have decided to undertake some regular quantitative studies on the occurrence of trehalose in this species.

EXPERIMENTAL

Material. Caterpillars, pupae and adult moths of *Celerio euphorbiae* were used. The determinations were done separately in haemolymph and in body after bleeding.

Identification of trehalose. It was accomplished by paper chromatographic separation, chromatographic elution, and estimation of reduction in the water eluate before and after hydrolysis. Three kinds of samples were applied on Whatman No. 1 paper: (I) trichloroacetic acid extracts of haemolymph and of tissues homogenate, (II) ethanol extracts at 75° deionized on Dowex 1 and 50, evaporated to dryness and dissolved in water (method described by Wyatt and Kalf [5]) and (III) ultrafiltrates from haemolymph.

The ascending chromatography was performed for 24 hr. at room temperture, *n*-butanol-acetic acid-water (4:1:1 v/v) being used as solvent. The spots were developed by silver nitrate method of Trevelyan *et al.* [4].

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Trehalose gives a slightly coloured spot, but quite sufficient for identification and localization of the compound.

Spots with an R_F corresponding with those of glucose and trehalose standards were found. The eluates of trehalose spots did not reveal any reduction in Hagedorn and Jensen method before, but did so after acid hydrolysis. Maltose had an R_F similar to that of trehalose when the same solvents were used, but the absence of reduction before hydrolysis in our case excluded the presence of this disaccharide.

Estimation of trehalose. Haemolymph and homogenate of the residue of the body was dialysed against distilled water for 24 hr. at room temperature, water being twice changed. The presence of reducing dialysable compounds was estimated by Hagedorn and Jensen method [2] in external fluids directly and after 90 min. hydrolysis in 1 N-sulphuric acid at 100° The results expressed as glucose were calculated for 100 g. of the dialysed tissue. Values found directly were subtracted from values obtained after hydrolysis giving contents of trehalose.

RESULTS

The trehalose contents in caterpillars of different age are given in Table 1. There is no trehalose in blood or in tissues in feeding caterpillars by our method, nor could trehalose be demonstrated chromatographically at this stage. The picture is rapidly changed when adult caterpillar ceases to feed and starts "running". The trehalose content in haemolymph on the first day of "running" is about 500 mg%, and reach as up over

Table 1

Trehalose in the haemolymph and in the tissues of Celerio euphorbiae caterpillars

Reduction was estimated in ultrafiltrates directly and after 90 min. hydrolysis in 1 N-H_2SO_4 at 100°. Values expressed as glucose (mg. per 100 g. of tissue)

Age of the caterpillar	Haemolymph			Tissues		
	directly	after hydrolysis	trehalose	directly	after hydrolysis	trehalose
Feeding	315	308	0	496	481	0
	330	367	0	426	442	0
	461	465	0	321	323	0
	440	443	0	412	414	0
Running: 1st day	205	752	547	288	280	0
	182	754	572	233	242	0
2nd day	205	1850	1645	—	—	—
	177	1470	1293	262	276	0
"Spinndle-form"	175	1520	1345	450	462	0
	252	1360	1108	332	337	0
	223	1530	1307	392	419	0

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1000 mg% on the second day. No trehalose is found in bleeded tissues at this stage. The further period of pupating, called "spindle-form" provides the similar data.

Table 2

Trehalose in the haemolymph and in the tissues of Celerio euphorbiae pupae

Reduction was estimated in ultrafiltrates directly and after 90 min. hydrolysis in 1 N-H_2SO_4 at 100°. Values expressed as glucose (mg. per 100 g. of tissue)

Age of the pupa	Haemolymph			Tissues		
	directly	after hydrolysis	trehalose	directly	after hydrolysis	trehalose
1 day	395	1680	1285	452	463	0
	363	1350	987	410	418	0
	395	885	490	336	314	0
	360	1254	894	397	395	0
2 days	362	1430	1068	470	477	0
9 days	134	1436	1302	290	212	0
10 days	124	1048	924	310	312	0
4 weeks	187	948	761	203	257	54
	200	940	740	215	307	92
9 weeks	372	1292	920	284	385	101
3 months	300	1170	870	335	435	100
6 months	232	1580	1348	311	524	213
	271	1285	1014	496	748	252
	243	1400	1157	442	735	293
	204	1390	1186	416	775	359
	216	1245	1029	403	753	350
	288	1418	1130	438	638	200
	270	1270	1000	470	720	250
first signs of wing	293	535	242	271	282	0
pigmentation	606	826	220	325	329	0
distinct wing pigmentation	545	676	131	298	300	0

Table 2 shows the trehalose content in pupae. Its level in haemolymph remains still about 1000 mg%, since pupating till the end of diapausis. During diapausis trehalose is present in bleeded tissues, too, in contrast with what is found during first week of pupal life. It appears at the end of the first month, reaches a concentration of about 300 mg% and disappears slowly at the end of diapausis. In the blood of pupae in full development the trehalose level decreases down to about 200 mg%, while it disappears completely from tissues.

Table 3 shows the results found in pupae one or two days before eclosion and in freshly emerged moths. Experiments were carried out on

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Table 3

Trehalose in the pupae and the adult moths of Celerio euphorbiae

Reduction was estimated in ultrafiltrates directly and after 90 min. hydrolysis in 1 N-H_2SO_4 at 100°. Values expressed as glucose (mg. per 100 g. of tissue)

Age	Directly	After hydrolysis	Trehalose
Pupa near to the eclosion	307	308	0
	420	430	0
	315	320	0
Moth immediately after emerging	283	283	0
	300	312	0
	226	226	0

homogenates of whole insects, since it is impossible to separate haemolymph at this stage. There is no trehalose in pupa just before eclosion nor in the adult moth.

We have examined *euphorbia* leaves which are the only food of *Celerio euphorbiae*. We have found a great amount of dialysable substances reducing in Hagedorn-Jensen test, and giving positive colour reaction with antron reagent corresponding to the amount of reduced ferricyanide. The content of this compound in leaves was about 1300 mg% expressed as glucose, while in the latex alone it was about 4000 mg%. The reduction did not increase after hydrolysis, thus the presence of trehalose was excluded. Trehalose was not found chromatographically, too.

The results presented point out once more the necessity of precaution in generalizations in insect biochemistry. The opinion that trehalose is the main sugar of haemolymph at larval stage does not hold for *Celerio euphorbiae*. Trehalose does not occur in feeding caterpillars of this insect, while its appearances can be regarded as a biochemical mark of the inset of pupating. The presence of trehalose is also typical for the next period of pupal quiescence whereas its decrease characterises the inset of imaginal development in the pupa. Pupa just before emerging as well as imago does not contain any trehalose at all. Trehalose is present in tissues during pupal diapausis, what seems to be a typical feature for this stage.

SUMMARY

The presence of trehalose has been stated in *Celerio euphorbiae* caterpillars only at the end of feeding period. It is also present in the pupal stage till the end of diapausis. Pupae during imaginal development and imago do not contain any trehalose. Trehalose occurs only in haemolymph except in pupae during diapausis when it can be also found in tissues.

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TREHALOZA U *CELERIO EUPHORBIAE*

Streszczenie

Wykazano obecność trehalozy u gąsienicy *Celerio euphorbiae* począwszy od zaprzestania żerowania oraz u poczwarki aż do końca okresu spoczynku. Gąsienica w okresie żerowania, poczwarka w rozwoju i motyl nie zawierają trehalozy. Trehaloza występuje tylko w hemolimfie, jedynie u poczwarki w diapauzie znajduje się ją także w tkankach.

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STUDIES OF REVERSIBLE PHOTOLYSIS IN OLIGO-AND POLY-URIDYLIC ACIDS

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In previous investigations from this laboratory it was shown that the loss in absorption of RNA and APA, provoked by irradiation at 2537 Å, could be partially restored in the "dark" by heating in neutral medium (Wierzchowski & Shugar, 1956; Shugar & Wierzchowski, 1957, 1958a). For deaminated RNA (Shugar & Wierzchowski, 1958a) as well as for poly-U and deaminated APA (Wierzchowski & Shugar, 1958) no such reversibility could be observed; but we have since found that, under suitable conditions, the photodecomposition of these polynucleotides can also be partially reversed in the "dark".

The obvious importance of the above observations in relation to the phenomenon of biological photoreactivation, which has now been demonstrated for isolated, biologically active, nucleic acids (Rupert, Goodgal & Herriot, 1958; Bawden & Kleczkowski, 1959) prompted us to extend them to a series of model oligonucleotides.

On the basis of the photochemical behaviour of purines and pryrimidines, particularly nucleosides and nucleotides of the latter, it was previously suggested that the reversible dark reaction exhibited by irradiated polynucleotide chains was most likely due to the reversible uptake of a water molecule by the 5, 6 double bond of some of the uracil and cytosine bases (see Shugar & Wierzchowski, 1958 for review). This was, however, based on the assumption that the behaviour of each aromatic ring in a nucleotide chain is independent of the presence of neighbouring rings. That such may not necessarily be the case is indicated by the fact that the photochemical behaviour of a nucleotide is dependent on the

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The following abbreviations are used throughout this text: RNA, ribonucleic acid; DNA deoxyribonucleic acid; APA, apurinic acid; Up, uridine-2′(3′)-phosphate; Up!, uridine-2′: 3′-phosphate; pUp, uridine-2′(3′), 5′-diphosphate; poly-U, poly-uridylic acid; *PR*, photoreactivation; *TR*, thermal reactivation

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position of esterification of the sugar hydroxyl(s) (Wierzchowski & Shugar, 1957), as well as the observation that the apparent quantum yield for deoxycytidylic acid in irradiated APA is higher by a factor of 3 than that for the free acid (Shugar & Wierzchowski, 1958a).

Additional evidence for appreciable interaction between aromatic rings in nucleotide chains is furnished by the hyperchromic effect (for review see Beaven, Holiday & Johnson, 1955) which, in the case of DNA, is due partially to hydrogen bonding between base pairs in the twin strands of the Watson-Crick model. However, appreciable hyperchromicity exists also in individual polynucleotide chains (Warner, 1957) and even in dinucleotides (Sinsheimer, 1954; de Garilhe & Laskowski, 1954). An examination of the quantitative variation of hyperchromicity with pH for small oligonucleotides has shown that hydrogen bonding is not the major source of this effect (Michelson, 1958) and that it is undoubtedly due in large part to interaction between π-electron orbitals of adjacent parallel rings with the formation of a more complex chromophore embracing several rings (Laland, Lee, Overend & Peacocke, 1954; Lawley, 1956; Michelson, 1958, 1959). It therefore follows that the behaviour of a given ring, upon excitation as a result of absorption of light, will depend on its position in the chain as well as the nature of neighbouring rings.

In view of the above it became desirable to examine in greater detail the behaviour of individual nucleotides in polynucleotide chains and, in accordance with a previous suggestion (Shugar & Wierzchowski, 1958a), we undertook the following investigation on the photochemistry of some homologous oligonucleotides of Up. These latter possess a distinct advantage over oligonucleotides of other nucleic acid derivatives in that poly-U exhibits only 5-6% hyperchromicity (Warner, 1957) so that spectral variations resulting from irradiation must be due practically entirely to modifications of the uracil rings.

MATERIALS

We are indebted to Dr. S. Ochoa for a sample of poly-U prepared by enzymatic synthesis (Grunberg-Manago & Ochoa, 1955) and to Dr. A. M. Michelson for several samples of chemically synthesized oligonucleotides of Up (Michelson, 1959) containing a mixture of 2′:5′ and 3′:5′ internucleotide linkages.

Di-, tri- and tetra-Up with terminal cyclic phosphate groups were prepared from poly-U by hydrolysis with ribonuclease, followed by chromatography, as described by Heppel, Ortiz & Ochoa (1957); the cyclic phosphate groups in these oligonucleotides were opened by acid treatment.

UpU was prepared, according to the procedure of Heppel, Whitfield

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& Markham (1955), using Up! prepared by a modification (Shugar & Wierzchowski, 1958c) of the procedure of Dekker & Khorana (1954) making possible the preparation of this compound in quantitative yield without column chromatography.

UpUp and UpUpUp were prepared from the chemically synthesized oligonucleotides of Up by separation on paper with the ethanol-ammonium acetate solvent of Heppel *et al.* (1957). UpUp containing only 2′: 5′ internucleotide linkages, was obtained by ribonuclease hydrolysis of the chemically synthesized oligonucleotides, followed by chromatography.

The procedure of Levene & Tipson (1934) was used for the preparation of isopropylideneuridine; the R_F value of this compound, relative to that of uridine, is 3.0 in ascending chromatography on Whatman No. 1 paper, using as solvent butanol saturated with a saturated solution of boric acid.

The 2′(3′), 5′-diphosphate of uridine was prepared according to the method of Hall & Khorana (1955), using as starting material uridine-2′(3′)--phosphate; yields were at least as good as those reported with uridine.

EXPERIMENTAL

The method of irradiation, with all attendant details, were as previously described (Wierzchowski & Shugar, 1957). Because of the small quantities of some of the materials at our disposal, many experiments were conducted in 10-mm. microcuvettes with a volume of 0.5 ml. in a Hilger Uvispek spectrophotometer in which a narrow exit slit was obtained by insertion of a suitable mask. Ordinary 10-mm. cuvettes were used for those experiments where larger quantities of material were available. For studying the influence of concentration on the course of a photochemical reaction, recourse was had to ordinary 1-, 2- and 5-mm. cuvettes as well as the thin (down to 0.05 mm) cylindrical cuvettes supplied with the Soviet SF4 spectrophotometer. All kinetic measurements were made at room temperature, about 22°. Solutions were irradiated in 0.02 M Sørensen phosphate buffer, pH 7.2; while pH control was by means of the glass electrode, using a Radiometer pH meter.

All calculations were based on changes in absorption which, because of the low hyperchromicity of oligonucleotides of Up, are directly proportional to changes in concentration of the number of Up residues in the solution. For the same reason concentrations of oligonucleotides of Up are expressed in terms of the equivalent concentration of mononucleotides, e. g. 10^{-4} M poly-U means that if the poly-U were hydrolyzed to mononucleotides, the concentration of the resulting solution of Up would be 10^{-4} M.

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RESULTS

The nature of the results obtained with Up as well as oligo- and poly-U were such that it was found necessary to examine in detail the kinetics for photolysis of 1,3-dimethyluracil and Up. For the sake of simplicity the data for these compounds are presented first.

1,3-dimethyluracil

The kinetics of photolysis of dimethyluracil were examined at room temperature (20°) at initial concentrations, C_0, of 10^{-2} to 10^{-4} M in 0.02 M-phosphate buffer [1]) pH 7.2 and the data analyzed by two different procedures:

(a) According to the general photochemical equation

$$-\frac{dD_c}{dt} = kI_0(1-10^{-D_a}) \qquad (1)$$

where D_c, the optical density of the solution being irradiated, and measured at the absorption maximum (in this case 2660 Å), is proportional to the concentration; D_a is the optical density at the wavelength emitted by the radiation source (2537 Å) and I_0 is the intensity of incident beam on the sample (cf. Sinsheimer, 1954b). Actually D_c in the above equation may be replaced by D_a, but we have found it more convenient to follow the course of irradiation at both wavelengths since this provides a more precise control of the nature of the reaction.

The rate of decrease of D_c was plotted against $(1-10^{-D_a})$ for those cases where the irradiated solution was optically "thin" enough to transmit some of the incident light.

For higher concentrations with $D_a > 2$, and with I_0 constant, as is usually the case, the above equation becomes

$$\frac{dD_c}{dt} = \text{constant}, \qquad (2)$$

if no concentration effects intervene.

(b) The reaction order was also estimated graphically, according to the difference method of van't Hoff. If k is the rate constant and n the order of a given reaction, then

$$-\frac{dD_c}{dt} = kD_c^n, \qquad (3)$$

[1]) Neither the buffer nor the ionic strength of the medium influence the reaction, since the results were the same in unbuffered solution.

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and n is obtained directly from the slope of the plot of log $(-dD/dt)$ vs. log D. For this purpose photolysis was usually carried to about 80% completion.

The course of photolysis of solutions of dimethyluracil with initial concentrations, C_0, in the range 10^{-5}—10^{-3} M is in good agreement with the general equation (1) given above (see Fig. 1) under conditions where the irradiated solution transmits some of the incident light. The quantum yield is, however, only approximately constant (see below) for the entire course of the reaction with an average value of about 10×10^{-3}, in reasonable agreement with that reported by Moore & Thomson (1956). At concentrations higher than 10^{-3} M the general equation is no longer obeyed (Fig. 1).

For optically "thick" solutions the kinetic data are not in accord with equation (2), as can also be seen from an analysis of the data by the dif-

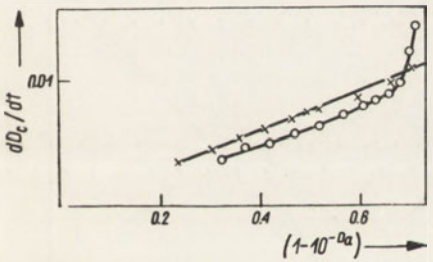

Fig. 1. Course of photolysis of 1,3-dimethyluracil in 0.02 M-phosphate buffer pH 7.2:
×, $C_0 \sim 10^{-3}$ M or 10^{-4} M
○, $C_0 \sim 10^{-2}$ M

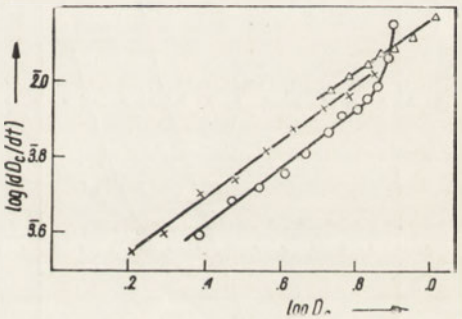

Fig. 2. Calculation of reaction order by difference method for photolysis of 1,3-dimethyluracil:
×, $C_0 \sim 10^{-4}$ M or 10^{-3} M; $D_0 < 1$; $n=0.77$
△, $C_0 \sim 10^{-3}$ M; $D_0=10$; $n=0.7$
○, $C_0 \sim 10^{-2}$ M; $D_0 < 1$; $n=0.75$

ference method (Fig. 2) from which it is apparent that the reaction course in the concentration range 10^{-5}—10^{-3} M is independent of the optical thickness of the irradiated solution. From Fig. 2 it will be observed that the reaction order is about 0.75. It follows that Ø cannot be constant and, in fact, a variation of about 10% prevails.

A 7-fold increase or decrease in incident light intensity was without effect on the reaction order. Furthermore, irrespective of the concentration irradiated, reversibility of the photoproduct to dimethyluracil in the dark was, in all instances, very high (98—100%) either by acidification to pH 1 (with a $t_{1/2}$ at room temperature of a couple of minutes) or by heating at neutral pH ($t_{1/2}$ at 80° about 15 mins.).

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Uridylic acid

The photochemical behaviour of this compound was examined over the same concentration range as methyluracil, 10^{-2}—10^{-5} M, in 0.02 M-phosphate pH 7 at 20°. Since the behaviour of the 2′ and 3′ isomers is similar as regards the forward reaction (Shugar & Wierzchowski, 1958a) the Up used was a commercial purified preparation.

The photolysis of Up was found to follow the general photochemical equation (1) only at concentrations of 10^{-4} M or less, as can be seen from Fig. 3. Above 10^{-4} M the relationship no longer holds and deviations from it increase with increasing concentration, the reaction rate rising for solutions exhibiting similar values of $(1—10^{-Da})$, as exhibited in Fig. 3.

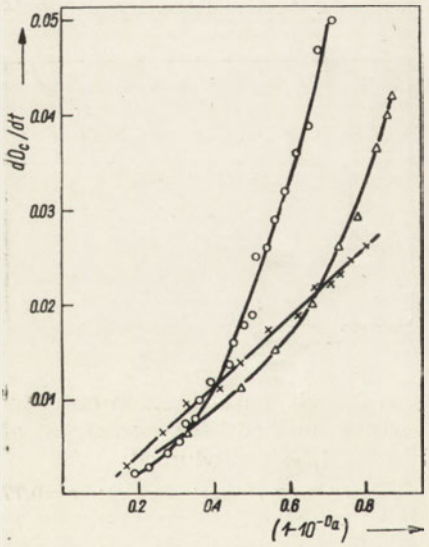

Fig. 3. Course of photolysis of Up in 0.02 M-phosphate buffer pH 7.2:
×, $C_0 \sim 10^{-4}$ M
△, $C_0 \sim 10^{-3}$ M
○, $C_0 \sim 10^{-2}$ M with incident dose about half that for: ×, △

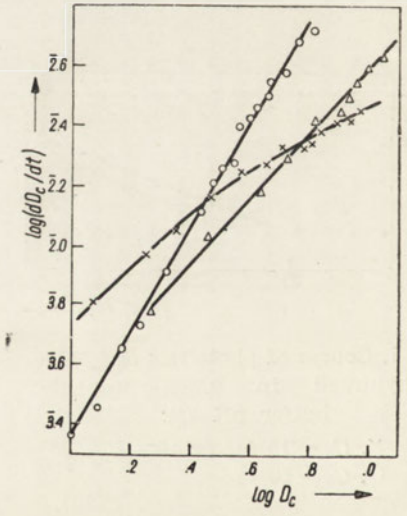

Fig. 4 Reaction order, by difference method, for photolysis of Up:
×, $C_0 \sim 10^{-4}$ M; $n_1 = 0.7$; $n_2 \sim 1$
△, $C_0 \sim 10^{-3}$ M; $n_1 = 1.1$; $D_0 < 1$ and $D_0 > 1$
○, $C_0 \sim 10^{-2}$ M; $n_1 = 1.7$; $D_0 < 1$ and $D_0 > 1$

Concurrently the quantum yield exhibits a concentration dependance, as one would expect. Only in the concentration range below 10^{-4} M is the quantum yield constant at about 20×10^{-3}, if we assume that the displacement of the curve along the abscissae by about 5% of the initial absorption, $(1—10^{-Da})$, is the result of absorption by the photoproduct which is, however, considerably more resistant to irradiation than Up itself.

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An analysis of the data by the difference method illustrates more clearly the concentration dependance of the reaction. From Fig. 4 it will be observed that the overall reaction order varies with concentration over the entire concentration range examined. In going from 10^{-2} to 10^{-3} M, the reaction order n decreases from 1.7 to 1.2; a further decrease in concentration to 5×10^{-4} M results in a further decrease in n to 0.7; and, finally, at 10^{-5} M the value of n increases slightly so that it is nearly unity. These results are entirely independent of whether the kinetic data were obtained on optically thin solutions or under conditions where total absorption prevailed.

An examination of the kinetic data by both of the above procedures can now be seen to indicate that the assumption of constant quantum yield at low concentrations of Up (see above) is unjustified, and that the explanation given above for displacement from the origin of the extrapolated curve of dD/dt vs. $(1—10^{-D_a})$ is likewise unacceptable. The variation of quantum yield for photolysis of Up at various concentrations is shown in table 1.

Table 1

Variation of quantum yield Ø for Up as a function of degree of photolysis

Exper. no.	C_0	% photolysis	$\emptyset\times10^3$ (mole·einstein^{-1})
1	0.89×10^{-4}M	6.0	19.5
		20.0	19.5
		31.0	18.8
		47.0	18.4
		64.5	18.2
		77.3	16.7
		83.5	14.4
		90.0	10.6
2	1.06×10^{-3}M	2.0	29.0
		10.0	28.2
		28.5	22.8
		38.5	21.5
		49.0	18.3
		61.0	16.0
		72.5	14.1
		82.5	11.5

From a plot of the initial rate of photolysis $(dD/dt)_i$ vs. $\log C_0$ for the concentration range 10^{-5}—10^{-3} M, the reaction order with respect to initial concentration C_0 was estimated. From 10^{-5} M to 5×10^{-4} M this is about 0.15; at higher concentrations the order increases and in the range 5×10^{-4} to 10^{-3} M attains the value 0.35.

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In view of the above results the absorption of solutions of Up was measured over a concentration range of 10^{-5} to 0.5×10^{-3} M; no deviations from the Beer-Lambert law were, however, observed.

Reversibility

Irrespective of the concentration used, reversion of the photoproduct to the original Up in the "dark" was practically quantitative under our conditions. Acidification to pH 2 or heating at neutral pH at 80° restored 95—100% of the original absorption at 2600 Å, irrespective of the concentration of the irradiated solution and in agreement with previous observations on thin solutions (Sinsheimer 1954b). Furthermore the rate of reversion of the photoproduct to the original Up was apparently independent of the concentration. Finally, the Up regenerated from the photoproduct by acidification or heating was chromatographically similar to the original Up.

Uridine

This compound was examined in the concentration range 10^{-5}—1.5×10^{-3} M, and the behaviour was found to be qualitatively similar to that for Up, i. e. the quantum yield Ø varies with concentration. In the concentration range 10^{-5}—10^{-4} M the photochemical behaviour is identical to that of Up: a straight line relationship according to equation (1) and a similar slope according to equation (2) with an initial n of 0.8 which, towards the end of the reaction, increases to 1.2.

For concentrations higher than 10^{-4} M the shape of both curves is analogous to those for Up but the slopes are different. The reaction order as estimated by the difference method is about 1.4.

Isopropylideneuridine

The course of photolysis of this compound in the concentration range 10^{-5}—10^{-4} M is practically identical with that for Up. The initial quantum yield is also the same. Thermal reversibility at 80° following 75% photolysis is $> 95\%$. There is, however, a marked difference in the rate of the reverse reaction, which is very rapid for this derivatives with a $t_{1/2}$ of less than 5 mins. Attention should be drawn to the fact that the rate of the reverse reaction is equally rapid for uridine-2′:3′-phosphate (Shugar & Wierzchowski, 1958a). In the case of cytosine nucleotides, not only the reverse reaction but also the rate of the photolytic reaction is influenced by the position of esterification of the carbohydrate moiety (Wierzchowski & Shugar, 1957).

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Uridine-2′ (3′), 5′-diphosphate

In the concentration range 10^{-5}—10^{-4} M the kinetic behaviour of this compound is completely analogous to that for Up; the quantum yields are also the same under similar conditions. The behaviour of pUp at higher concentrations up to 10^{-3} M does not differ from that at lower concentrations; since, however, the solutions contained, in addition to phosphate buffer, also 0.1 M-sulphate ions, a direct comparison with Up under comparable conditions is not warranted because of the possible influence of ionic strength of the medium.

Thermal reversibility of photolysed pUp is as high as for Up, i. e. 95—100%.

Di-uridylic acids

We have examined the photochemical behaviour of a variety of dinucleotides of Up, including UpU, UpUp, UpUp! as well as UpUp in which the internucleotide linkage is 2′, 5′, again under conditions similar to those used for Up. Due, however, to the small quantities of material available, it was possible to examine the effect of concentration only in the case of UpUp containing an equal mixture of 2′, 5′, and 3′, 5′ internucleotide linkages, over the range 10^{-5} to 10^{-3} M.

In view of the fact that all the above were found to exhibit similar photochemical behaviour, only some representative results will be included here.

A plot of dD_c/dt vs. $(1—10^{-Da})$ for the above compounds gives a curve consisting of two intersecting straight lines with markedly different slopes (Fig. 5), the change in slope occurring approximately at the point where 50% of the aromatic rings have undergone reaction. Because of the fact that neither of the straight-line portions of the curve pass through the origin, the quantum yields vary during the course of the reaction. Fig. 6 exhibits the quantum yield Ø as a function of the proportion of the compound which has already reacted photochemically, for UpUp. The initial quantum yield is found to be twice as large as the initial quantum yield for a solution of Up containing an equal concentration of aromatic rings.

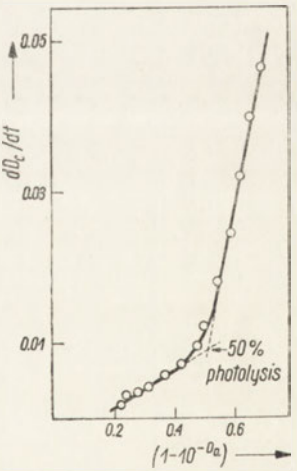

Fig. 5. Course of photolysis of UpUp (equimolar mixture of internucleotide linkages 2′, 5′ and 3′, 5′) in 0.02 M-phosphate buffer pH 7.2, with $C_0 \sim 10^{-4}$ M or $\sim 10^{-3}$ M

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The reaction order, as determined by the difference method, is shown in Fig. 7. As before we find a discontinuity at a point corresponding to that where approximately 50% of the aromatic rings have been photolysed. Up to this point the reaction order n is about 2.5, following which it drops to practically unity, actually about 1.1.

By contrast to Up, however, percentage reversion of the photoproduct to the original compound is dependent on the extent of the photochemical reaction. A closer study of this dependance was made for isomeric UpUp (i. e. containing a mixture of 2′, 5′ and 3′, 5′ internucleotide linkages) because more of this material was available, and because it was found that the individual isomers behaved similarly as regards both the forward and backward ("dark") reactions.

The experimental findings are included in Table 2. It will be observed that the extent to which the reaction is reversible in the dark (thermal reactivation or *TR*) increases with the degree of photolysis. Up to the point where 50% of the uracil rings have reacted, the % *TR* does not exceed 50. However, beyond the point where 50% of the aromatic rings

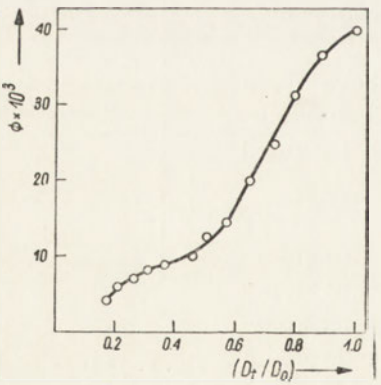

Fig. 6. Variation of quantum yield Ø with course of photolysis of UpUp (conditions as in Fig. 5)

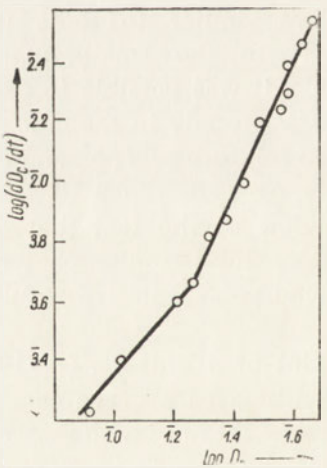

Fig. 7. Determination of reaction order for photolysis of UpU by difference method: $C_0 \sim 10^{-4}$ M; $n_1 = 2.4$; $n_2 = 1.1$

have undergone photolysis, the % *TR* begins to increase and attains values as high as 80%. This is perhaps best illustrated by irradiating a given solution of the dinucleotide with increasing doses and reactivating thermally after each dose. From these results it is clear that, following a 50% decrease in absorption as a result of irradiation, further decreases in absorp-

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Table 2

Course of photolysis and % thermal reactivation (% TR) at 80° of isomeric UpUp, as well as % TR following repeated photolysis of regenerated photoproduct

A solution of UpUp, concentration c at time 0, has an optical density D_c^0 ; after irradiation for time t its optical density drops to D_c^t. The % decrease in optical density, or % photolysis is therefore $\left(1-\frac{D_c^t}{D_c^0}\right)\times 100$. The solution is then heated until the optical density attains a final value D_c^f; the % *TR* is then $\left(\frac{D_c^f - D_c^t}{D_c^0 - D_c^t}\right)\times 100$. The thermally reactivated solution is now again irradiated until its optical density has fallen to the next lower value in the D_c^t column and then again heat reactivated and the % *TR* calculated in the same way

Exper. no.	D_c^0	D_c^t	% photolysis	D_c^f	% *TR*	% repeated *TR*
1	0.695	0.422	39	0.550	47	
		0.235	66	0.540	66.5	97.0
		0.136	80.5	0.540	72.5	100
2	0.690	0.650	6	0.655	12.5	
		0.528	23.5	0.555	16.7	20.3
		0.460	33.5	0.510	21.8	52.5
		0.382	44.5	0.460	25.3	61
		0.302	56.0	0.460	40.7	100
		0.130	81.3	0.460	59.0	100
3	0.620	0.092	85.2	0.500	77.5	
4	0.612	0.096	84.3	0.490	76.5	

tion resulting from additional irradiation are completely reversible in the dark.

Kinetic data for *TR* presented according to the difference method also emphasize the differences in *TR* prior to, and following, 50% photolysis, as can be seen from the difference in slopes of the curves before and after 50% photolysis (Fig. 8). Furthermore, in those instances where photolysis has not affected more than 50% of the aromatic rings, the *TR* curve is very steep; where photolysis has exceeded 50%, that portion of the *TR* curve corresponding to the initial 50% photolysis exhibits a similar steep slope.

Tri-uridylic acid

The photochemical kinetics for this compound in the concentration range 10^{-5}—10^{-4} M resemble those for UpUp, with the exception that the change in the reaction order, as well as the nature of the dependance of rate of photolysis on the absorption of the irradiated solution, occurs only after about 60% of the aromatic rings have reacted.

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The degree of thermal reactivation for the trinucleotide is similar to that for the dinucleotide, i. e. about 80%, provided the photochemical reaction has been carried to the point where 80% of the rings have reacted. There are, on the other hand, some slight differences in behaviour, e. g. early in the stage of photolysis the degree of reactivation is higher than for dinucleotides, while at the later stages of the reaction the % *TR* approaches that for the dinucleotide.

Poly-Uridylic acid

Qualitatively the photochemical behaviour of poly-U resembles that for the mononucleotide in that the absorption maximum at 2600 Å decreases in height, along with a small drop in absorption in the neighbourhood of the minimum at 2300 Å (Fig. 9). There is, however a marked difference in kinetic behaviour and, in fact, the kinetics of photolysis of oligonucleotides more and more begin to resemble that for poly-U as the number of residues in the chain increases.

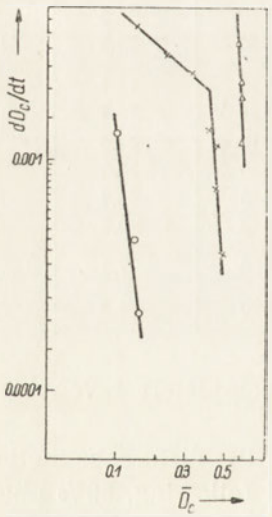

Fig. 8. Course of thermal reactivation at 80° for Up Up:
△ after 16% photolysis
○ „ 47% „
× „ 85% „

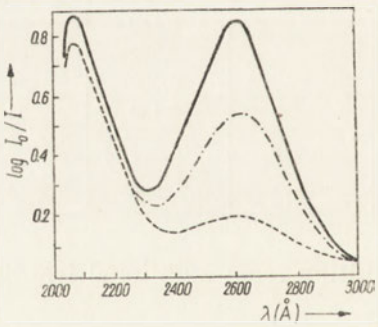

Fig. 9. Photolysis and thermal reactivation of poly-U 10^{-4} M with respect to Up in 0.02M-phosphate buffer pH 7.2:
——— before irradiation
– – – after 1 hour irradiation
–·–·– irradiated solution heated for 3.5 hours at 80°

The course of photolysis of poly-U over the concentration range 10^{-5}—10^{-3} M is apparently independent of concentration, and exhibits a straight-line relationship according to equation (1) only to a point where

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about 50% of the uracil rings have reacted. Beyond this point the course of the reaction begins to deviate continuously from the straight line, as shown in Fig. 10.

The quantum yield consequently varies during the course of the reaction, the initial value being twice that for a solution of Up of the same concentration (i. e. containing the same number of uridylic acid residues) but similar to that for di- and higher oligonucleotides under

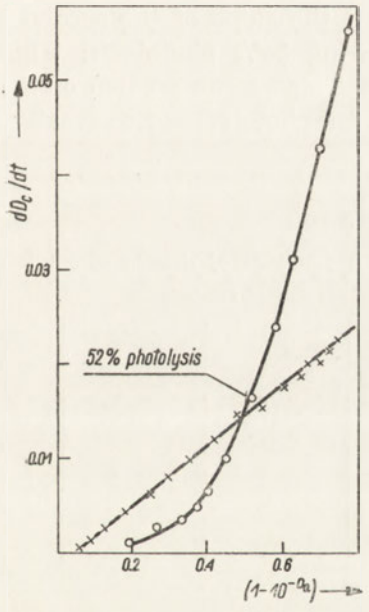

Fig. 10. Course of photolysis of high molecular weight poly-U, $C_0 \sim 10^{-4}$ M or $\sim 10^{-3}$ M with respect to Up:
○ poly-U
× after enzymatic hydrolysis to mononucleotides

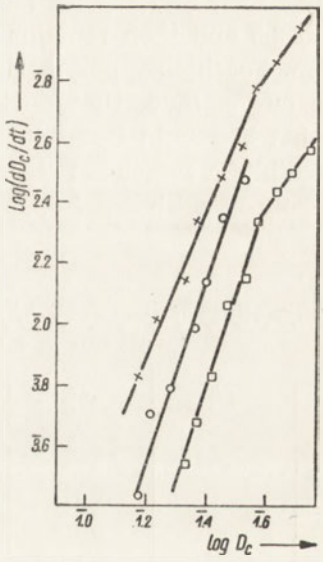

Fig. 11. Determination of reaction order for photolysis of highly polymerized poly-U:
×, $C_0 \sim 10^{-4}$ M; $n_1 = 1.35$; $n_2 = 2.5$.
○, same solution as above following *TR*; $n = 3.0$
□, $C_0 \sim 10^{-3}$ M in D_2O, pD = 7.0; $n_1 = 1.35$; $n_2 = 3.3$

similar conditions. To exclude the possibility of artifacts, the poly-U was hydrolyzed with RN-ase until only mononucleotides were present; the photochemical behaviour of such a solution was now found to exactly duplicate that for Up (Fig. 10).

Because of this marked difference in Ø between poly-U and Up, the photochemical behaviour of both of these was examined in heavy water at a pD of 7 (cf. Wierzchowski & Shugar, 1956, 1957; Shugar & Wierzchowski, 1957, 1958b). For poly-U the isotope effect $Ø_{H_2O}/Ø_{D_2O}$ was found

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to be about 1.2 initially, and this value increased gradually until it reached 2.8, which is comparable to that of 3.0 found for Up.

The kinetic data calculated according to the difference method are shown in Fig. 11. The slope of the curve for highly polymerized poly-U is about 1.35 up to 35% photolysis, following which there is a break, with a change in slope to 2.5 until completion of the reaction.

For shorter oligonucleotides of Up the initial portion of the curve is slightly flatter and shorter (~15% photolysis), following which the slope rises to 2.5 and then remains unchanged during the entire course of the reaction. For highly polymerized poly-U the slope of the second portion of the curve (i. e. that portion following 35% photolysis, Fig. 11) is somewhat higher in D_2O where $n \sim 3.3$. This second portion of the curve also exhibits a somewhat steeper slope for photolysis of thermally reactivated poly-U, with $n \sim 3.0$.

Table 3

Reversibility of photolysis of poly-U as a function of degree of photolysis

See title to Table 2 for explanation of symbols

Exper. no.	D_c^0	D_c^t	% photolysis	D_c^f	% *TR*	% repeated *TR*
1	0.655	0.540	17.5	0.580	35.0	
		0.317	51.5	0.432	34.0	43.5
2	0.625	0.312	50.0	0.422	35.0	
		0.170	73.0	0.420	55.0	100
3	0.560	0.140	75.0	0.370	55.0	
		0.140	75.0	0.370	55.0	100

The photolysis of poly-U may be reversed by heating in neutral solution or by acidification at room temperature, and, for the highly polymerized sample 55% reactivation may be obtained if not more than 80% of the uracil rings have reacted photochemically. If the reaction is not carried this far, the degree of reversibility is lower, as may be seen from Table 3. However, even when the photochemical reaction has proceeded for 20% of its course, the degree of reversibility is still about 35%; hence although the attainable degree of reversibility for poly-U is less than for dinucleotides, it is higher for the former following short periods of photolysis. If a sample of poly-U is photolyzed to half-completion of the reaction and then thermally reactivated, the degree of reactivation attains 36%; if it is now again irradiated to the point where 73% of the initial extinction at 260 mμ has disappeared, thermal reactivation of this second drop attains a value of 100%.

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The rate of the reverse reaction at 80° is dependent on the ionic strength and is reduced 3-fold in 0.1 M-NaCl ($t_{1/2} \sim 40$—45 mins.) as compared to that in the absence of any salt ($t_{1/2} \sim 100$—120 mins.). In acid the reverse reaction is very slow; at pH 1.3 and room temperature, $t_{1/2}$ is about 17 hrs. Because of this the degree of reactivation was estimated from the effect of heating at neutral pH.

DISCUSSION

The measured decrease in absorption of an irradiated solution clearly represents the secondary photochemical process which controls the overall reaction rate. For the simple uracil derivatives (e. g. DMU, Up) this secondary reaction most likely involves a single mechanism since, whatever the conditions used, the reaction is completely reversible in the dark. Bearing in mind that the reaction mixture involves only a uracil derivative and water, the various reaction steps may be represented as follows:

$$U + h\nu \xrightarrow{v_1} U^* \qquad (4)$$

$$U^* \xrightarrow{v_2} U \qquad (5)$$

$$U^* + H_2O \xrightarrow{v_3} U \cdot H_2O \qquad (6)$$

$$U \cdot H_2O \xrightarrow{v_4} U + H_2O \qquad (7)$$

where the v's represent velocities. Reaction (4) is the primary photochemical process leading to the formation of excited molecules, (5) the return of excited molecules to the ground state via fluorescence or internal conversion, (6) the secondary photochemical process leading to formation of the photoproduct, and (7) the spontaneous decomposition of the photoproduct.

Under conditions where the incident light intensity is constant and completely absorbed in the reaction system, $D_a > 2$, we have $v_1 = I_a =$ = const. and hence [U*] is constant.

We then have

$$v_1 - v_2 = v_3 - v_4$$

which, bearing in mind the stability of the photoproduct during the period of observation, so that $v_4 \ll v_3$, becomes

$$v_3 = v_1 - v_2$$

or

$$\frac{d[U]}{dt} = k_3 [U^*] = I_a - k_2 [U^*] = \text{const.}$$

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The rate of decrease in absorption will be zero order and the quantum yield should be constant and equal to

$$\emptyset = \frac{k_3 [U^*]}{I_a} = \text{const.} \quad (8)$$

Since the observed reaction order is not zero, while the quantum yield varies during the course of the reaction, it is pertinent to examine the validity of the assumptions leading to the formulation of equation (8).

The assumption of a stationary state concentration of excited molecules, although not open to question, does not take into account the possibility of transitions from the initial to other excited states in which the molecules is more capable of uptake of a water molecule; as well as the possible dependence of such transitions on the concentration. In the absence of supplementary experimental data, there is little to be gained by any detailed discussion of this point. It would obviously be desirable to examine the excited states of some of the above derivatives by the method of flash photolysis.

It is also conceivable, in view of the concentration dependance of reaction rate, that some interaction occurs between the photoproduct and excited molecules such that the latter impart energy to the former by collisions of the second kind, thus provoking the elimination of a water molecule (energy of activation required is about 20 kcal./mole for dimethyluracil (Moore & Thomson, 1956) and probably more for Up). Since the frequency of collisions of the second kind increases with concentration, this effect should become more pronounced as the concentration of photoproduct increases as well as with higher absolute concentrations of substrate and photoproduct. The first of these suppositions is in agreement with our observations; however the second is contrary to the results obtained since, with increasing absolute concentrations of the reactants, the reaction rate increases.

Di-uridylic acids: The kinetic behaviour of dinucleotides differs markedly from that observed for Up and other uracil monomer analogues. The lack of any differences in behaviour between the various di-uridylic acids UpU, UpUp, UpUp! and U2′p5′U2′p clearly suggests that the principal source of this difference between dinucleotides and monomers is the presence in the former of two uracil rings with a configuration such that there is interaction between their π-electron orbitals (Laland *et al.*, 1954; Lawley, 1956; Michelson, 1958, 1959), notwithstanding the fact that the low hyperchromicity of poly-U has been interpreted as indicating the absence of any such interaction (Warner, 1958).

The two-fold higher initial quantum yield as compared to uracil monomers, the characteristic changes in reaction order, the slope of the curve

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obtained according to equation (1) following photolysis of approximately 50% of the uracil rings, and the similar changes in the reverse, dark, reaction all lead to the following conclusion, *viz.* that in a dinucleotide one of the aromatic rings reacts initially and the second ring undergoes reaction only when, to a first approximation, all the first have reacted.

However, it is of some significance that about 25% of the uracil rings, of any steric effects resulting from the mutual interaction between rings a reversible photoproduct. In view of the *TR* results for irradiated poly-U following treatment with ribonuclease or acid (see below) it would appear that the lack of reversibility of 25% of the uracil rings is not the result of any steric effects resulting from the mutual interaction between rings in a dinucleotide. On the other hand we have no direct chemical evidence to suggest that they have been irreversibly degraded. One may visualize the possibility of a simultaneous absorption of two quanta, one by each of the rings in a dinucleotide; if during this process, there is a transfer of energy from one ring to another (and such a transfer has actually been demonstrated for a molecule such as DPNH which is analogous to a dinucleotide (Weber, 1958)), the additional energy concentrated in one of the rings may be sufficient to provoke ring rupture instead of formation of an excited state capable of uptake of a water molecule.

If, as postulated, the 50% of the uracil rings which first undergo reaction comprise mainly one of the rings in each dinucleotide molecule, the reaction course for the remaining 50% should then be similar to that for Up and pUp. Such, in fact, does appear to be the case; the reaction order for the remaining 50% is the same as that for a similar concentration of Up, while the quantum yield is also approximately the same (actually somewhat lower). Some minor differences in behaviour could logically be expected since each ring is still linked to a hydroxyhydrouracil ring which may modify its behaviour, but to a considerably lesser extent than an adjacent aromatic ring.

Such intramolecular interaction appears to be the only cause for the rapid decrease in quantum yield, as compared to Up, during the course of the reaction in view of the lack of dependence of reaction rate on concentration in the range 5×10^{-6} to 5×10^{-4} M. Photosensitized induction of the reverse reaction by energy transfer is also a possibility and this, if it occurs at all, would be considerably more efficient intramolecularly and could explain the lower quantum yields observed as compared to Up.

The reaction of the first of the two rings in each molecule corresponds to a reaction order of about 2.5 by the difference method, or about twice that for the second ring. It is therefore of some interest that, if the reaction is conducted in heavy water, an isotope effect is observed only when the second ring in each molecule is reacting. An analogous behaviour

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prevails for the course of *TR*. The question therefore arises, whether the reversible reaction observed involves addition of a water molecule to the pyrimidine ring; whether, even where this is so, some additional reaction is the rate-determining step, or whether some other process is involved leading to saturation of the 5,6 double bonds, which is the experimental criterion used for following the reaction (on the assumption that simple ring rupture is not involved).

Higher oligonucleotides: The behaviour of the higher polymers of Up (irrespective of chain length or the presence of 2′, 5′ internucleotide linkages in place of 3′, 5′) under the influence of irradiation differs only in minor respects from that for dinucleotides insofar as the forward reaction is concerned, in that the slope of the initial portion of the curve is lower. It would therefore appear reasonable to assume that, irrespective of chain length, the behaviour of any one of the uracil rings initially reacting is determined largely by the presence of a neighbouring ring.

The only real, significant, difference in behaviour is in the reverse reaction, involving a gradual decrease in *TR* with increase in chain length to about 55% for highly polymerized poly-U. Bearing in mind, however, that the number of pairs of adjacent aromatic rings increases with chain length, this phenomenon is readily understandable in the light of the results for di-uridylic acid.

It remains to be established what has happened to those residues which do not exhibit *TR*. Beukers, Ylstra & Berends (1958), on the basis of some experiments on the photolysis of nucleic acid derivatives in frozen solution, suggest that bound water in a highly polymerized chain such as DNA may markedly alter the course of the reaction for the individual bases. If such is indeed the case it does not, on the other hand, explain the absence of 100% reversibility for UpUp.

Non-reversible photolysis: An adequate understanding of the nature of the non-reversible reaction undergone by the one-half of the 50% of the uracil rings initially reacting in an oligonucleotide is obviously of primary importance for an interpretation of the general mechanism involved. In effect, the radiation doses used in these experiments were such that degradation of the reversible photoproducts of Up would not be expected. Hence, either the influence of an adjacent ring results in some of the residues undergoing irreversible photolysis or some other processes are involved (see above). Clearly, the most effective procedure for elucidation of this question would be hydrolysis of an irradiated oligonucleotide to mononucleotides, followed by chemical and physico-chemical analysis of the latter. This may not be an easy problem since the degradation procedure to mononucleotides may irreversibly modify the nature of the initial photoproducts.

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One attempt has, however, been made to attack this problem in the following manner. We have observed that poly-U may be completely hydrogenated on a rhodium catalyst (Cohn & Doherty, 1956) and that, following hydrogenation, the resulting polymer is degraded by ribonuclease, presumably to monomers of dihydrouridylic acid (Janion & Shugar, unpublished). A solution of poly-U was therefore irradiated until about 75% of the uracil rings had undergone photolysis. The photoproduct was then submitted to the action of ribonuclease overnight, following which *TR* was attempted. The results, however, were identical with those obtained for *TR* of the photoproduct immediately following irradiation. Exposure of the photoproduct to 1 N-HCl, which might also be expected to hydrolyze the polynucleotide to monomers, likewise resulted in no greater degree of reversibility than *TR* of the photoproduct directly. While these results are not by any means conclusive, they do lend weight to the argument that the reaction undergone by those rings which do not subsequently revert to uracil involves more than a simple uptake of a water molecule at the 5,6 double bond.

Biological photoreactivation. Earlier experiments demonstrating partial reversibility of photolysis in polynucleotides involved the use of RNA and APA. Any arguments that might have been raised against the validity of these findings, on the grounds that hyperchromicity was not adequately accounted for, are now effectively disposed of by the results for uridylic oligonucleotides. It is even of some significance that the maximum degree of *TR* previously observed for polynucleotides from natural sources (of the order of 50%) is almost identical with that prevailing for highly polymerized poly-U.

The existence of *TR* for photolysed RNA and APA led to the suggestion that reversible photolysis of pyrimidine nucleotides provides an experimental model to account for biological *PR*. Supporting evidence for such an hypothesis has since been forthcoming from the demonstrations of biological *PR* in isolated nucleic acid preparations such as transforming DNA (Rupert *et al.*, 1958) and infectious RNA from TMV (Bawden & Kleczkowski, 1959).

The results for oligonucleotides of Up further substantiate this hypothesis, if we consider *TR* and *PR* to represent merely different forms of energy for the photoreactivating process. Of the various agents which have been found to be effective in the reversal of the effects of irradiation, it has been pointed out that *TR* is the one which most closely resembles *PR* (Jagger, 1958).

It would obviously be desirable now to examine the behaviour of dinucleotides of Up and Cp in which the second nucleotide residue is either Ap or Gp. A clarification of the behaviour of the individual pyri-

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midine nucleotides in polynucleotide chains is now a possibility, and the information so gained might be expected to lead to a localization of the sites of action of irradiation in nucleic acids.

SUMMARY

A study has been made of the kinetics of reversible photolysis at 2537 Å of a number of uracil derivatives, with particular emphasis on 1,3-dimethyluracil and uridine-2′(3′)-phosphate, and on oligo- and polynucleotides of Up.

For dimethyluracil and uridylic acid the kinetics of the photolytic reaction are markedly concentration dependent, a finding which is difficult to interpret in view of the fact that for both of them (as well as the other uracil monomers investigated) the photoproducts may be completely regenerated to the original compound in the "dark" by acidification or by heating at neutral pH.

The kinetics of photolysis of di-uridylic acid, and the degree of reversibility as a function of the number of Up residues which underwent photolysis, suggest that one of the two Up residues first reacts preferentially and that only one half of these form reversible photoproducts. The photoproduct formed by the second ring in the dinucleotide reverts completely to Up in the dark. The total degree of reversibility for the dinucleotide, following extensive photolysis, may therefore attain about 75%. The same behaviour is exhibited by UpU, UpUp! and by UpUp with a 2′,5′ internucleotide linkage.

The results for UpUp assist in the interpretation of the photochemical behaviour of higher oligonucleotides. The degree of reversibility decreases with increasing chain length until, for highly polymerized poly-U, it is about 50—55%.

The overall results substantiate previous findings to the effect that the photolysis of pyrimidine nucleotides in nucleic acids is partially reversible and provide an experimental model for biological photoreactivation.

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[26] Warner R. C., — 1958 — *IVth Intern. Congress of Biochemistry, Vienna, Symposium* No. IX.
[27] Weber G., — 1958 — *J. Chim. Phys.* **55**, 878.
[28] Wierzchowski K. L. & Shugar D., — 1956 — *Annual Congress „Chemische Gesellschaft DDR"*, Leipzig.
[29] Wierzchowski K. L. & Shugar D., — 1957 — *Biochim. Biophys. Acta* **25**, 355.
[30] Wierzchowski K. L. & Shugar D., — 1958 — *IVth Intern. Congress of Biochemistry, Vienna, Communication* No. 3—35.

Addendum: Since submission of the above text, a paper has appeared (F. Millich & G. Oster, J. Am. Chem. Soc., **81**, 1357, 1959) on the photoreduction of acridine dyes, in which it was found that the photochemical behaviour of 3,6-diaminoacridine with respect to concentration resembles that reported here for uracil monomers. The quantum yield for diaminoacridine increases 10-fold with increase in concentration from 10^{-6} to 10^{-5} M; the dye also exhibits strong self-quenching of fluorescence in this same low concentration range. These facts were interpreted as indicating a transition of an electronically excited single-state species to a long-lived state, induced by the dye molecules in the ground state. Although we have no direct evidence for the existence of long-lived excited states for uracil monomers, the results of the above authors support our contention that the initial excited state may undergo an induced transition to a metastable state which can more readily take up a water molecule.

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BADANIA NAD ODWRACALNOŚCIĄ FOTOLIZY KWASÓW OLIGO- I POLIURYDYLOWYCH

Streszczenie

Zbadano kinetykę odwracalnej fotochemicznej przemiany (2537 Å) szeregu pochodnych uracylu, przede wszystkim 1,3-dwumetylouracylu, 2′(3′)-monofosforanu urydyny oraz oligo- i polinukleotydów Up.

Stwierdzono znaczny wpływ stężenia roztworu na kinetykę przemiany fotochemicznej 1,3-dwumetylouracylu i kwasu urydylowego. Fakt ten jest trudny do interpretacji w świetle pełnej odwracalności badanej przemiany obu związków (jak i innych badanych monomerów uracylu). Fotoprodukt daje się ilościowo przeprowadzić w wyjściowy związek w wyniku zakwaszenia lub ogrzania obojętnego roztworu.

Kinetyka fotochemicznej reakcji kwasu dwu-urydylowego oraz charakter zależności pomiędzy odwracalnością reakcji a stopniem przereagowania (licząc na reszty urydylowe) sugeruje, że przemianie ulega przede wszystkim jeden z dwu pierścieni uracylowych i tylko połowa z nich tworzy odwracalny fotoprodukt. Fotoprodukt utworzony przez drugi z kolei pierścień w cząsteczce dwunukleotydu w wyniku ciemnej reakcji odtwarza ilościowo resztę kwasu urydylowego. Jeżeli więc przemiana fotochemiczna została doprowadzona wystarczająco daleko, wówczas jej odwracalność może osiągnąć około 75%. UpU, UpUp! oraz UpUp z 2′ 5′ międzynukleotydowymi wiązaniami zachowują się w pełni analogicznie do kwasu dwuurydylowego.

Wyniki dla UpUp stanowią podstawę do interpretacji fotochemicznego zachowania się wyższych oligonukleotydów. Stopień odwracalności przemiany fotochemicznej oligonukleotydów maleje wraz ze wzrostem długości łańcucha polinukleotydowego. Dla wysokospolimeryzowanego poli-U spada on do wartości około 50—55%.

Całość wyników potwierdza wcześniejsze wnioski o częściowej odwracalności fotochemicznej przemiany nukleotydów pirymidynowych w kwasach nukleinowych. Odwracalna przemiana fotochemiczna oligonukleotydów może więc stanowić model biologicznej fotoreaktywacji.

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A. M. MICHELSON

SOME ASPECTS OF THE CHEMISTRY OF POLYNUCLEOTIDES AND NUCLEOTIDE CO-ENZYMES

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NUCLEOTIDE CO-ENZYMES

Knowledge of the biological significance of a number of nucleoside derivatives of pyrophosphoric acid — collectively known somewhat loosely as nucleotide co-enzymes, has expanded rapidly over the past decade. The biochemistry of such compounds is now quite extensive and has been reviewed recently by Baddiley and Buchanan [3]; at the same time the organic chemistry of pyrophosphate esters has been developed, largely by the Cambridge School under the direction of Sir Alexander Todd, so that a number of methods are now available whereby virtually any known nucleotide co-enzyme or analogue, may be synthesised by purely chemical means.

The first synthesis of a nucleotide co-enzyme, that of adenosine-5′ pyrophosphate by Baddiley and Todd in 1947 [8], employed the classical method of preparing mixed anhydrides — treatment of the salt of an acid with an acid chloride. Treatment of the silver salt of adenosine-5′ benzyl phosphate with dibenzyl phosphorochloridate gave the fully esterified adenosine-5′ tribenzyl pyrophosphate from which benzyl groups were readily removed by catalytic hydrogenolysis, to give adenosine-5′ pyrophosphate in 55% yield [7].

Repetition of this method, making use of a quaternisation procedure developed by Baddiley, Clarke, Michalski and Todd [6], to remove one

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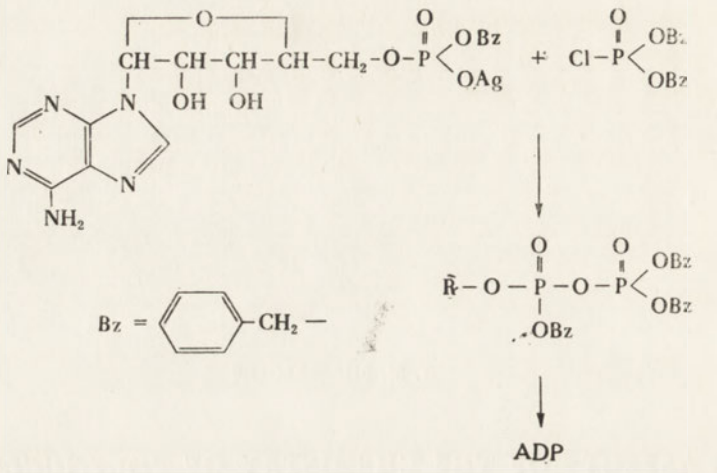
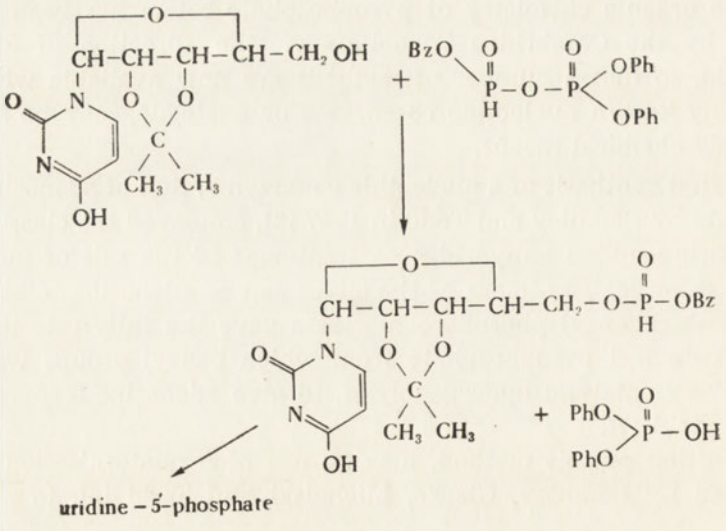


benzyl group from the fully esterified pyrophosphate, yielded adenosine-5′ triphosphate (ATP) [7]. In an alternate synthesis adenosine-5′ triphosphate was obtained, presumably *via* a 5′-cyclic trimetaphosphate intermediate, on treating adenosine-5′ phosphate directly with excess dibenzyl phosphorochloridate followed by hydrogenolysis [77a].

Since this early work, the introduction of a number of techniques has greatly facilitated the development of newer and more convenient methods of synthesis. Nevertheless, in spite of considerable inherent disadvantages, the classical approach has had a fair degree of success, particularly when Kenner, Todd and co-workers developed the use of *O*-benzyl-phosphorous di-*O*-phenyl phosphoric anhydride as a mixed anhydride reagent for preparing nucleoside benzyl phosphites [28].



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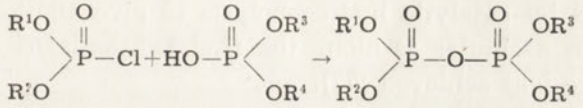
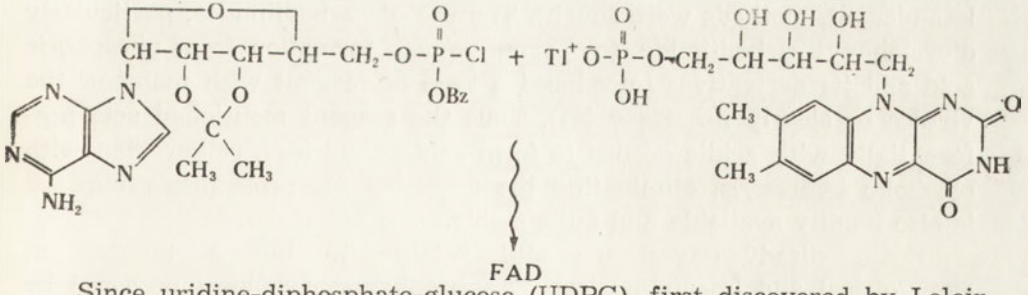
Like the model compound dibenzyl phosphite, these derivatives could be oxidised readily to the phosphorochloridate by the action of N-chlorosuccinimide, to give extremely useful intermediates. All practical variations of the classical method have now been employed. Examples are the synthesis of uridine-5′ pyrophosphate [45] ($R^1 = R^3 = R^4 =$ benzyl, $R^2 =$ = nucleoside), of thymidine-5′ pyrophosphate [36] ($R^1 = R^3 =$ benzyl, $R^2 =$ nucleoside, $R^4 = H$) and of adenosine-5′ pyrophosphate [69] ($R^1 =$ $= R^2 =$ benzyl, $R^3 =$ nucleoside, $R^4 = H$), protecting groups being removed by hydrogenolysis.



Of greater interest however was the application to the synthesis of flavin adenine dinucleotide, the co-enzyme of a variety of flavoproteins active in hydrogen transport in biological systems. Apart from the usual technical difficulties, the problem was further complicated in this case by the presence of a hydroxyl group which could be attacked by the pyrophosphate (a mixed anhydride) to give riboflavin 4′, 5′ cyclic phosphate as indeed occurs when FAD is treated with aqueous ammonia [31]. Nevertheless, Christie, Kenner and Todd were able to prepare FAD by treating the monothallous salt of riboflavin-5′ phosphate with 2′, 3′ isopropylidene adenosine-5′ benzyl phosphorochloridate in phenol solution [26]. Phenol debenzylation, followed by mild acid treatment to remove the isopropylidene residue, gave a complex mixture containing some 6% of the co-enzyme from which pure FAD was isolated by chromatographic methods.



FAD

Since uridine-diphosphate-glucose (UDPG), first discovered by Leloir and his collaborators in 1949 as co-enzyme for the conversion of *α*-D-galactose 1-phosphate into *α*-D-glucose 1-phosphate in galactose adapted yeast [18], is even more unstable than FAD, it was clear that if the phosphorochloridate route were to be used, the isopropylidene group would be unsuitable for protection, because of the acidic conditions necessary for its removal. Benzyl groups were therefore used since their removal by hydrogenolysis would be unlikely to disrupt the co-enzyme molecule.

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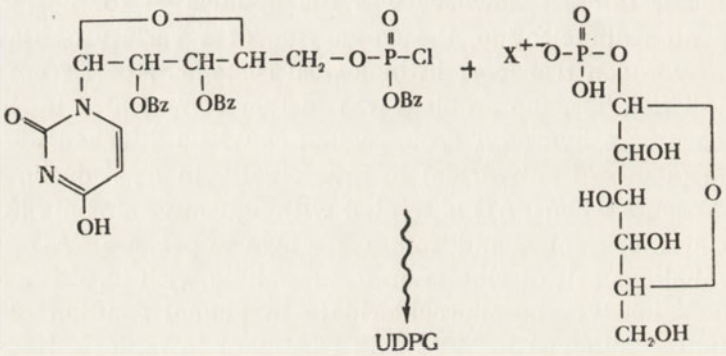
Solubility difficulties on the part of the *a*-D-glucose-1-phosphate moiety were circumvented by use of salts with long chain amines. Benzylation of 5′-*O*-trityluridine followed by removal of the trityl group gave 2′, 3′-*OO*-dibenzyluridine which with *O*-benzylphosphorus *OO*-diphenylphosphoric anhydride, yielded 2′, 3′-*OO*-dibenzyluridine-5′ benzyl phosphite, converted in the usual way to the phosphorochloridate with N-chlorosuccinimide. Reaction with *a*-D-glucose 1-(tri-*n*-octylamine hydrogen phosphate) in benzene solution in the presence of tri-*n*-butylamine gave a product from which the pyrophosphate benzyl and ether benzyls were removed by catalytic hydrogenolysis to give UDPG in some 15% yield [77]). In a similar fashion, the first synthesis of uridine-diphosphate-galactose was achieved [77].



Since this approach was of limited general application, each co-enzyme presenting a set of problems peculiar to its synthesis, more effective, if less elegant, methods were sought. The use of carbodiimides, particularly dicyclohexyl carbodiimide, for pyrophosphate formation from phosphoric acid and its derivatives introduced a type of reagent with many of the virtues so clearly necessary [47]. Thus the reagent mentioned acts preferentially with acidic groups to form acid anhydrides, rather than with alcoholic hydroxyls, eliminating the necessity for protecting groups; it is also readily available and quite stable.

Some disadvantages are still retained [1]), but a number of symmetrical and unsymmetrical pyrophosphates have been prepared by means of this reagent. While for the synthesis of certain co-enzymes the carbodiimide approach has failed, dicyclohexyl carbodiimide has proved strikingly successful for the synthesis of co-enzymes I and II by Hughes, Kenner and Todd [41], and for such newer co-enzymes as cytidine diphos-

[1]) Indicated perhaps by the numerous publications describing modifications of the method as applied to the relatively simple syntheses of nucleoside pyrophosphates and triphosphates and of nucleoside cyclic phosphates.

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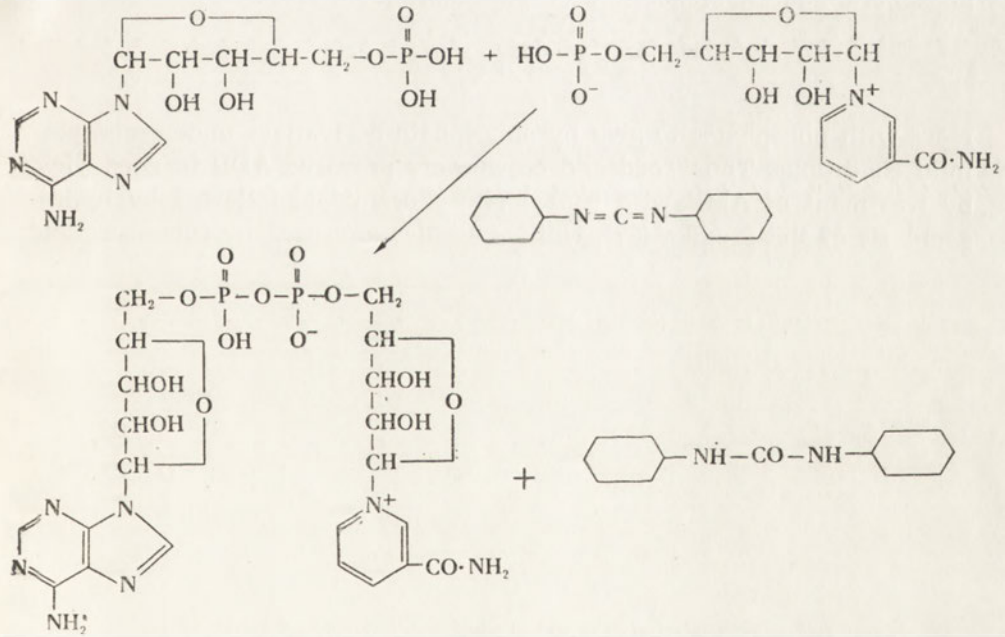
phate choline [43], cytidine diphosphate glycerol [5] and cytidine diphosphate ribitol [4], as well as a multitude of acyl adenylates (anhydrides of adenosine-5′ phosphate) [81]. Normally however, complex mixtures are obtained containing all possibilities of anhydride formation from the mixture of acids used. In the case of a mixture of nicotinamide N-D-ribofuranoside-5′ phosphate and adenosine-5′ phosphate with dicyclohexylcarbodiimide in dimethylformamide solution, the reaction product contained cozymase (Co-enzyme I) as the major component, with rather less di(adenosine-5′) pyrophosphate, and very little of the di-(nicotinamide nucleoside-5′) pyrophosphate. Ion exchange chromatography gave crude cozymase containing largely the β form, together with some of the α. The β form was reduced in a yeast alcohol dehydrogenase system to the alkali-stable dihydro form, and the unchanged labile quaternary α isomer destroyed with alkali, followed by reoxidation to give the pure β form of cozymase identical with the natural co-enzyme. A similar process, replacing adenosine-5′ phosphate with a mixture of adenosine-2′, 5′ and -3′, 5′ diphosphates gave co-enzyme II of some 15% purity.



Several reagents similar to carbodiimides have been developed, among which may be mentioned imidoyl phosphates [2], ketenimines [24], cyanamide and dialkylcyanamides [44] .These show a varying performance against dicyclohexylcarbodiimide as standard, to some extent depending on the substrates used. Thus cytidine-5′ pyrophosphate was prepared in good yield by heating cytidine-5′ phosphate and phosphoric acid with

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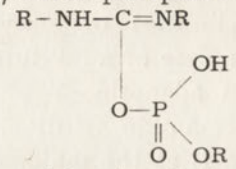
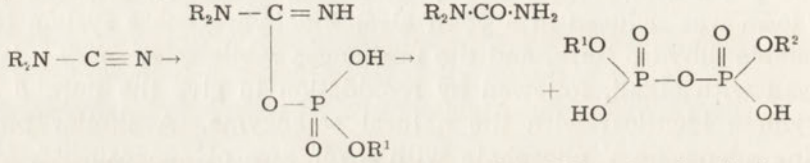
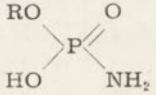
dimethylcyanamide in aqueous dimethylformamide [44]. However, until a reagent of this type is found, whereby it is possible to segregate the first step — formation of a ψ -urea phosphate



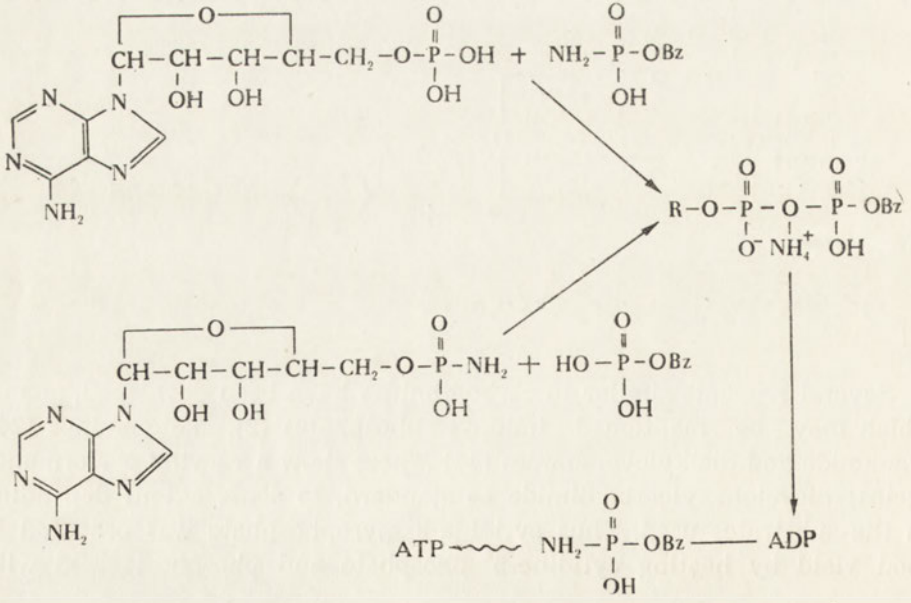
from the secondary reaction with phosphate anions, a specific method has still to be devised for the preparation of unsymmetrical pyrophosphates directly from the phosphate esters.



A third approach to the pyrophosphate bond has involved the use of phosphoramidates. Monoesters of phosphoramidic acids



react with phosphates to give pyrophosphate derivatives under relatively mild conditions. Thus Todd and co-workers prepared ADP in good yield by treatment of AMP with benzyl phosphoramidate followed by hydrogenolysis of the product [27], while a similar approach by Chambers and



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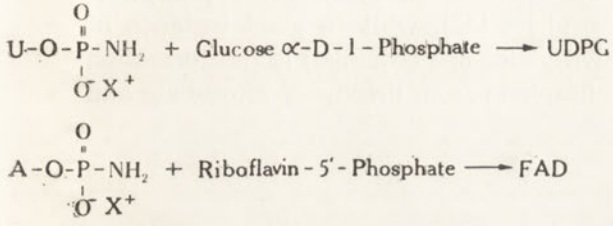
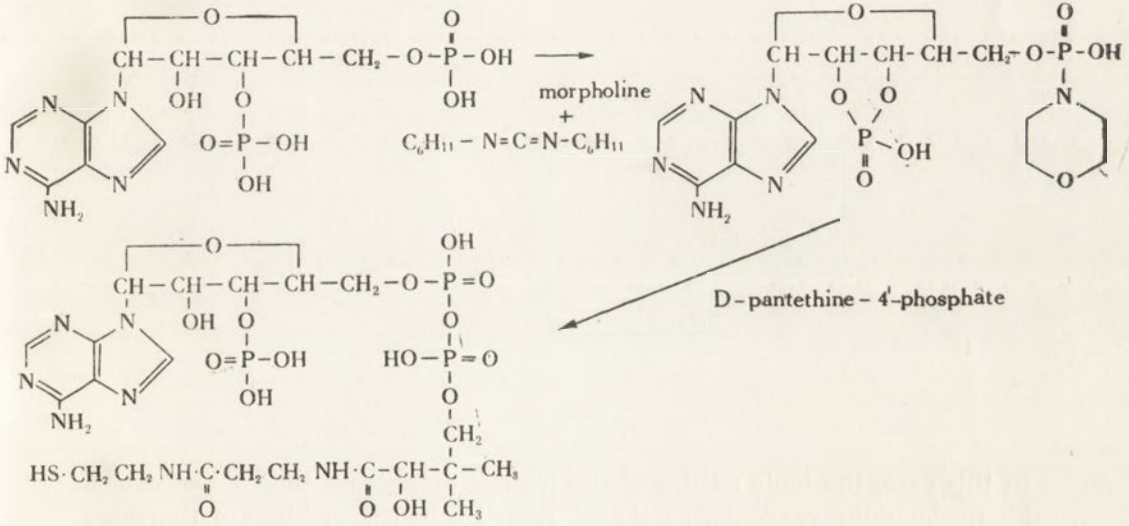
co-workers using the nucleoside phosphoramidate (from AMP, NH_3 and dicyclohexylcarbodiimide) and benzyl phosphoric acid likewise gave ADP [22].

Application to the synthesis of UDPG and FAD was equally successful [80], while the synthesis of co-enzyme A using essentially the same method, has been reported recently [46].





The biological functions of these nucleoside-5′ pyrophosphate derivatives are too numerous and varied to be reviewed here. However, as has been pointed out many times by Sir Alexander Todd [102], the naturally occurring compounds have one significant feature in common — they are all unsymmetrical esters of pyrophosphoric acid, i.e. they are mixed anhydrides. I would like to suggest that it is now more convenient to interpret the properties of these compounds not in terms of the "energy rich bond" [61] concept [2]), but rather in the light of the known behaviour of esterified anhydrides of phosphoric acid with regard to exchange

[2]) A well reasoned criticism of this concept was published some years ago by Gillespie, Maw and Vernon (*Nature* 1953, **171**, 1147).

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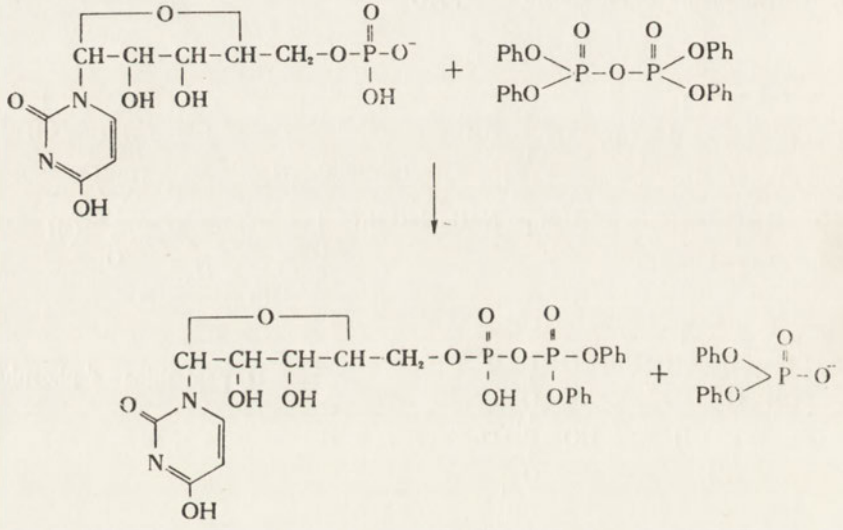
reactions and as acylating agents. The work of the Cambridge School has shown that esterified pyrophosphates are powerful phosphorylating agents for alcohols and amines and also for acids, i. e. they readily undergo exchange reactions with anions [25, 65]. With unsymmetrically substituted anhydrides, in general the weaker acid acylates, producing esters (from alcohols) or new anhydrides (from anions). A chemical example of phosphorylation by such methods is the synthesis of guanosine-5′ phosphate using tetra *p*-nitrophenyl pyrophosphate [23], while a good instance of anion exchange (in that quantitative yields are obtained) is the formation of P^1 uridine-5′ P^2 diphenyl pyrophosphate from uridine-5′ phosphate and tetraphenylpyrophosphate [69].



In this case, the ionic nature of the triester diminishes the ease of attack by nucleophilic reagents, so that further exchange to liberate the more stable anion (i. e. that of the stronger acid) with formation of P^1P^2 di(uridine-5′) pyrophosphate, does not occur. With fully esterified derivatives the second exchange takes place more readily. Thus tetraphenyl pyrophosphate reacts with dibenzyl phosphoric acid (a weaker acid) to produce P^1 diphenyl P^2 dibenzyl pyrophosphate which then undergoes further nucleophilic displacement by the dibenzyl phosphate anion to give tetrabenzyl pyrophosphate [65] (see top of next page).

The position of equilibrium in any exchange reaction will of course depend to a large extent on the relative nucleophilic characteristics of the anions concerned as well as environmental conditions (such as pH, which would have pronounced effect over the range pH 6-8 covering the secondary phosphoryl dissociation), while with a triphosphate A-PPP-B (where A is

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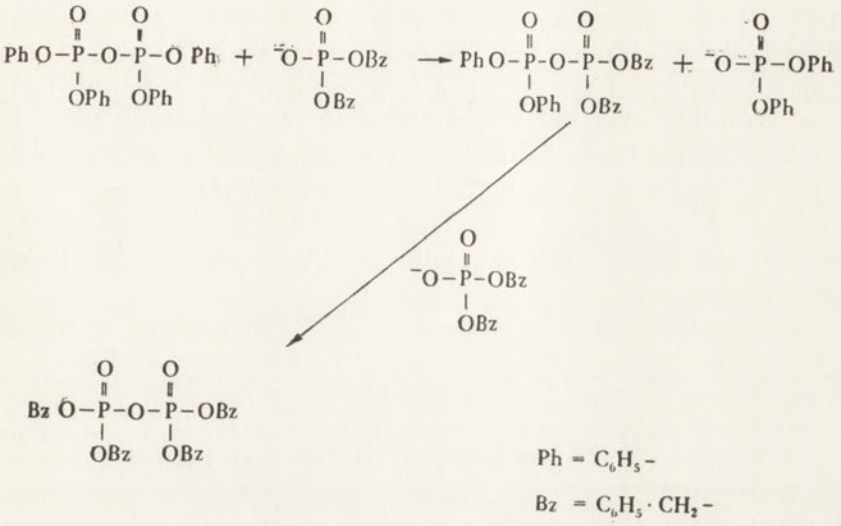
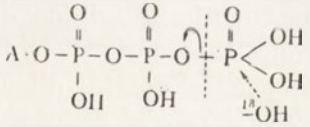


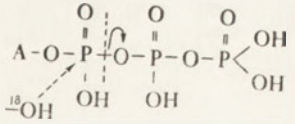
Ph = C_6H_5-

Bz = $C_6H_5 \cdot CH_2$-

a nucleoside and B either hydrogen or an alkyl group) exchange can occur at two loci, i. e. A-P÷PP-B or A-PP÷P-B. Since pyrophosphates are stronger acids than phosphates, this greatly increases the range of exchange and acylation reactions possible with the simpler anhydrides A-PP-B (where B is either hydrogen or an alkyl group). Thus in the simplest instance, hydrolysis of ATP to ADP in the presence of $H_2{}^{18}O$ should give inorganic phosphate containing ^{18}O and ADP free of ^{18}O, i. e.



while hydrolysis to AMP and inorganic pyrophosphate should proceed to yield ^{18}O in the nucleotide only, i. e.



To some extent the point of fission will be controlled by the presence or absence of metal chelation with the enzyme; ligand formation would presumably tend to favour mononucleotide donation rather than phosphorylation, an extreme case being the polymerisation of nucleoside-5′ pyrophosphates. (The stability of divalent metal complexes is in the order triphosphate > pyrophosphate > monophosphate). On this basis one would not expect the biochemical synthesis of ribose-5 phosphate-α-1-pyrophosphate to proceed by the postulated [46a] mechanism of pyrophosphory-

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lation of the sugar hydroxyl at Cl (that is nucleophilic attack at a phosphorus atom) but rather by nucleophilic attack at the sugar carbon *with inversion,* i.e. the substrate is β-D-ribose-5 phosphate and not the α anomer. In this case ^{18}O would appear in the resultant adenosine-5′ phosphate. Another type of attack may be involved in the enzymic synthesis of glycogen [60a] and of "active methionine" [17a].

Biochemical examples of the type of reaction discussed above are legion and include the enzymic syntheses summarised in the following expressions:

A-PPP+B-P ⟷ A-PP-B+PP	Synthesis of FAD, Co I, UDPG and CDP glycerol [91, 48, 103, 93]
A-PPP+B-P ⟷ B-PP+A-PP	Nucleoside monophosphokinases [39]
A-PPP+B-PP ⟷ B-PPP+A-PP	Nucleoside diphosphokinases [14]
A-PP-B+C-P ⟷ A-PP-C+B-P	Uridyl transferase (UDPG + GalP ⟷ UGPGal+GP) [60]
A-PPP+Amino acid ⟷ A-P-Amino acid+PP	Acyl amino adenylates [13]
A-PPP+B ⟶ B-P+A-PP	Phosphokinases [17]
A-PPP+RNA ⟶ RNA-P-A+PP	End group incorporation [110]
n (A-PPP) ⟶ (A-P)n+n PP	DNA synthesis [49]
n A-PP ⟷ (A-P)n+n PP	Polynucleotide phosphorylases [38]

The last examples, acylation with mixed anhydrides (analogous to the chemical polymerisation of ribonucleoside-2′, 3′ cyclic phosphates) [70] are essentially "anhydrous" reactions in aqueous solution, that is, they involve nucleophilic attack by hydroxyl groups. It would seem that a major function of the enzyme is to position the substrates so that the pyrophosphate moiety is closer to a sugar hydroxyl than to molecules of water. That such stereochemical considerations can play an important part in *apparent* reactivity is shown by comparison of the mixed anhydrides obtained by treating uridine-5′ phosphate and uridine-3′ phosphate with ethyl chloroformate — the former is relatively stable, while the latter has only a transient existence, being converted to uridine-2′, 3′ cyclic phosphate, even in aqueous solution [74]. Similar examples are the breakdown of UDPG [85] and FAD [31] to give glucose 1,2 cyclic phosphate and riboflavin 4′, 5′ cyclic phosphate, respectively.

POLYNUCLEOTIDES

While the development of chemical methods for the synthesis of polypeptides has been vigorously pursued since Fischer's work at the turn of the century, the corresponding problems connected with the synthesis of nucleic acids, first isolated by Miescher in 1869 [78] received little attention until lately and it was not until 1955 that the first compound containing a true internucleotide linkage was prepared by Michelson and

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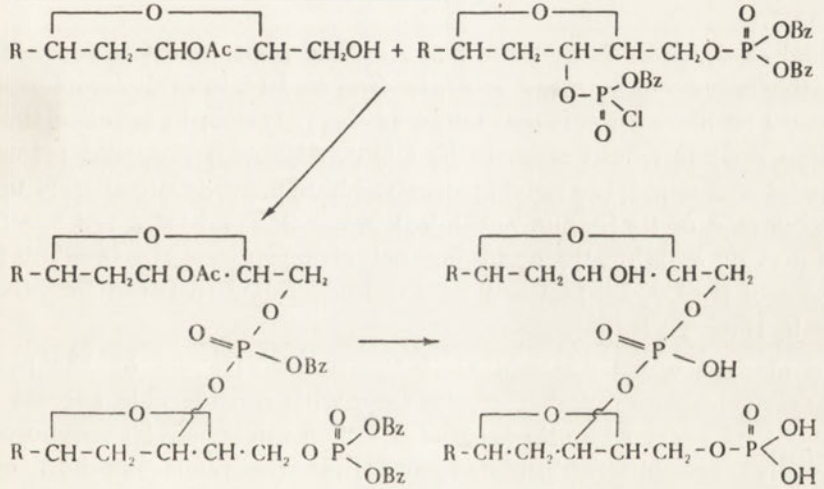
Todd [3]). Treatment of 3′-*O*-acetylthymidine with 5′-*O*-acetylthymidine-3′ benzyl phosphorochloridate gave a fully protected dinucleoside phosphate from which the benzyl group was removed by catalytic hydrogenolysis and the acetyl groups by treatment with mild alkali, to yield dithymidine: 3′,5′: phosphate [76]. An analogous procedure using thymidine-3′ benzyl phosphorochloridate-5′ dibenzyl phosphate to phosphorylate 3′-*O*-acetylthymidine gave the dinucleotide dithymidine 5′: 3′,5′: bis phosphate, isolated as the crystalline calcium salt [76], while extension to the riboside series gave adenosine 2′ uridine 5′ phosphate from 3′,5′-di-*O*-acetyladenosine-2′ benzyl phosphorochloridate and 2′,3′-di-*O*-acetyluridine, followed by removal of protecting groups [75].



Although this classical approach demonstrated the feasibility of synthesis of polynucleotides, it was clear for a number of reasons that any extended synthesis — say of a tetranucleotide — would require alternative simpler and more effective methods. The polymerisation of glycerophosphoric acid to polyglycerophosphoric acid, possibly the simplest analogue of ribonucleic acid, was therefore examined, using a mixed anhydride intermediate [68]. Treatment of glycerol 1 (or 2) phosphate with tetraphenyl pyrophosphate (or diphenyl phosphorochloridate) gave glycerol 1,2 cyclic phosphate *via* the initially formed P^1 glycerol-1 (or 2) P^2 diphenyl pyrophosphate. Addition of more diphenyl phosphorochloridate (or tetraphenyl pyrophosphate) then caused rapid polymerisation to poly-

[3]) Tener and co-workers (*J. Amer. Chem. Soc.*, 1958, **80**, 6223) have recently described the synthesis in low yield of small polythymidylic acids by methods presumably involving nucleoside metaphosphate intermediates (Todd, *Proc. Nat. Acad. Sci.* U. S. 1959, Sept.).

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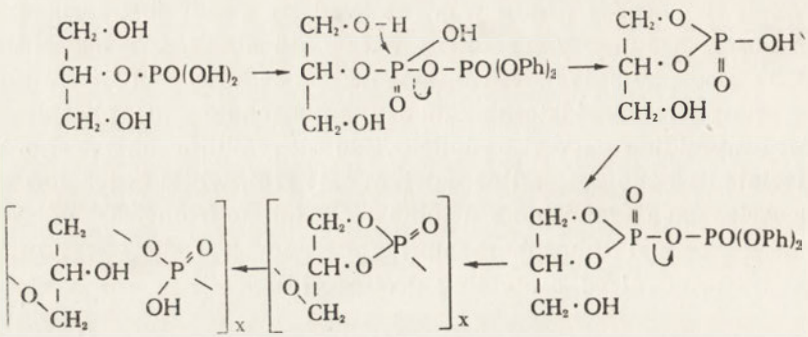
glycerophosphoric acid, presumably *via* a second unsymmetrical pyrophosphate, P^1 glycerol-1,2 cyclic P^2 diphenyl pyrophosphate.



The final cleavage of one of the bonds of the phosphate triester to form the diester can occur in three ways, leading to 1 : 3 interglycerophosphate linkages, 2 : 3 linkages or degradation of the polymer; in practice the degradative process would seem to be slight. Although prepared primarily as a model compound, the polyglycerophosphate proved immediately useful for comparison of its immuno-chemical properties and infra red spectrum with those of a naturally occurring polyglycerophosphate isolated from *Streptococci* by Dr. M. McCarty of the Rockefeller Institute for Medical Research, New York [66].

The method was then applied to the polymerisation of the readily available nucleoside-2′ and-3′ phosphates, with considerable success [73]. Treatment of yeast adenylic acid with either tetraphenyl pyrophosphate or diphenyl phosphorochloridate gave first the nucleoside-2′, 3′ cyclic phosphate, and then quantitative polymerisation to polyadenylic acids of various chain lengths ranging from dinucleotide to approximately icosanucleotide. In a similar fashion polyguanylic, polyuridylic and polypseudouridylic acids were prepared. In the case of cytidylic acid, polymer material was also formed. However chemical, enzymic and spectroscopic examination showed that this "stable" polycytidylic acid did not have phosphate linkages analogous to those in the natural nucleic acids (i. e. between a secondary sugar hydroxyl of one nucleoside and the primary hydroxyl of another) but probably between the 2′ and 3′ hydroxyls and the 6 amino group of the cytosine moiety [74]. Cytidine-2′, 3′ cyclic phosphate was therefore selectively acetylated at the 6 amino group and the resultant N^6 acetyl derivative polymerised in the usual way, followed by very mild alkaline treatment (pH 9.6) to remove acetyl groups from the polymer.

Under the polymerisation conditions generally used, about 20% of the crude product dialysed against water, leaving a residue with an average chain length of 5—6 nucleotides. Further dialysis against 2 M-sodium

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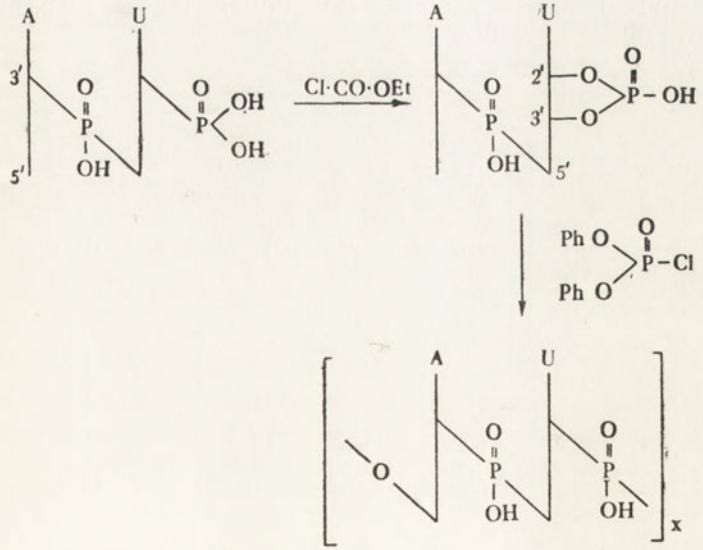
chloride gave a final dialysis residue (35—40% of the original mixture) with an average molecular weight of 3500 to 4500. While the average chain length of this fraction was 10—12, the molecular species varied from polymers approximately 6 units long, to those with up to about 20 nucleotides. Variation of solvent and reaction conditions could possibly give material of higher molecular weight as could substitution of say tetra *p*-nitrophenyl pyrophosphate for the tetraphenyl pyrophosphate used. Under suitably dilute conditions, products containing relatively large amounts of small oligonucleotides were obtained. These were shown to be a homologous series of polynucleotides ending with a 2′, 3′ cyclic phosphate [73]. Apart from homopolymers of the five major ribonucleotides, several co-polymers of the various nucleotides were prepared, including a co-polymer of all the nucleotides — a synthetic "ribonucleic acid" similar in many respects to nucleic acids isolated from yeast. Polymerisation of mixtures of adenylic and uridylic acids in various proportions gave the mixed polynucleotides, with a more or less random distribution of adenine and uracil, while partial polymerisation of adenylic and uridylic acids separately (to an average chain length ~ 3) followed by copolymerisation of the mixture of oligonucleotides, gave polynucleotides with tracts of purines and pyrimidines [73]. Yet a third form of adenylic-uridylic co-polymer was obtained by polymerising the dinucleotide adenosine uridine: 3′, 5′: 3′ bisphosphate, to give polynucleotides with a defined arrangement of alternate adenylic and uridylic acids [74].



Similar polymerisation of G3′P5′C3′P and of A3′P5′A3′P5′C3′P (in these cases the 6 amino group of cytosine had to be first protected by acetyla-

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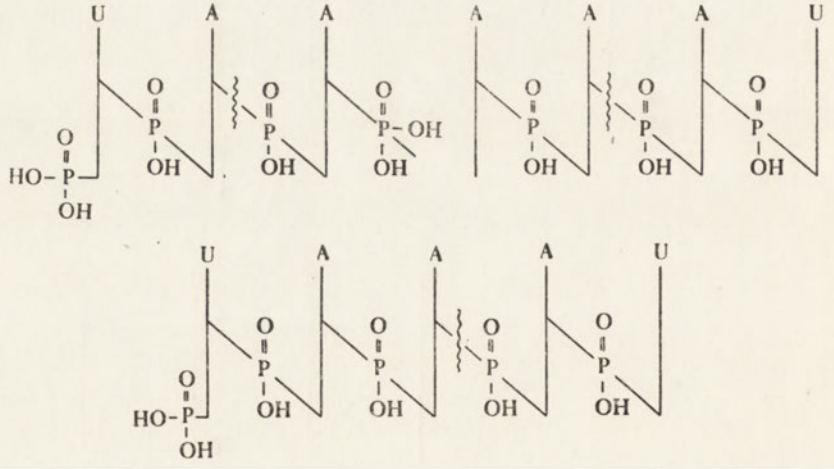
tion) gave the corresponding polymers with a defined, repeating order of bases.

The synthetic polynucleotides prepared in this fashion were linear polymers containing nucleosides combined through 2′-5′ and 3′-5′ phosphodiester linkages, with a terminal 2′,3′ cyclic phosphate which could be opened to the 2′(3′) phosphate. Separation of the homologues in a given series was obtained by gradient elution from columns of ECTEOLA cellulose ion exchanger, using aqueous lithium chloride at pH 7.

Since some nucleic acids can be regarded as polynucleoside-5′ phosphates, it was of interest to synthesise oligonucleotides with a terminal 5′ phosphate, that is, to prepare polymers with specific head and tail residues.

Treatment of uridine-2′ (or 3′) : 5′ diphosphate with tetraphenyl pyrophosphate (or diphenyl phosphorochloridate) yielded P^1-uridine 2′, 3′-(cyclic phosphate)5′-P^2-diphenyl pyrophosphate which was copolymerised with adenylic acid in the usual way, the uridine derivative acting as a chain terminator. Mild alkaline treatment cleaved the triesterified pyrophosphate, to give polyadenylic acid ending with uridine-5′ phosphate at one end and adenosine-2′(3′) phosphate at the other. Similarly, polymerisation of adenylic acid in the presence of 2′, 3′-di-*O*-acetyl uridine followed by mild alkaline removal of the acetyl groups gave polymer with a terminal uridine, while polymerisation in the presence of both uridine derivatives gave products with terminal uridine and uridine-5′ phosphate units, i.e. a polynucleoside-5′ phosphate [73].



The next stage was clearly the stepwise synthesis of oligonucleotides containing a number of different bases in a defined order. Uridine-2′

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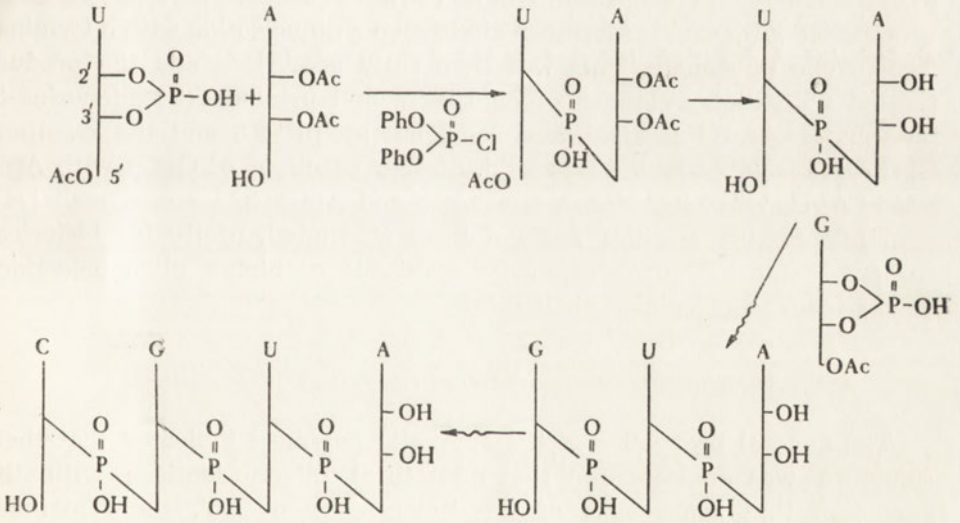
(or 3′) phosphate was converted to the cyclic phosphate by the action of ethyl chloroformate and base in aqueous solution [74]. The anhydrous nucleotide was then fully acetylated and 5′-*O*-acetyluridine-2′, 3′ cyclic phosphate treated with diphenyl phosphorochloridate and base in the presence of 2′, 3′-di-*O*-acetyladenosine. Acylation of the 5′ hydroxyl of adenosine by the intermediate $P^1$5′-*O*-acetyluridine-2′, 3′ cyclic P^2 diphenyl pyrophosphate gave a good yield of the protected dinucleoside phosphate from which the acetyl groups were removed with mild alkali, to give a mixture of uridine-2′ adenosine-5′ phosphate and uridine-3′ adenosine-5′ phosphate. Similar reactions between various nucleotide and nucleoside derivatives gave a number of dinucleoside phosphates, isomeric pairs of which could be separated by ion exchange chromatography. Approximately 50% of the material in each case was 3′-5′ linked, the remainder being 2′-5′ [74].



For the synthesis of trinucleoside diphosphates, advantage was taken of the greatly disparate reactivities of the primary and secondary hydroxyls of ribonucleosides and their derivatives [72]. Treatment of adenosine-3′ uridine-5′ phosphate with 5′-*O*-acetylguanosine-2′,3′ cyclic phosphate and diphenyl phosphorochloridate, followed by removal of the acetyl group, gave GpApU (where p represents a phosphate linkage from the 3′ (or 2′) hydroxyl of the nucleoside on the left, to the 5′ hydroxyl of that on the right). In similar fashion GpUpA, UpGpA, CpUpA and CpApU were prepared from the appropriate acetylated nucleoside-2′, 3′ cyclic phosphate and dinucleoside phosphate [74].

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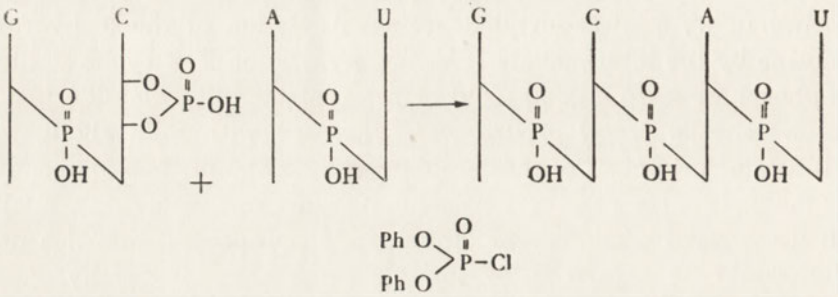
Repetition of this process, using an excess of N^6, 5′-*O*-diacetylcytidine-2′, 3′ cyclic phosphate on GpApU gave CpGpApU and in like manner CpGpUpA and CpUpGpA were synthesised in good yield from the respective trinucleoside diphosphates [74].



An alternate approach to the stepwise synthesis of oligonucleotides with a defined order of bases, used acylation of a dinucleoside phosphate by a dinucleotide. Guanosine cytidine : 3′, 5′ : 3′ bisphosphate (G3′P5′C3′P) was treated with ethyl chloroformate to give a dinucleotide with a terminal 2′, 3′ cyclic phosphate. This was then fully acetylated and the product treated with diphenylphosphorochloridate and uridine-2′(3′) adenosine-5′ phosphate. Acetyl groups were removed at pH 9.8 and the resultant GpCpUpA isolated as the free acid. Similar reactions of GpCp with ApU and of ApUp with GpC yielded GpCpApU and ApUpGpC, respectively [74].

While further addition has not been attempted, partly for historical reasons, extension to the stepwise synthesis of higher oligonucleotides would seem to offer few difficulties.

PROPERTIES OF THE SYNTHETIC POLYNUCLEOTIDES

The general properties of the synthetic polymers indicated that their structure was analogous to that of natural oligonucleotides with the exception that some 50% of the linkages were 2′-5′, the remainder being 3′-5′.

Chemical. Mild acid treatment opened the terminal cyclic phosphate to give polynucleotides with tail units containing a 2′ (or 3′) phosphate, while more vigorous acid-degradation gave either purines and/or pyrimidine nucleoside-2′ and 3′ phosphates, depending on the polymer. Aqueous sodium hydroxide caused complete breakdown to mononucleotide (and terminal nucleoside-2′ (or 3′), 5′ diphosphate or nucleoside, where these were present). All of the homo-polymers (and co-polymers) on treatment with protamine sulphate in aqueous solution gave precipitates which were partially soluble in 2 M-sodium (or lithium) chloride, re-precipitation occuring on dilution.

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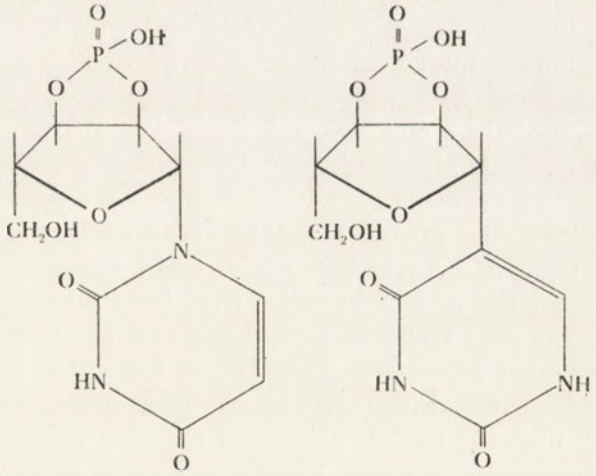
Biochemical. Prostatic monoesterase removed the terminal phosphate from mild acid treated homopolymers, to give a homologous series of oligonucleotides deficient in phosphate, i.e. $(NP)_{n-1}N$ where N is nucleoside. Rattlesnake (*crotalus atrox*) venom caused degradation to the nucleoside and terminal nucleoside-2′ (or 3′), 5′ diphosphate. While dinucleotides (with a terminal 2′ or 3′ phosphate) were quite resistant to the mixture of monoesterase and diesterase present in the venom, trinucleotides were slowly attacked; higher oligonucleotides were more rapidly degraded. When the polymer lacked a terminal 2′ or 3′ phosphate (e.g. CpUpA) then degradation to nucleoside was very fast.

While polyadenylic and polyguanylic acids were resistant to the action of pancreatic ribonuclease, the pyrimidine compounds — polycytidylic, polyuridylic and polypseudouridylic acids were broken down to mixtures of nucleoside-3′ phosphate and di-, tri- and tetra-nucleotides containing 2′-5′ linkages exclusively. That polypseudouridylic acid should be attacked, is perhaps not surprising, since both the overall shape and relative arrangement of functional groups (e.g. for hydrogen bonding to the protein) are maintained [27a].



In a similar fashion, polyadenylic acid was cleaved by spleen diesterase to adenosine-3′ phosphate and enzyme-resistant oligonucleotides containing 2′-5′ internucleotide linkages exclusively [73].

Biological. While polyuridylic acid had no action on tissues in culture, polyadenylic acid was an effective inhibitor [73]. This marked difference has been previously noted in studies on the effect of purine and pyrimidine mononucleotides on tumours in mice [86].

The synthetic polyguanylic acid has been shown by Dr. D. Shugar and Miss H. Tomerska to be highly active in inducing Streptolysin S formation

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in *Streptococci*, while Dr. L. A. Heppel of the National Institutes of Health, Bethesda, U. S. A. has found that the synthetic polymers greatly stimulated the enzymic polymerisation of nucleoside-5′ pyrophosphates by polynucleotide phosphorylase, although, lacking a free 3′-hydroxyl group for esterification, they were not incorporated into the biosynthetic polymer, as were the normal poly (nucleoside-5′ phosphate) primers.

Physical. Degradation of ribonucleic acids by alkali, ribonucleases and other reagents and of deoxyribonucleic acids on treatment with deoxyribonucleases or on denaturation is accompanied by an increase in ultraviolet absorption, normally measured in the region of 260 mμ, the absorption maximum for nucleic acids. This hyperchromic effect has been observed frequently [51, 84, 83, 52, 104, 63, 62, 99, 100, 64, 40, 33, 34, 79, 89], and indeed it may be concluded that all the recorded values of the molar absorptivities (ε max.) for intact nucleic acids are lower than the summation of the absorptivities of the component mononucleotides [10]. Thus Magasanik and Chargaff [63] found a 24—37% increase in absorption on total alkaline hydrolysis of a number of ribonucleic acid specimens. Similar increases have been noted by Holden and Pirie [40] and by Reddi [89] on treating tobacco mosaic virus nucleic acid with leaf ribonuclease, which causes much more extensive degradation than pancreatic ribonuclease, treatment with which gave a 15% increase in absorption. Since the hyperchromic effect was relatively large in fragments with a high guanine content, Magasanik and Chargaff [63] suggested that it might be associated particularly with guanylic acid residues. Similar generalisations involving guanylic and cytidylic acid residues [79] and polypurine segments [89] have been advanced, and occasionally the elementary mathematics of weight ratios have been disregarded in order to make the ribonuclease resistant core account for the major part of the increase in ultraviolet absorption on complete degradation of the intact ribonucleic acid, even when the core has exhibited a smaller hyperchromicity than the nucleic acid from which it was derived.

The hyperchromicity of some biosynthetic high molecular weight polynucleotides has also been described [30, 105]. Alkaline hydrolysis of polyadenylic acid was accompanied by a 55% increment in ultraviolet absorption at the maximum, while with polycytidylic, polyinosinic and polyuridylic acids, the increases were 40%, 60% and 5% respectively [105].

In view of the profound importance of the anomalous ultraviolet absorption characteristics of nucleic acids in relation to the fine structure of the macromolecule, the hyperchromic effect on alkaline degradation of relatively simple synthetic polynucleotides was studied. The results are given in tables 1 and 2, alkaline hyperchromicity being defined as the percentage increase in absorption at the maximum on degradation,

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Table 1

Compound	Average Chain Length	% Alkaline Hyperchromicity
Poly U	11.9	8.6
Poly U	6.6	6.5
Poly U	3.5	4.8
Poly A	13.6	36.7
Poly A	7.7	31.8
Poly A	3.3	19.6
Adenosine-2′(3′) phosphate	—	0
Diadenylic	—	15.1
Triadenylic	—	22.8
Tetraadenylic	—	30.7
Pentaadenylic	—	32.9
Poly G	12.5	0
Poly G	5.4	0
Poly G	3.2	0
A2'P5'U	—	12.8
A2'(3′)P5′T	—	12.7
A3′P5′C3′P (from RNAase digest)	—	7.2
Poly AU (4.5:1) Random	5.2	21.1
Poly AU (4.5:)1 Random	11.5	32.0
Poly AU (1.4:1) Random	8.3	13.4
Poly AU (1.4:1) Random	13.1	22.0
Poly AU (0.8:1) Random	6.8	9.1
Poly AU (3.1:1) Tracts	5.3	22.9
Poly AU (0.12:1)Tracts	4.3	8.5
Poly AG (2.0:1) Random	6.1	19.0
Poly AG (1.9:1) Random	3.3	18.1
Synthetic "RNA"	12.5	14.3
Yeast RNA	12.2	12.0
RNAase resistant cores	6.2	10.9
P5′U poly A	11.9	32.0
P5′U poly A	6.7	30.5
P5′U poly A	4.0	23.1
P5′U poly U	5.4	7.8
P5′U poly U	3.4	7.7
Dicytidylic	—	8.7
Tricytidylic	—	13.9
Tetracytidylic	—	15.3
C2′P5′CP	—	11.0
C2′P5′C2′P5′CP	—	15.7
Polycytidylic	4.4	13.9
Polycytidylic	9.8	15.9
Poly ψ uridylic	6.2	10.5

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Table 2

Compound	% Alkaline Hyperchromicity
A3′P5′A3′P5′C3′P (natural)	22.5
A3′P5′A3′P5′C ,,	23.3
A3′P5′A ,,	10.7
A3′P5′C3′P ,,	8.0
A3′P5′C ,,	7.6
G3′P5′C3′P ,,	3.8
G3′P5′C ,,	3.0
A3′P5′U3′P ,,	4.1
A3′P5′U ,,	1.4
Poly (AAC) 2M-NaCl residue	28.8
Poly (AU) 2M-NaCl residue	17.2
GPCPGPCP	3.1
U2′(3′)P5′A	4.2
U2′P5′A	5.2
U3′P5′A	3.2
A2′P5′U	11.1
A3′P5′U	4.1
C2′(3′)P5′U	8.3
C2′P5′U	7.9
C3′P5′U	8.7
C2′P5′C	10.8
C2′P5′C	7.4
G2′P5′A	5.0
G3′P5′A	3.3
A2′P5′A	18.4
A3′P5′A	13.3
C2′P5′A	9.7
C3′P5′A	10.2
A3′P5′C (natural)	7.6
UpGpA	6.8
GpApU	11.3
GpUpA	5.3
CpApU	12.5
CpUpA	7.5
CpUpGpA	7.7
CpGpApU	12.9
CpGpUpA	6.2
GpCpApU	12.3
GpCpUpA	6.6
ApUpGpC	8.6
Poly ACGU	12.1

absorption measurements being made in alkali at zero time and again in alkaline solution after complete hydrolysis to mononucleotide.

It can be seen that appreciable values are obtained even for dinucleotide derivatives, in agreement with the observations of Sinsheimer

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[95] and of de Garilhe and Laskowski [33] that isolated oligodeoxynucleotides have about a 10% hyperchromic effect. It may also be noted that in a given homologous series hyperchromicity rapidly reaches a limiting value at chain lengths of 5—6, and that the various generalisations of the special effects of purines or of pyrimidines are not in fact correct. It is clear that the increase in ultraviolet absorption on degradation of a nucleic acid is a function, not of any particular tract of nucleotides, but of the total molecule, determined not only by the composition but also the order of bases in the polymer.

Table 3

	Hyperchromicity		
	0·01N-HCl	H_2O pH 7	0·2N-NaOH
Diadenylic	0	—	15.1
Triadenylic	6.8	—	22.8
Tetraadenylic	13.2	—	30.7
Pentaadenylic	16.4	—	32.9
Polyadenylic (13.6)	22.9	—	36.7
Polyguanylic (3.2)	9.0	10.3	0
Polyguanylic (5.4)	14.6	16.5	0
Polyguanylic (12.5)	18.0	23.0	0
Polyadenylicguanylic (6.2)	14.7	36.7	19.0
A2′ P5′ U	12.3	16.0	12.8

In view of the zero hyperchromicity of polyguanylic acids at alkaline pHs, the variation of hyperchromicity with pH was examined for a number of derivatives (Table 3). The results indicate that as with the hyperchromic effect itself, the effect of pH on the ultraviolet absorption of a large nucleic acid, relative to that of its component mononucleotides, will be a composite of many different curves, characteristic not only of the nucleotide composition, but also of the distribution of bases.

Polymerisation can also cause pronounced changes in the apparent p*Ka* values of the bases, particularly with polyguanylic acids. Spectrophotometric titrations indicated that the apparent pK of 2.32 for guanosine-2′ (or 3′) phosphate was shifted to 2.63 for polyguanylic acid with an average chain length of 5.4 units, while in the alkaline region, shifts from 9.33 to 10.76 (for polymer average chain length 5.4 nucleotides) and to 11.20 (for polyguanylic acid, average chain length 12.5 units) were noted. A similar shift (to 10.20) of this guanine dissociation was observed on polymerisation of the dinucleotide G3′P5′C3′P i.e. the effect is not dependent on the juxtaposition of guanine residues. This type of shift has been noted previously in a number of deoxynucleic acids [59].

A reasonable interpretation [71] of these results is that even in the relatively small synthetic polymers, the purine and/or pyrimidine bases

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are stacked in layers above each other and that interaction of π electrons of adjacent rings, with the formation of electron orbitals extending over more than one unit, orientated normal to the plane of the ring systems [4]), causes essentially a new electronic species with an ultraviolet absorption characteristic of the entire molecule, rather than a simple summation of the independent absorptions of the component mononucleotides. This interaction of the π electron systems, evident in the altered absorption spectra, would also have an effect on the ionisable groups of the purines and pyrimidines, since they participate directly in the chromophoric systems [5]). As a corollary, perturbation of these groups — by hydrogen bonding or by ionisation, would affect the π electron interaction of the total chromophoric system. Variation in ionic strength or other factors [105, 20, 32, 15, 101, 57], changing the macromolecular configuration and superstructure would likewise have an effect on this basic interaction, and hence on the ultraviolet absorption. Any process which increases the interaction of the purine and/or pyrimidine rings (e.g. contraction of the macromolecule, metal chelation between rings) will increase the hyperchromic effect, while a further diminution in ultraviolet absorption will be caused by hydrogen bonding between helices [105, 53, 11] or between different parts of a single helix.

The influence of the macromolecular superstructure of undenatured nucleic acids seems to account for some 50% of the total hyperchromicity as shown on denaturation by acid, alkali and heat [57], a process presumably due to the breaking of hydrogen bonds cross linking either two helical chains as in the Watson-Crick model of DNA, or between turns of a single chain in a spiral with the bases parallel to the long axis of the molecule as in the model for tobacco mosaic virus nucleic acid described by Ginoza [35]. Degradation of this latter polymer by ribonuclease to an extent corresponding to one break per molecular weight of 7000 is accompanied by a 15% increase in ultraviolet absorption at 260 mμ. [34]. The hyperchromic effect of a dinucleotide is of the order 10%. On the basis of one internucleotide break per twenty nucleotides this would average out as a maximum hyperchromicity of 1%, rather than 15%, implying that such a break is sufficient to destroy any macromolecular configuration which the biologically active nucleic acid may have in solution [34], and which is responsible for part of the abnormal ultraviolet absorption. This also suggests that minimum chain

[4] A somewhat distant analogy may lie in such compounds as ferrocene and dicyclopentadienylmagnesium where the metal bond is to the ring system as a whole, rather than to one particular atom.

[5]) Such an interaction might also be expected in poly-L-tyrosine (Katchalski and Sela, *J. Amer. Chem. Soc.*, 1953, **75**, 5284).

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lengths of twenty to forty nucleotides are necessary for the natural occurrence of helical structures in nucleic acids, though with certain homopolymers it is likely that helical structures can be formed at much shorter chain lengths. A possible significance for the presence of fully methylated purine components in certain ribonucleic acids may be to prevent such intra-helical hydrogen bonding, that is, the ribonucleic acid has a discontinuous ordered structure of stretches of helix linked by non-helical portions.

Table 4

Paper Electrophoresis

Movement (cm.) towards the anode is tabulated, for Whatman No 1 paper, with I. 0.02 M-Na_2HPO_4 (pH 8.9); II. 0.02 M-KH_2PO_4 (pH4.5); both at 10v/cm. for 2 hr.

	I	II		I	II
A2′ (3′) P	9.3	6.9	A2′ P5′ A	5.0	4.5
C2′ (3′) P	10.5	8.2	A3′ P5′ A	4.3	3.7
G2′ (3′) P	9.6	7.4	A3′ P5′ A (natural)	4.3	3.7
U2′ (3′) P	11.1	8.8	C2′ P5′ A	5.4	4.7
Ψ U2′ (3′) P	10.8	8.0	C3′ P5′ A	4.8	4.6
			A3′ P5′ C (natural)	4.8	4.7
U2′ (3′) P5′ A	5.7	5.0	A2′ P5′ U	5.3	6.0
C2′ (3′) P5′ U	6.5	5.9	A3′ P5′ U	5.2	4.9
G2′ P5′ A	5.5	5.0	A3′ P5′ U (natural)	5.2	4.9
G3′ P5′ A	5.0	4.3	A5′ P5′ U	5.6	5.7
UpGpA	6.9	7.6	CpUpGpA	8.7	7.9
GpApU	6.9	7.3	CpGpApU	8.9	8.5
GpUpA	6.5	6.8	CpGpUpA	8.5	6.9
CpApU	7.7	7.6	GpCpApU	8.7	8.0
CpUpA	7.2	7.3	GpCpUpA	8.3	7.6
			ApUpGpC	8.3	7.6

Further evidence of limited freedom of movement about the internucleotide linkage even at the dinucleotide level, under normal conditions, was provided by an examination of isomeric dinucleoside phosphates. In general the 2′-5′ isomer has a higher hyperchromicity than the 3′-5′ derivative (Table 2) while differences in electrophoretic mobility suggest that the 2′-5′ compounds are more compact or "streamlined" than the 3′-5′ isomers, i.e. that stronger interaction occurs between the bases to restrict rotation about the internucleotide linkage. In each case examined, the 2′-5′ isomer had a greater electrophoretic mobility, although the 3′-5′ dinucleoside phosphate was the stronger acid (Table 4).

It is also significant that A2′P5 U showed a hyperchromicity twice that of the isomeric U2′P5′A, i.e. the effect is dependent on the order of the bases. That the synthetic polynucleotides, although relatively small, have

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definite configurations involving the stacking of purines and/or pyrimidines parallel to each other in aqueous solution, was further demonstrated by the hypochromic effect on the absorption spectra of dyestuffs in combination with the polymers.

The binding of certain planar basic dyes by nucleic acids has been studied extensively [67, 29, 21, 54, 55, 108, 97], as has the interaction of amino acridines [58, 42, 16, 82, 87]. Basic dyes which exhibit metachromasy are characterised by their failure to obey Beer's Law in aqueous solution, due to aggregation of the dye molecules, causing an alteration in the π electron system of the chromophores. Binding of the dye by nucleic acids causes similar effects [108] and indeed the induced metachromasy of toluidine blue has been used [29] to show that the uncombined phosphate groups in calf thymus nucleoprotein occur in sequences. The binding of rosaniline by nucleic acids has received much attention [21, 54, 55], while similar spectral changes accompanying the binding of acridine orange by high molecular weight biosynthetic polyadenylic acids have been described by Steiner and Beers [97] who also report a quenching of the fluorescence of this dyestuff similar to that due to dimerisation [88]. That the metachromasy of such dyes due to concentration effects is a direct parallel to the hyperchromicity of polynucleotides is further suggested by work which has shown that the forces operating between the rings of the neighbouring ions of the dye aggregate have an equilibrium distance of 3—4 Å [88, 94].

The effect of some low molecular weight synthetic polymers (average chain length approximately 6) on the absorption spectra of rosaniline, toluidine blue and acridine orange is shown in Figs. 1 and 2. Equimolar amounts of dye and polymer phosphate were used to avoid "monomeric" binding [56] to phosphate groups, which would presumably arise if a large excess of polymer was present. In all cases the effects due to the synthetic polymers were smaller than those observed with yeast nucleic acid. Since the linkages in the synthetic materials are mixed 2′-5′ and 3′-5′ the arrangement of phosphate groups along the backbone is not as uniform as in the natural material so that while all sites may have been occupied, only where tracts of 2′-5′ or of 3′-5′ linkages occurred would any metachromasy result, giving rise effectively to "dimeric" binding, rather than "polymeric", using Lawley's definition of the terms [56]. It can be seen that the nature of the purine or pyrimidine has a profound effect on the induced metachromasy of the dye, i.e. that secondary stereospecific requirements for binding sites are also necessary. The influence of the chain length of the polynucleotide on interaction with dyestuffs is indicated (Figure 3); minimum chain lengths of 8—10 nucleotides would seem to be necessary for significant interaction to occur.

Marked quenching of the fluorescence of acridine orange was also

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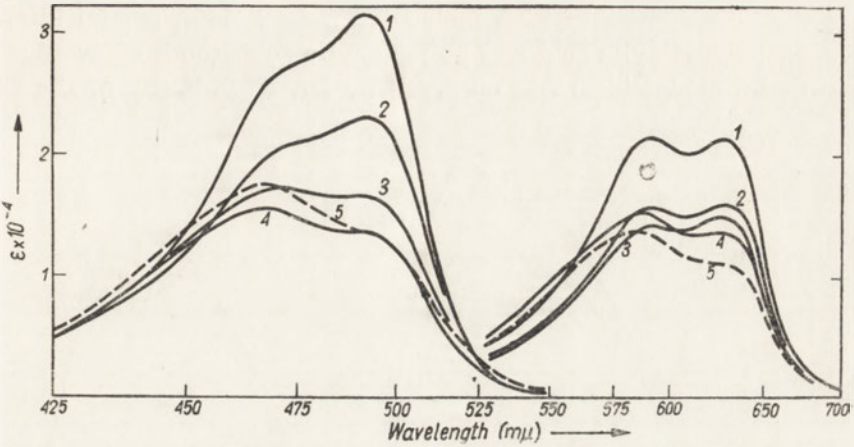

Fig. 1. Action of Polynucleotides 3×10^{-5} M (P) on the absorption spectra of Acridine Orange (3×10^{-5} M) (left) and Toluidine Blue (3×10^{-5} M) (right) in 0.01 M-ammonium acetate at pH 6.8. 1, Dye alone; 2, 3, 4 and 5, with added polyuridylic, polyadenylic, polyguanylic, and yeast ribonucleic acid, respectively.

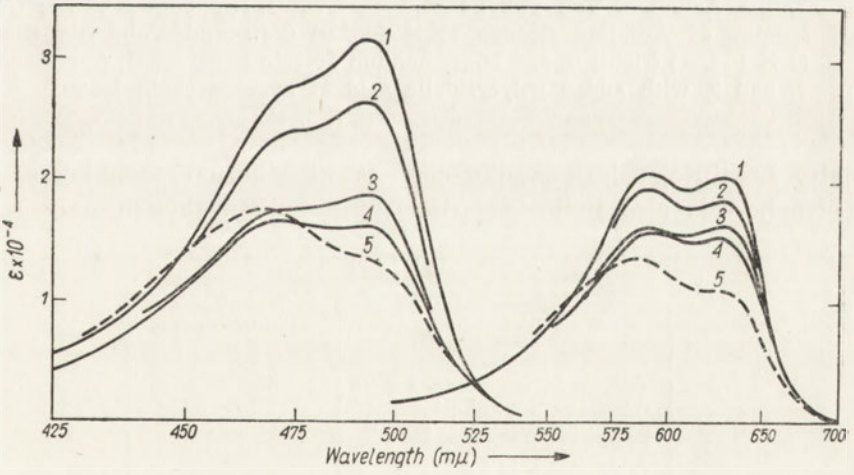

Fig. 2. Action of Polynucleotides 3×10^{-5} M (P) on the absorption spectra of Acridine Orange (3×10^{-5} M) (left) and Toluidine Blue (3×10^{-5} M) (right) in 0.01 M-ammonium acetate at pH 6.8. 1, Dye alone; 2, 3, 4, and 5, with added poly ACGU, poly AG, ribonuclease resistant "cores" from yeast nucleic acid, and yeast ribonucleic acid, respectively.

observed, particularly by polyguanylic acid and to a lesser extent by polyuridylic acid (Table 5). Polyglycerophosphate with an average chain length of 12.1 glycerophosphate units was quite without effect on the absorption of these dyes or on the fluorescence of acridine orange.

While no evidence was found of interaction between the synthetic polyadenylic and polyuridylic acids, similar to that observed with

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biosynthetic polymers of high molecular weight [105], partial interaction between polycytidylic acid and polyguanylic acids (both of average chain length ~ 6 nucleotides) was observed by Dr. K. S. Kirby of the Chester

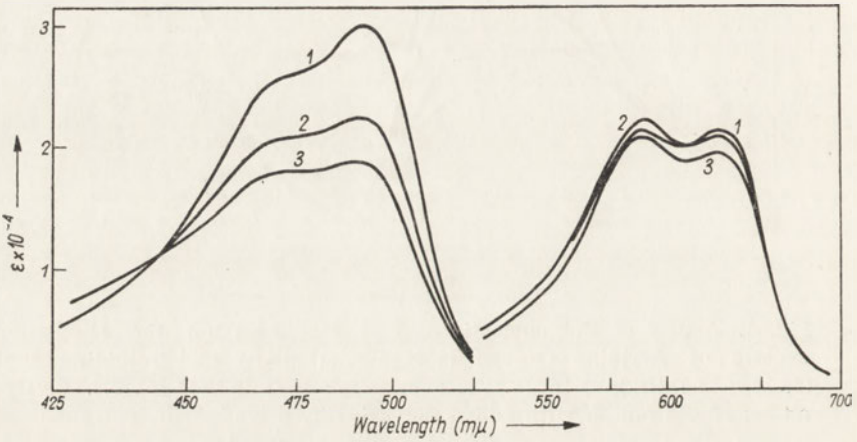

Fig. 3. Action of Polycytidylic acid 3 × 10^{-5} M (P) on the absorption spectra of Acridine Orange (3 × 10^{-5} M) (left) and Toluidine Blue (3 × 10^{-5} M) (right) in 0.01 M-ammonium acetate at pH 6.8. 1, Dye alone; 2, and 3, with added polycytidylic acid of average chain length ~ 6 and ~ 10 nucleotides respectively.

Beatty Institute, using counter-current distribution techniques. Paper electrophoresis also indicated partial interaction, though only a slight

Table 5

Substance	Fluorescence %
Acridine orange alone	100
Polyglycerophosphate	99.5
Adenosine-2′ (3′) phosphate	99
A2′ P5′ U	99
A3′ P5′ C3′ P	98
Poly U	86
Poly ACGU	75
Poly A	62
Poly G	52
Poly AG	46
RNAase resistant core	37
Yeast RNA	30

decrease in absorbance (3%) occurred on mixing solutions of the two polymers. It seems probable that hydrogen bonding between the cytosine

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6 amino group and the guanine-NH-CO-group is stronger than that involved between adenine and uracil [6]). This interaction, though only partial (presumably only the longer polynucleotides in the mixture are effective) is a final demonstration that relatively small oligonucleotides have a definite ordered configuration with restricted rotation about the internucleotide linkages, previously suggested by the hyperchromic effect, the shift in apparent pK*a*s, the electrophoretic mobilities of isomeric dinucleoside phosphates and the interaction with planar basic dyes.

That the configuration favouring interplanar interaction may be of lower energy than other configurations has been shown by the effect of adenosine on the fluorescence of riboflavin (particularly in flavin adenine dinucleotide) [106] and by the quenching effect of purines on the fluorescence of certain hydrocarbons [107]. In any nucleic acid one might therefore expect two opposing effects — strain set up by the highly charged sugar phosphate backbone, counteracted to some extent by binding between purines and pyrimidines. If this stabilising effect (perhaps evident in the exceptional stability to alkali of di- and tri-adenylic acids) [53a] reaches a limit at a relatively low number of nucleotide units, then the spontaneuos degradation of high molecular weight ribonucleic acids to much smaller polymers may possibly be expected. The protective action of high ionic strength [101, 19] and in particular the greater efficiency of divalent cations such as Mg^{++}, Ca^{++} and Ba^{++} in reducing ε values [92] would be interpreted as due to the formation of ion pairs along the phosphate backbone. It is of interest that the binding constant for dye-nucleic acid complexes is lowered by excess sodium ions, and that divalent cations are some thirty times more effective than monovalent [54].

Because of this restricted rotation, small oligonucleotides could act as templates (c/f the interaction of low molecular weight polyguanylic and polycytidylic acids) — a factor of possible significance for speculations on the chemical origin of life. Since these small polynucleotides precipitate with protamine and bind planar basic dyes, it is possible that protection by small peptides would occur at relatively short chain lengths, giving rise to a process of natural selection at the molecular level and evolution from primitive oligonucleotide-peptides, to the present day nucleoproteins. Some biological properties of proteins and nucleic acids are not entirely dependent on extremely high molecular weight.

In this region of pure speculation one might also mention the possibility of identical replication (rather than complementary) with some of the simpler polynucleotides, as suggested by the work of Rich [90] and others

[6]) Marmur and Doty have recently (*Nature* 1959, **183**, 1427) shown that the denaturation temperature of DNA is a function of the guanine-cytosine content.

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on the formation of double and treble strands from enzymically prepared homopolymers. Identical replication may also be involved in the interesting single stranded DNA of bacteriophage ΦX 174, described by Sinsheimer [96].

Recent work has strongly suggested that RNA is formed in the nuclei, then transferred to the cytoplasm [109] where it is required for protein synthesis. This is perhaps quite reasonable in that the metabolic stability of RNA is such that the information carrier must be renewed continuously, a necessary situation if Mendelian genetics are to be maintained undistorted by extensive cytoplasmic inheritance, which would perhaps occur were stable forms of RNA (or nucleoprotein) abundant. The difference between plant viruses such as tobacco mosaic virus (RNA) and the bacterial viruses and transforming factors (DNA) is after all, a matter of degree, rather than absolute. In each case information for self replication is carried, as well as an ability to cause specific visual and biochemical effects, though of course this replication is intimately connected with the metabolism of the frequently extremely specific host. It is perhaps not surprising that specialisation and differentiation occur so soon (e.g. the phosphatase activity and contractile nature of the protein tail of T2 bacteriophage) [50]. While the one gene — one enzyme situation may well hold in biochemistry [9], at a lower level there is no *a priori* reason for assuming one molecule — one function. Harris has shown that the synthesis of protein and RNA are prerequisites for DNA synthesis [37], in agreement with the mechanism of DNA replication devised by Stent [98]. In this scheme the double helical DNA molecule serves as a template (through hydrogen bonding to the complementary base pairs) for synthesis of nucleoprotein containing a single RNA strand, which now contains the information of the parent DNA double helices. Reversal of this process, that is alignment of complementary pairs of deoxyribonucleotide derivatives on single bases of the RNA strand by specific hydrogen bonds, followed by polymerisation, gives DNA. *In toto* this provides not only a mechanism for the synthesis of DNA but also an attractive method for transfer of information from the chromosomal DNA, to RNA involved in wider metabolic activities.

In conclusion I should like to quote Prof. W. T. Astbury [1] — "More dimly still, but now not nearly so as it appeared only a short while ago, a day can be envisaged when we may be able, in a process analogous with infection by a bacteriophage, or with any fertilization, to add a synthetic polynucleotide to a collection of synthetic substrates and thereby set going a little hive of 'synthetic life'." He then goes on to say that there is something seriously wrong with dreams such as this. That "systems of proteins and nucleic acids and accessory molecules with

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nothing else can scarcely keep on thinking and asking questions about themselves and experimenting on themselves until they have at last succeeded in making themselves. Reason apart, the halt will surely be called by an ultimate indeterminancy principle which says that it is impossible to place the required components in their correct places all at the same time".

This may well be wishful thinking. In any case, it is perhaps a more worthy objective than the abuse of ducks [12], or for that matter, man.

SUMMARY

Chemical methods for the synthesis of nucleotide coenzymes are described. These include the reaction of an acid chloride with the salt of an acid, the use of carbodiimide reagents and reaction of monoesters of phosphoramidic acids with phosphates. The chemistry of esters of pyrophosphoric acid is related to the biological functions of the naturally occurring derivatives.

The chemical polymerisation of mononucleotides through intermediate mixed anhydrides, gives rise to linear polymers analogous to those of natural origin, except that some of the linkages are 2′5′ rather than 3′5′. Using this method homopolymers of the five major ribonucleotides, as well as various copolymers, have been prepared, including three different types of copolymer of adenylic and uridylic acids containing random distribution of bases, tracts of bases, or alternating adenylic and uridylic acid units. The stepwise synthesis of oligonucleotides containing a number of different bases in a defined order is also described. Some of the synthetic polymers show biological activity.

The physical properties of these synthetic polynucleotides of relatively low molecular weight show that they have ordered structures involving the "stacking" of purines and/or pyrimidines, with restricted rotation about the internucleotide linkages, even at the dinucleotide level. These physical properties include the hyperchromic effect on degradation of the oligonucleotides, the variation in pK values of the bases on polymerisation, the interaction of the polymers with certain basic dyes, a comparison of the hypochromicity and electrophoretic mobility of isomeric dinucleoside phosphates, and the interaction between synthetic polyguanylic and polycytidylic acids.

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Z POGLĄDÓW NA CHEMIĘ POLINUKLEOTYDÓW I KOENZYMÓW NUKLEOTYDOWYCH

Streszczenie

Opisano chemiczne metody syntezy koenzymów nukleotydowych. Obejmują one reakcje chlorków kwasowych z solą kwasu, użycia odczynników karbodwuimidowych oraz reakcji monoestrów kwasów fosforoamidowych z fosforanami. Starano się powiązać własności chemiczne estrów kwasu pyrofosforowego z funkcjami biologicznymi ich pochodnych występujących w przyrodzie.

Chemiczna polimeryzacja poprzez mieszane bezwodniki prowadzi do powstania łańcuchowych polimerów analogicznych do występujących w przyrodzie, z tym jednak, że niektóre spośród wiązań są raczej 2′5′ niż 3′5′. Stosując tę metodę otrzymano homopolimery pięciu zasadniczych rybonukleotydów a także różne kopolimery, a w tym trzy różne rodzaje kopolimerów kwasów adenilowych i urydylowych, różniące się rozmieszczeniem zasad; jedne z nich mają zasady rozmieszczone przypadkowo, w innych następują po sobie ciągi tej samej zasady, w trzeciej wreszcie grupie kwas adenilowy i urydylowy idą na przemian. Opisano również stopniową syntezę oligonukleotydów zawierających pewną ilość różnych zasad w określo-

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nym porządku. Niektóre syntetyczne polimery wykazywały aktywność biologiczną.

Fizyczne własności tych syntetycznych polinukleotydów o stosunkowo niewielkiej masie cząsteczkowej wykazują, że mają one uporządkowane struktury, przy czym pierścienie purynowe i pirymidynowe są „usztywnione" z ograniczoną możnością rotacji dokoła wiązania międzynukleotydowego, nawet w przypadku dwunukleotydów. Te fizyczne właściwości obejmują wzrost barwliwości przy postępującej degradacji oligonukleotydów, zmianę wartości pK zasad w miarę polimeryzacji, reagowanie polimerów z pewnymi barwikami zasadowymi, równoległość wzrostu barwliwości i ruchliwości elektroforetycznej izomerycznych fosforanów dwunukleozydowych, a także reagowanie ze sobą syntetycznych kwasów poliguanilowych i policytydylowych.

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B. DROŻDŻ

ODRÓŻNIENIE KSYLOZY OD ARABINOZY I INNYCH CUKRÓW

Zakład Farmakognozji A. M. w Poznaniu
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Do odróżniania pentoz tak w analizie kroplowej, jak i na chromatogramach nadają się roztwory kwasu barbiturowego i kwasu tiobarbiturowego w kwasie octowym lodowatym. Wspomniane kwasy znane są już w analityce węglowodanów. Jäger i Unger [7] wykorzystali zdolność kwasu barbiturowego do tworzenia trudno rozpuszczalnych połączeń z aldehydami aromatycznymi dla ilościowego oznaczania furfurolu powstającego z pentoz. Dox i Plaisance [2] z jeszcze lepszym wynikiem zastosowali kwas tiobarbiturowy. Eder i Sack [4] oznaczyli zawartość glicyryzyny w surowcach i preparatach farmaceutycznych strącając kwasem barbiturowym furfurol powstający z kwasu glikuronowego. Drożdżowa i Drożdż [3] stwierdzili przydatność roztworu kwasu barbiturowego w mieszaninie kwasu octowego i mlekowego w mikroanalizie surowców roślinnych dla wykazania błon zdrewniałych.

Badając działanie roztworów kwasu barbiturowego, lub tiobarbiturowego w kwasie octowym lodowatym na cukry stwierdzono występowanie barwnych plam. Barwne plamy są charakterystyczne dla poszczególnych grup cukrów, a co więcej umożliwiają rozróżnienie pentoz, ponieważ w przypadku ksylozy pojawia się ciemnozielone, a arabinozy i rybozy brązowawe zabarwienie.

Analiza kroplowa

Najlepsze wyniki szybkiego odróżnienia ksylozy od arabinozy i innych węglowodanów osiągnięto przy zastosowaniu metody kroplowej na bibule chromatograficznej Whatman 1.

Na bibułę nanoszono od 1 do 10 μg cukrów w roztworach (plama wielkości 20 do 50 mm^2), po wysuszeniu spryskiwano bibułę 1% roztworem kwasu barbiturowego w kwasie octowym lodowatym przygotowanym na zimno, lub 0,5% roztworem kwasu tiobarbiturowego w kwasie octowym

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Tablica 1

Reakcje barwne cukrowców z kwasem barbiturowym i tiobarbiturowym

Cukrowiec	Zabarwienie plam w świetle	
	dziennym	ultrafioletowym
A. Kwas barbiturowy		
Aldopentozy:		
L-ksyloza, D-ksyloza	ciemnozielone	brunatnozielone
L-arabinoza, D-ryboza	jasnobrązowe	jasnopomarańczowe
Aldoheksozy:		
D-glikoza, D-galaktoza D-mannoza	żółtopomarańczowe	ciemnożółtozielone
Ketoheksozy:		
L-sorboza, D-fruktoza	pomarańczowe	żółte
6-dezoksyheksozy:		
L-ramnoza, L-fukoza	jasnożółte	zielonożółte
Kwasy uronowe:		
kwas D-galakturonowy	jasnozielone	żółtozielone
Disacharydy:		
maltoza, laktoza	żółtopomarańczowe	żółtozielone
B. Kwas tiobarbiturowy		
Aldopentozy:		
L-ksyloza, D-ksyloza	ciemnooliwkowe	brązowe
L-arabinoza D-ryboza	szarobrązowe	jasnobrązowe
Aldoheksozy:		
D-glikoza, D-galaktoza D-mannoza	żółtopomarańczowe	pomarańczowe
Ketoheksozy:		
L-sorboza, D-fruktoza	ciemnopomarańczowe	pomarańczowe
6-dezoksyheksozy:		
L-ramnoza, L-fukoza	żółte	zielone
Kwasy uronowe:		
kwas D-galakturonowy	szare	żółtozielone
Disacharydy:		
maltoza, laktoza	żółte (b. słabo widoczne)	zielonkawe

lodowatym. Bezpośrednio po spryskaniu, bibułę umieszczano na przeciąg 10 do 15 minut w suszarce w temperaturze 125°. Na białym tle pojawiały się wówczas żółte plamy, które po krótkim trzymaniu bibuły nad wrzącą wodą przyjmowały zabarwienie charakterystyczne dla poszczególnych cukrów, jak to wykazuje załączona tablica.

Cgrzewanie. W temperaturze niższej od 120° plamy pojawiają się wolniej. W temperaturze 90—100° podobną intensywność osiągają dopiero

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po kilku godzinach ogrzewania. Plamy ketoz i dwusacharydów w temperaturze poniżej 100° są prawie niewidoczne.

W temperaturze powyżej 130° reakcja przebiega szybko, ale tło zabarwia się również na kolor żółtawy, a same plamy cukrów nie wykazują już tak wyraźnego zróżnicowania barw, jak w przypadku wywoływania w temperaturze 120—130°.

Nawilgacanie plam. Przeprowadzając próby zaobserwowano, że żółte plamy poszczególnych cukrów zmieniają po pewnym czasie swoje zabarwienie przyjmując charakterystyczne odcienie. Proces różnicowania barw przyspieszono umieszczając bibułę bezpośrednio po ogrzaniu nad wrzącą wodą. Po kilku minutach uzyskiwano rezultat, który w normalnych warunkach osiągano po kilku względnie nawet kilkunastu godzinach.

Trwałość plam. Plamy poszczególnych cukrów wywołane podanymi odczynnikami zachowują się jeszcze po upływie kilku miesięcy, zmienia się jednak ich natężenie i zabarwienie.

Zastosowanie w chromatografii

Roztwory kwasu barbiturowego i tiobarbiturowego w kwasie octowym lodowatym nadają się do uwidoczniania plam cukrów na chromatogramach. Stosując podany sposób wywoływania bezspornie identyfikowano poszczególne cukry w ilościach do 5 μg, po ich rozwinięciu różnymi zestawami rozpuszczalników [1, 5, 6].

Badane cukry pochodziły z firmy S. Hoffmann — La Roche, Basel. Dodatkowo przebadano L-ksylozę z firmy Eastman Kodak i D-ksylozę otrzymaną z Podhurtowni Odczynników Katowickiej Hurtowni Farmaceutycznej, a deklarowaną jako „D(+)-ksyloza cz. przep. import. Niemcy".

STRESZCZENIE

Zastosowano roztwory kwasu barbiturowego i tiobarbiturowego w kwasie octowym lodowatym do wykrywania ksylozy, arabinozy i innych cukrów w analizie kroplowej i na chromatogramach bibułowych. Szybkie i dobre zróżnicowanie barw otrzymuje się, wywołując plamy w 125° przez 15 minut i następnie nawilgacając je nad wrzącą wodą.

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IDENTIFICATION OF XYLOSE, ARABINOSE AND OTHER SUGARS

Summary

The solutions of barbituric and tiobarbituric acid in glacial acetic acid were used to detect xylose, arabinose and other sugars by means of drop analysis as well as in paper chromatography. The quick and clear differentiation of colours was obtained when the spots were developed at 125° for 15 min. and then moistened over boiling water.

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