ACTA BIOCHIMICA POLONICA

QUARTERLY

Vol. XI
No. 4

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GLUCOSE, LACTATE AND PYRUVATE IN THE BLOOD OF GUINEA PIGS AFTER ELECTRICAL STIMULATION OF THE BRAIN

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The present work was initiated under the guidance of the late Professor Dr. Bolesław Skarżyński, our former Teacher, and member of the Scientific Council of the Department of Experimental Pathology of the Polish Academy of Sciences, to whom we owe a dept of deepest gratitude for his unfailing help and interest.

In 1955 - 1957 Maśliński & Karczewski [7, 8] on applying lethal doses of histamine to guinea pigs have observed lower mortality among those animals which had been subjected to electrical stimulation of the brain. This stimulation is known to cause contractions of skeletal muscles and apnoea, but the mechanism of its protective effect in histamine shock has not been elucidated.

The present work is concerned with the changes in the concentration of glucose, lactate and pyruvate in the blood of guinea pigs after electrical stimulation of the brain.

MATERIALS AND METHODS

For experiments male guinea pigs weighing 300 - 430 g. were used. Electrical stimulation of the brain was performed according to Maśliński & Karczewski [7, 8], the time of stimulation in the first series of experiments being 30 sec., and in the second one 11 - 13 min. depending on the weight of the animal.

Before the experiment 0.1 ml. of heparine solution (500 I.U.) was injected to the animal, then two 2.3 ml. samples of blood were taken from the *arteria carotis communis*, one sample just before and another immediately after stimulation, the total amount collected being about 16% of the blood volume of the guinea pig.

Glucose was assayed with glucose oxidase according to Hugget .

& Nixon [5]; the extinction was determined in a Coleman photocolori-
meter at 430 mμ. Lactate and pyruvate were assayed with lactate de-
hydrogenase according to Horn & Bruns [3] for lactate, and to Thorn
et al. [14] for pyruvate, the extinction being determined in a Hilger
spectrophotometer at 340 mμ.

Special reagents used were: lactate dehydrogenase, glucose oxidase,
peroxidase, NAD and NADH (Boeringer, Mannheim). Other organic
reagents were from the same source. Perchloric acid (Merck, Darmstadt)
was used for deproteinization of the blood.

Table 1

*The concentration of glucose and lactate in the blood of normal guinea
pigs, depending on the loss of blood*

Three samples of blood, 2.3 ml. each, were taken at the indicated time intervals
between the collecting of the first and second and the second and third samples.
The results of 7 separate experiments are given.

Sample...	Glucose (mM)			Lactate (mM)		
	I	II	III	I	II	III
Interval...		30 sec.	11—13 min.		30 sec.	11—13 min.
Expt. no. 1	5.3	5.4	7.8	10.0	10.1	11.7
2	5.0	5.0	8.2	7.1	7.2	11.2
3	5.1	5.0	7.9	10.4	9.8	14.6
4	4.4	4.3	6.7	2.1	2.2	
Interval...		11—13 min.	30 sec.		11—13 min.	30 sec.
Expt. no. 5	4.6	4.6	7.5	8.9	9.6	15.7
6	5.1	5.2	7.9	7.2	7.0	10.4
7	4.5	4.5	7.6	3.8	3.9	

Since it is known that a loss of a large quantity of blood results in
changes in lactate and glucose concentrations, in preliminary experi-
ments three samples of blood were taken from non-stimulated animals
at intervals of 30 sec. and 11 - 13 min. or 11 - 13 min. and 30 sec. between
the first, second and third sample (Table 1). The obtained results
indicated that there were no changes in glucose and lactate concentra-
tion between the first and second samples, while in the third one their
concentration was increased. Therefore in the proper experiments only
two samples of blood were taken from each animal.

RESULTS AND DISCUSSION

The changes in the concentration of glucose, lactate and pyruvate
in the blood of guinea pigs subjected to electrical stimulation of the
brain are shown in Table 2. The values for the so-called excess lactate
(*XL*) were calculated according to Huckabee [4].

After electrical stimulation of the brain, both for 30 sec. and for 11 - 13 min., in all animals a slight increase in glucose and pyruvate concentration and a marked increase in lactic acid were observed. There was an excess of lactate as well.

Also in other types of shock hyperglycaemia has been observed [9, 1, 12] and is thought to be due to the effect of shock on liver metabolism. In the presented experiments, the slight increase in glucose concentration was accompanied by a marked increase of lactate while the concentration of pyruvate remained almost unchanged. The ratio of lactate to pyruvate concentration expressed as excess lactate was increased. This accumulation of lactate seems to be due to hypoxia. Also the results of Łyszczarz [6] who under similar experimental conditions observed a decrease in oxygen content in the arterial blood, seem to indicate that electrical stimulation of the brain causes hypoxia.

Table 2

The concentration of glucose, lactate and pyruvate, and excess lactate in the blood of guinea pigs subjected to electrical stimulation of the brain

Excess lactate was calculated according to Huckabee [4] from the equation:

$$XL = (L_n - L_0) - (P_n - P_0) \times (L_0/P_0)$$

where L_0 and P_0 are the concentrations in the blood of, respectively, lactate and pyruvate before electrical stimulation of the brain, while L_n and P_n, their concentrations after stimulation.

Expt. no.	Time of stimulation	Glucose (mM)		Lactate (mM)		Pyruvate (mM)		Excess lactate
		Control	Stimulated	Control	Stimulated	Control	Stimulated	
1	30 sec.	5.8	6.3	12.9	21.0	0.3	0.4	3.8
2		4.6	6.3	11.8	21.6	0.4	0.5	6.8
3		5.2	6.7	7.3	16.8	0.4	0.5	6.1
4		5.0	5.8	15.4	22.0			
5	11—13	5.4	7.4	13.0	18.4	0.4	0.4	5.4
6	min.	6.0	9.4	7.7	19.3	0.2	0.4	3.9
7		5.3	5.7	8.1	18.2			
8		5.4	6.1	8.2	20.2	0.4	0.5	10.0
9				4.2	16.5			
10		5.2	5.8	10.2	16.7			
11				4.7	17.8			
12		4.7	6.2	11.1	18.7	0.3	0.4	3.9

Similar results concerning the changes in concentration of lactate and pyruvate in animal blood were obtained in various types of shock [1, 11, 2, 10]. The authors believe that in shock there is a predominance

of anaerobic metabolism of carbohydrates resulting from insufficient oxygen supply. Engel *et al.* [1] supposed that the chemical changes in the blood in shock result from disturbances in liver function caused by early hypoxia of the liver itself and by subsequent hypoxia of peripheral tissues.

The similarity in chemical changes in the blood observed both in various types of shock and in the present experiments suggests that electrical stimulation of the brain acts as a shock. Therefore it seems permissible to assume that its protective effect in histamine shock may have a mechanism somewhat similar to the effect described by Schorr *et al.* [13] who demonstrated lower mortality in haemorrhagic shock in those rats which had been previously subjected to a traumatic shock of another kind. The results obtained so far seem to indicate that hypoxia of the organism may play a role in the mechanism of the protective effect of electrical stimulation in histamine shock.

SUMMARY

The changes in glucose, lactate and pyruvate concentration in the blood of guinea pigs subjected to electrical stimulation of the brain were studied. Only a marked increase of lactate was observed, suggesting the occurrence of hypoxia during electrical stimulation of the brain.

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GLIKOZA, KWAS MLEKOWY I KWAS PIROGRONOWY WE KRWI ŚWINEK
MORSKICH PO DRAŻNIENIU MÓZGU PRĄDEM ELEKTRYCZNYM

Streszczenie

Przebadano zachowanie się poziomu glikozy, kwasu mlekowego i pirogronowego we krwi świnek morskich po drażnieniu mózgu prądem elektrycznym. Stwierdzono wyraźny przyrost stężenia kwasu mlekowego. Wyniki wskazują na możliwość występowania niedotlenienia ustroju po drażnieniu mózgu prądem elektrycznym.

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QUANTITATIVE DETERMINATION OF DEOXYRIBONUCLEIC ACID IN BRAIN TISSUE

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The reaction of deoxyribose with diphenylamine according to Dische [6] is commonly used for quantitative determination of DNA in tissues. The reaction is carried out either in the whole extract of nucleic acids [1] or after separation of RNA from DNA [17]. Deoxyribose can be also determined using the indole reagent [5, 18]. DNA separated from RNA and other phosphorus compounds after Schmidt & Tannhauser [17] can be determined by total phosphorus content as well as spectrophoto-metrically at 260 mµ according to Logan, Mannell & Rossiter [10] or according to Tsanev & Markov [20] by measuring ΔE at 268 and 284 mµ.

The reliability of Dische's reaction for determination of DNA has lately been questioned [9], and Løvtrup & Roos observed the destruction of deoxyribose during acid extraction [11, 12].

In brain tissue, the determination of DNA is impaired by the presence of a large amount of non-nucleotide phosphorus compounds not easily separable from DNA. Moreover, as it has been shown by Tsanev & Markov [20] and Fleck & Munro [7], during extraction of DNA from cerebral tissue some non-nucleotide compounds are released which interfere with UV absorption measurements.

In the present work, the effect of varying conditions of DNA extraction from the brain was studied using different methods of determination.

EXPERIMENTAL

Methods

Adult albino Wistar rats were decapitated, the head being immediately immersed in acetone chilled to —60°. Then the cerebral hemispheres and cerebellum were taken out and treated as previously described [15, 2]. The tissue homogenate was extracted with 0.6 N-perchloric acid (PCA) at 0° to remove the acid-soluble compounds, and the residue was

delipidated by hot ethanol treatment. From the obtained dry tissue powder RNA was removed according to Schmidt & Tannhauser [17] by alkaline hydrolysis in 0.2 N-NaOH at 37° for 16 hr. Then the hydrolysate cooled to 0° was acidified with 60% PCA and centrifuged. The sediment containing DNA and protein was extracted with 1.5 N-PCA at various temperatures and for various time lengths, and after cooling the protein sediment was centrifuged off. In the supernatant the following determinations were made : (1), deoxyribose with diphenylamine after Dische as described by Burton [4], and in some cases also using the indole reagent [5, 18]; (2), total phosphorus according to Briggs [3] in the modification of Macheboeuf [14]; (3), estimation of extinction at 260 mµ according to the method of Logan *et al.* [10], in which $\varepsilon(P)$, the atomic extinction coefficient with respect to P, is used for calculations; and (4), ΔE at 268 and 284 mµ according to Tsanev & Markov [20]. The extinction was measured in a Hilger spectrophotometer in 1 cm. quartz cuvettes. All the results are presented in relation to DNA phosphorus.

Determination of DNA in standard preparation

For experiments, a commercial preparation of herring-sperm DNA (B.D.H., London) was used; it contained 6.7% P and had the $\varepsilon(P)_{260}$ value of 10 100 and E_{260}/E_{280} ratio of 1.315. A 5.0 mg. sample of herring-sperm DNA placed in a wide tube was added with 15 ml. of 1.5 N-PCA and
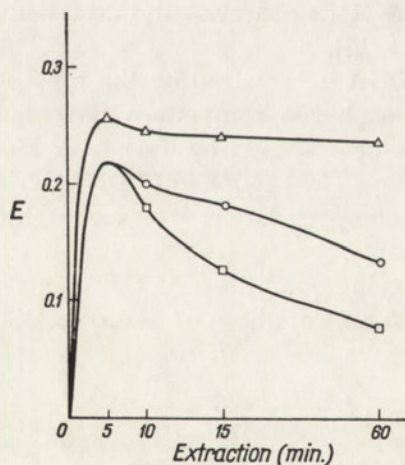
Fig. 1   Fig. 2

Fig. 1. The effect of temperature and time of extraction of standard DNA with PCA solution, on the diphenylamine reaction. Extraction: ($\triangle$), at 70°; ($\bigcirc$), at 80°; ($\square$), at 90°.
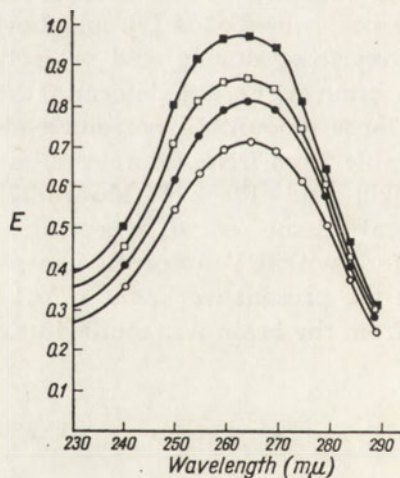
Fig. 2. The effect of temperature and time of extraction with PCA solution, on the UV-absorption spectra of standard DNA extracts. Extraction: ($\bigcirc$), 5 min. at 80°; ($\bullet$), 30 min. at 80°; ($\square$), 5 min. at 90°; ($\blacksquare$), 60 min. at 90°.

incubated with constant stirring in a water thermostat; samples for analysis were taken at determined time intervals. From the data presented in Fig. 1 and Table 1 it is evident that the colour reaction with diphenylamine is dependent on the temperature and time of extraction, in agreement with the results of Løvtrup & Roos [11, 12] on salmon-sperm DNA. The decrease of the colour reaction was most marked when the extraction had been carried out at 90°; after 1 hr. only 40% of DNA present in the sample could be determined. The temperature of 70° had a smaller effect as even after 1 hr. the colour reaction was reduced only by 10%. With concentrations of PCA lower than 1.5 N it was difficult to obtain a satisfactory colour reaction because of developing turbidity.

Table 1

Determination of DNA in the standard solution using different methods

Total P was determined after mineralization, deoxyribose according to Burton [4], E_{260} according to Logan *et al.* [10]. ΔE, the difference in extinction at 268 and 284 mμ, was calculated according to Tsanev & Markov [20]. Extraction with 1.5 N-perchloric acid.

Extraction time (min.)	DNA concentration (μg. P/ml.)			
	Total P	Deoxyribose	E_{260}	ΔE
Extraction at 70°				
5	22.4	22.3	22.4	24.3
15	22.4	21.5	22.5	25.1
30	22.7	21.3	23.3	26.6
60	24.5	20.0	25.6	28.5
Extraction at 80°				
5	22.4	20.1	20.5	23.8
15	22.4	17.6	22.4	25.1
30	23.1	15.5	22.4	26.1
60	23.8	12.4	23.7	27.7
Extraction at 90°				
5	22.4	19.4	21.8	24.4
15	22.8	15.0	22.0	24.6
30	24.1	11.2	22.0	26.9
60	25.9	6.0	26.8	29.0

In the determinations of total P, the slight increase observed with time of extraction seems to be due to the concentration of the sample by evaporation. Determinations of $E_{260 \text{ m}\mu}$ corresponded very closely with the actual content of DNA in the sample while the values obtained by the ΔE method were somewhat higher.

The effect of temperature and time of DNA extraction on the UV-
-absorption spectrum are presented in Fig. 2. It may be seen that the
extinction increased with time and temperature of extraction, but this
could be due as well to the appearance of the hyperchromic effect [8].
Too high values obtained by the ΔE method of Tsanev & Markov may
be accounted for by the fact that the increase in time and temperature
of extraction increased the extinction at 265 mμ quite distinctly but at
284 mμ only slightly.

Next, the effect of 1.5 N-PCA extraction was compared with that
of 10% trichloroacetic acid (TCA) used in Schneider's method [19].
It was found (Table 2) that extraction with TCA at 90° caused smaller
destruction of deoxyribose than that caused by PCA. The estimations
of deoxyribose with diphenylamine and with the indole reagent gave
the same values.

Table 2

*The effect of extraction with PCA or TCA on determination of DNA
by different methods*

The methods applied were as described in Table 1; deoxyribose was estimated
also with the indole reagent. Extraction with 1.5 N-perchloric acid or 10% trichloro-
acetic acid at 90°.

Extraction time (min.)	Extractant	DNA concentration (μg.P/ml.)				
		Total P	Deoxyribose		E_{260}	ΔE
			by diphenyl-amine	by indole reagent		
5	PCA	22.4	19.4	19.4	21.8	24.4
	TCA	22.4	21.0	21.3	21.8	24.4
15	PCA	22.8	15.3	15.2	22.0	24.6
	TCA	22.8	20.5	20.5	21.8	24.6
30	PCA	24.1	11.2	12.0	22.0	26.9
	TCA	24.1	18.6	18.1	22.0	26.5
60	PCA	25.9	6.0	6.8	26.8	29.0
	TCA	25.9	17.4	16.8	25.4	30.0

From the presented experiments it may be concluded that the results
of herring-sperm DNA determinations performed by different methods
were in best agreement when DNA had been hydrolysed in 1.5 N-PCA
for 5 min. at 70°.

For the evaluation of the extracted DNA, a calibration curve for DNA
estimated by the Dische reaction was made using the standard prepara-
tion hydrolysed for 5 min. at 70°.

Determination of DNA in cerebral tissue dry powder

Extractability of DNA varies considerably with the type of tissue. Løvtrup & Roos [11] have shown that complete extraction of DNA from brain or liver dry powder may be achieved within 40 or 50 min. The effect of time of extraction on determination of DNA from the cerebellum and cerebral hemispheres was followed using different conditions and methods (Table 3). On increasing the time of extraction, an increase in DNA determined by all the methods applied was observed, except for the diphenylamine method which gave the highest values after 15 min. extraction and lower ones if the extraction was continued for a longer period.

Table 3

Determination of DNA in brain tissue using different methods

The methods applied were as described in Table 1. Extraction with 1.5 N-perchloric acid at 70°. The concentration of DNA is expressed as µg. P per 100 mg. of dry delipidated tissue powder.

Extraction time (min.)	Total P	Deoxyribose	E_{260}	ΔE
Cerebellum				
5	138.8	128.0	141.2	120.0
15	153.4	141.0	149.0	130.4
30	160.0	128.0	166.0	143.0
60	199.0	102.4	204.0	152.8
Cerebral hemispheres				
5	12.3	16.8	26.5	17.7
15	27.0	28.0	30.2	20.2
30	35.0	21.0	36.0	21.5
60	38.5	20.0	51.3	26.5

The DNA extracted from cerebral powder was contaminated with non-nucleotide compounds which may account for the differences observed in particular determinations of the cerebellum and brain hemisphere extracts.

The values for DNA determined by the spectroscopic method increased with time and temperature of extraction. The results calculated according to Tsanev & Markov differ from those calculated according to Logan *et al.*; this seems to be due to the release, on prolonged extraction, of non-nucleotide UV-absorbing compounds. The UV spectra for
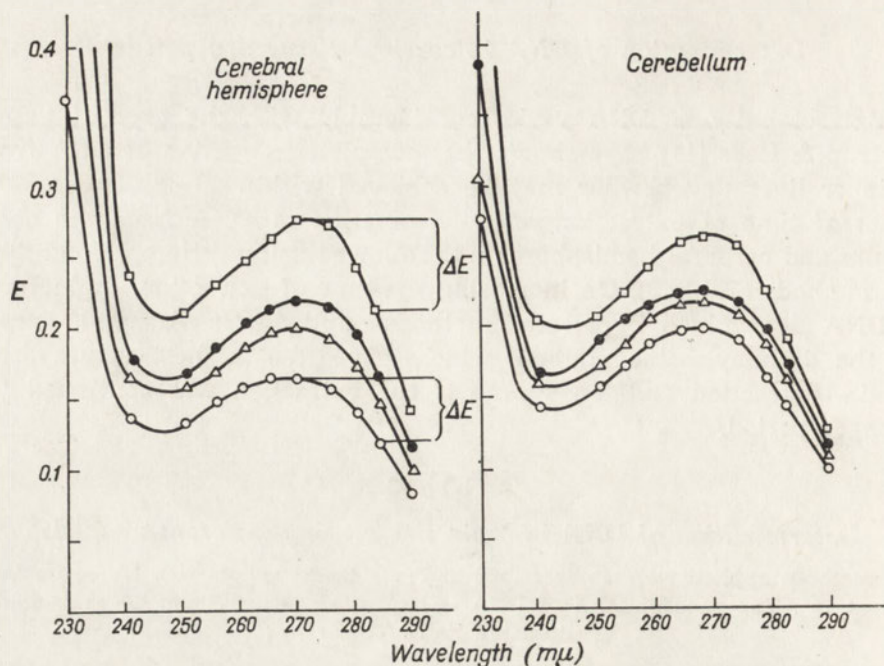
Fig. 3. The effect of time of extraction with PCA solution at 70° on the UV-
-absorption spectra of DNA from the cerebral hemispheres and cerebellum.
ΔE, difference in extinction at 268 and 284 mμ according to Tsanev & Markov [20].
Time of extraction: (O), 5 min.; ($\triangle$), 15 min.; (●), 30 min.; ($\square$), 60 min.

DNA extracts from the cerebral hemispheres and cerebellum (Fig. 3)
have a maximum at about 270 mμ irrespective of the time and tem-
perature of extraction. Therefore it may be concluded that the method
of Tsanev & Markov introducing the correction for the absorption due
to protein degradation products, permits to estimate with reasonable
accuracy the concentration of DNA in tissues.

DISCUSSION

Our studies on the determination of DNA in cerebral tissues were
performed on the delipidated rat brain powder, DNA being separated
from RNA according to Schmidt & Tannhauser [17]. Extraction with
hot PCA caused a decrease of the colour reaction of deoxyribose with
diphenylamine. The destruction of deoxyribose, first reported by Løvtrup
& Roos [11, 12], was confirmed by us using the indole reagent [5, 18]
in addition to the diphenylamine reaction. Therefore it appears that
Burton's procedure commonly used for determination of tissue DNA is
not a fully satisfactory method. It should be added that Løvtrup
& Roos [11, 12] have analysed the kinetics of deoxyribose destruction
by PCA and proposed a method of calculation permitting to evaluate

the initial amount of DNA. However, since the destruction of deoxyribose by PCA in the standard preparation of DNA is not the same as for DNA extracted from cerebral tissue, it does not seem possible to adopt a constant correction coefficient.

In the cerebral tissues the determination of DNA separated from RNA, based on the estimation of total P, presents special difficulties, since the concentration of non-nucleotide phosphorus increases with temperature and time of extraction. This is most marked in the cerebral hemispheres in which the concentration of DNA phosphorus is relatively low as compared with that of other phosphorus compounds, mainly phosphoproteins.

When the time of extraction was increased from 30 to 60 min., the results of DNA determinations obtained by the method of Logan et al. [10] increased markedly, while those obtained by the method of Burton [4] and after Tsanev & Markov [20] increased but slightly. The high values obtained by the method of Logan et al. seem to be due to protein degradation products interfering in extinction measurements. This, however, may be corrected for by using Tsanev's coefficient.

Although the method of Tsanev & Markov for RNA determination has been criticized by Fleck & Munro [7], it seems to be fully satisfactory with respect to DNA determination. The results of Łucenko & Promysław [13] also support this view. From the comparison of results obtained by several methods it appears that the extraction with 1.5 N-perchloric acid for 30 min. at 70° causes the least destruction of components of DNA and seems to be sufficient for complete hydrolysis of DNA from tissue samples.

SUMMARY

The effect of time and temperature of extraction on the determination of DNA from brain tissue was studied using several methods. It was demonstrated that extraction with hot PCA causes a marked destruction of deoxyribose. Reliable results can be obtained by measuring the extinction at two wavelengths according to Tsanev & Markov.

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ILOŚCIOWE OZNACZANIE KWASU DEZOKSYRYBONUKLEINOWEGO W TKANCE MÓZGOWEJ

Streszczenie

Przeprowadzono badania nad wpływem czasu i temperatury ekstrakcji na ilościowe oznaczanie DNA w tkance mózgowej. Wykazano, że ekstrakcja kwasem nadchlorowym na gorąco powoduje znaczną destrukcję dezoksyrybozy.

Porównanie wyników otrzymanych kilkoma metodami wykazało, że najbardziej wiarygodne wyniki dla tkanki mózgowej otrzymuje się przy zastosowaniu pomiaru absorpcji w dwóch długościach fal zgodnie z metodą Tsaneva i Markova.

J. UMIASTOWSKI

AMP AMINOHYDROLASE IN THE MUSCLES OF SOME VERTEBRATES AND INVERTEBRATES

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AMP aminohydrolase (EC 3.5.4.6) is known since 1928 [16], and has been obtained in crystalline form in 1957 by Lee [6, 7, 8]. In skeletal muscle it occurs as a complex with myosin [6, 2]. Deamination of adenylic acid seems to be connected in some way with the muscular work [11, 12], attempts to demonstrate deamination during a single muscle twitch gave, however, rather controversial results [18, 1]. The enzyme is present also in some other tissues [19, 13] and there are reasons to suppose that it takes part in the urine ammonia production [9, 10]. In the present work the occurrence of AMP aminohydrolase in the muscles of different animals has been investigated.

MATERIALS AND METHODS

Chemicals. AMP, ATP, and histidine were L. Light & Co. Ltd., Colnbrook (England) products. The remaining chemicals were purchased from Fabryka Odczynników Chemicznych, Gliwice (Poland).

AMP aminohydrolase assays. The animals were killed by decapitation; the muscle was taken as soon as possible and a 5% homogenate in 3 mM-EDTA was prepared, a Potter-Elvehjem homogenizer chilled with ice and water being used. Human muscle was taken in the course of a surgical operation. The patients were in ether anaesthesia; in all cases but one they had received "Flexedil" which caused muscle flaccidity.

Spectrophotometric estimations were made according to Kalckar [5]. The incubation mixture contained 0.2 ml. of muscle homogenate and 3 ml. of 0.15 mM-AMP in 50 mM-sodium succinate buffer, pH 6.0. The reaction was started by addition of the protein; after 5 min. at 25° the incubation was stopped by the addition of 1 ml. of 15% perchloric acid. Then the sample was made up to 10 ml. with water and centrifuged. Extinction at 265 mμ was estimated in the supernatant fluid both in the proper sample and in the blank sample deproteinized prior to incubation.

The decrease in extinction of the proper sample was taken as the measure of deamination.

When no AMP aminohydrolase activity was demonstrated by the Kalckar procedure, further estimations were made using higher substrate concentration and the chromatographic technique. The incubation mixture contained: 0.2 ml. muscle homogenate, 1 ml. of 50 mM-sodium succinate buffer, pH 6.0, and 0.2 ml. of 50 mM-AMP (final concentration of AMP 7.1 mM). Incubation was carried out at 25° for 60 min. The reaction was started by adding protein and stopped by inserting the test tube into boiling water bath for 5 min. The protein precipitate formed was removed by filtration and the clear solution was shaken with chloroform for 25 min. to remove proteins remaining still in the solution [17]. After centrifuging, the protein-free aqueous layer was subjected to descending chromatography on Whatman no. 1 paper. Isopropanol - saturated ammonium sulphate - water (2:79:19, by vol.), was used as the solvent. The chromatograms were photographed in ultraviolet light and the UV-absorbing spots were eluted with 0.1 N-HCl for 24 hr. Nucleotide concentrations were estimated spectrophotometrically (Unicam SP 500) taking the molar extinction according to Deutsch & Nilsson [3] (molar extinction for inosine derivatives 13 200 at 250 mµ, and for adenosine derivatives 14 200 at 260 mµ).

ATPase activity assay. This was made on clam *Unio pictorum* only. The shells were opened and the adductor muscles cut off as close to one shell as possible. The adductor hanging at the other shell was cut parallel to the fibres into slices weighing about 100 mg. (Fig. 1). Each slice was homogenized in 0.05 M-KCl to obtain a 5% homogenate. ATPase activity was assayed by the method of Bailey as described by Rüegg [14], 0.1 ml. of the homogenate being taken for each sample. Phosphate was estimated according to Gomori [4] and nitrogen by the Kjeldahl procedure.

RESULTS AND DISCUSSION

Table 1 presents the results of spectrophotometric estimations of AMP aminohydrolase activity in the muscles of 6 invertebrate and 12 vertebrate species. Under the conditions used, i.e. at low substrate concentration and a short time of incubation, no activity was observed in any of the invertebrates investigated except the snail, nor in the muscles of tortoise, lizard, and in three out of four human muscles.

The muscles in which the attempt to demonstrate AMP aminohydrolase activity by the Kalckar procedure [5] was unsuccessful, were subjected to longer incubation with a higher AMP concentration and the products were separated chromatographically. The results of these experiments are presented in Table 2. As may be seen the clam *Unio pictorum* is the only animal in which no AMP aminohydrolase could be detected.

Table 1

AMP aminohydrolase activity followed spectrophotometrically in the muscles of different animals

Substrate concentration 0.15 m$_M$. Incubation 5 min. at 25°.

Species	No. of expts.	AMP decomposition	
		μmoles/1 g. of tissue/5 min.	μmoles/1 mg. of nitrogen/5 min.
Annelida			
Earthworm (*Lumbricus terrestris*)	2	not detected	
Mollusca			
Clam (*Unio pictorum*)	4	not detected	
Snail (*Helix pomatia*)	3 a	36.0	1.8
	b	24.2	1.3
	c	38.5	1.7
Crustacea			
Crayfish (*Carcinus affinis*)	2	not detected	
Crab (*Rhithropanopeus harrisi*)	2	not detected	
Cyclostomata			
Lamprey (*Lamperta fluviatilis*)	4	not detected	
Pisces			
Carp (*Cyprinus carpio*)	4 a	15.7	0.6
	b	5.7	0.3
	c	20.8	0.7
	d	16.7	0.6
Eel (*Anguilla anguilla*)	2 a	8.6	0.4
	b	8.6	0.4
Trout (*Salmo irideus* Gibbons)	2 a	9.2	0.4
	b	5.0	0.2
Vimba (*Vimba vimba*)	1	17.0	1.1
Amphibia			
Frog (*Rana esculenta*)	2 a	19.2	1.7
	b	14.2	1.4
Reptilia			
Tortoise (*Testudo graeca*)	2	not detected	
Lizard (*Lacerta agilis*)	1	not detected	
Viper (*Vipera berus berus*)	1	7.1	0.2
Aves			
Pigeon (*Columba livia*)	3 a	27.8	1.3
	b	5.9	0.3
	c	7.1	0.3
Mammalia			
Rat (*Rattus norvegicus*)	4 a	19.3	1.0
	b	23.3	1.1
	c	35.5	1.0
	d	29.0	0.9
Guinea pig (*Cavia porcellus*)	3 a	16.2	0.6
	b	28.7	0.9
	c	13.0	0.6
Man	4 a, b, c*	not detected	
	d	8.7	0.5

* One of the human subjects showing no AMP aminohydrolase activity received no "Flexedil" which was given *intra operationem* to the other three subjects.

Table 2

AMP aminohydrolase activity followed chromatographically in the muscles of animals in which no activity was detected by the Kalckar spectrophotometric method

Substrate concentration 7.1 m$_M$. Incubation 1 hr. at 25°.

Species	No. of expts.	AMP decomposed (μmoles/1 g. of tissue/1 hr.)
Earthworm (*Lumbricus terrestris*)	2	53; 119
Clam (*Unio pictorum*), adductor muscle	2	0; 0
Crayfish (*Carcinus affinis*)	2	99; 172
Crab (*Rhithropanopeus harrisi*)	2	106; 106
Lamprey (*Lamperta fluviatilis*)	2	292; 397
Tortoise (*Testudo graeca*)	1	240
Lizard (*Lacerta agilis*)	1	1000
Man	3	505; 384; 635

It is worth to mention that also Saito *et al.* [15] did not find any AMP aminohydrolase activity in the mollusc squid.

Rüegg [14] has shown that the adductor muscle of the marine clam *Pecten maximus* consisted of a smooth and striated muscle; the most peripheral part of the smooth muscle had a very low ATPase activity and its central part a higher activity. A similar distribution of ATPase activity was found in the adductor muscle of *Unio pictorum* (Fig. 1).
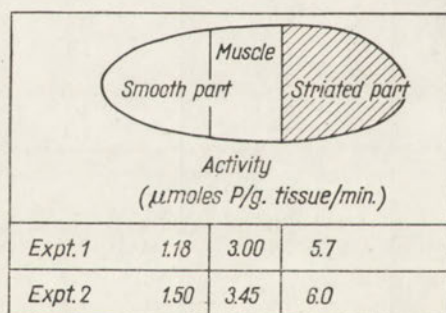
Fig. 1
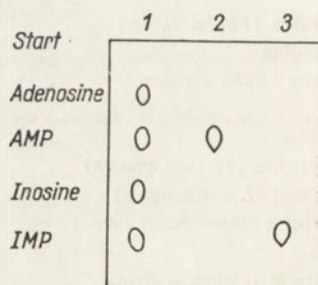
Fig. 2

Fig. 1. ATPase activity in the adductor muscle of the clam *Unio pictorum*.

Fig. 2. Chromatographic identification of 5′-adenylic acid extractable with 0.05 $_M$-succinate buffer, pH 6.0, from crayfish muscle. (1), Standards; (2), crayfish muscle extract; (3), crayfish muscle extract incubated with rat muscle homogenate.

On incubating the muscle of crayfish at low AMP concentration it was found that the extinction at 265 mμ did not decrease but increased. This observation suggested that some endogenous nucleotide may be eluted from the muscle during incubation. To prove this supposition

0.2 ml. of crayfish muscle homogenate was incubated with 1 ml. of succinate buffer at 25° for 1 hr. An UV-absorbing spot of R_F corresponding to AMP appeared on the chromatogram of the incubation mixture. This spot had an absorption maximum at 258 mμ which corresponds to the maximum of AMP. In order to identify this compound, crayfish muscle extract was incubated with rat muscle homogenate serving as a source of AMP aminohydrolase. After incubation the mixture was subjected to paper chromatography and a spot of IMP was found instead of AMP (Fig. 2). These facts indicate that the UV-absorbing compound eluted during incubation from the crayfish muscle homogenate is 5′-adenylic acid. The concentration of AMP in the extracts of three crayfish muscles determined spectrophotometrically [3] amounted to 3.7; 3.8; and 4.3 μmoles per gram of fresh tissue.

From the obtained results it appears that AMP aminohydrolase is abundant in the muscles of very different animal species, although its activity shows large differences.

SUMMARY

1. AMP aminohydrolase activity in muscles of 12 vertebrate and 6 invertebrate species has been investigated; it was present, although with different intensity, in all the animals tested except the freshwater clam *Unio pictorum*.

2. In the adductor muscle of *Unio pictorum* a considerable ATPase activity was observed.

3. In the muscle of the crayfish *Carcinus affinis* the presence of 5′-adenylic acid extractable with 0.05 M-succinate buffer, pH 6.0, has been shown; its concentration was about 4 μmoles per gram of tissue.

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AMP AMINOHYDROLAZA W MIĘŚNIACH NIEKTÓRYCH KRĘGOWCÓW I BEZKRĘGOWCÓW

Streszczenie

1. Oznaczono AMP aminohydrolazę w mięśniach 12 gatunków kręgowców i 6 gatunków bezkręgowców; aktywność enzymu u badanych zwierząt wykazywała duże różnice. Nie stwierdzono jej u szczeżuji.

2. W mięśniach zwieraczach szczeżuji wykazano aktywność ATPazy.

3. W mięśniu raka wykazano obecność kwasu 5′-adenilowego, dającego się ekstrahować 0.05 M-buforem bursztynianowym o pH 6.0, w ilości około 4 μmoli na gram tkanki.

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MOVEMENT OF IODIDE, BROMIDE AND CHLORIDE ACROSS THE GASTRIC WALL IN THE RAT

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In the course of our previous experiments on the accumulation of iodide in the stomach [9] it appeared of interest to study under similar conditions the behaviour of other halogens, namely bromide and chloride. The present paper describes the experiments concerning the distribution of these halogens in rat tissues and their accumulation in, and absorption from, the rat stomach surgically separated *in vivo* from other parts of the digestive tract.

MATERIALS AND METHODS

Adult Wistar albino rats, weighing 250 - 280 g. each, were used; for 24 hr. preceding the experiments the animals received no food but only water. Sodium salts of ^{131}I (Institute of Nuclear Research, Poland) and ^{82}Br and ^{36}Cl (The Radiochemical Centre, Amersham, England) were used throughout. The preparation of $Na^{131}I$ was carrier free, the specific activity of $Na^{82}Br$ exceeded 30 mc/g. Br, and that of $Na^{36}Cl$ was 400 µc/g. Cl. The individual doses applied to the animals in 1.0 ml. of physiological saline solution were 18 µc of ^{131}I and ^{82}Br, and 5 µc of ^{36}Cl; they did not exceed 1 mµg. of iodide and 1 mg. of bromide or chloride.

The distribution of halogens in rat organs and tissues was examined after subcutaneous injection of the respective isotope dissolved in 1.00 ml. of 0.9% NaCl.

In experiments concerning the accumulation of halogens in the stomach, it was surgically separated from other parts of the digestive tract in the following way. Under ether anaesthesia, ligatures were put on the oesophagus near the cardia and on the duodenum just beyond the pyloric end of the stomach; then the oesophagus and duodenum were cut off and the abdominal wall was sutured, the stomach being left inside attached to its intact blood vessels. The control animals were submitted to a sham operation (opening of the abdominal wall without

separation of the stomach). Soon after the operation the animals awoke, and to study the penetration of halogen ions from the blood into the stomach, 1 ml. of the respective solution was injected subcutaneously.

To examine the absorption of halogen ions from the stomach, 1.00 ml. of the radioisotope solution was introduced, through a very thin needle, into the isolated stomach during the operation, before suturing of the abdominal wall.

In all three series of experiments, 60 - 90 min. after the injection the animals were anaesthetized with ether and samples of the blood were taken by heart puncture; samples of other tissues to be tested were also taken and weighed with the accuracy of $\pm$ 0.01 g. The radioactivity of the tissue samples containing ^{131}I or ^{82}Br was measured directly with the scintillation counter. The samples containing ^{36}Cl were first added with 5 ml. of 20% NaOH solution in 50% methanol, heated, added with water to 25 ml. and measured with a Geiger liquid counter.

A part of each isotope solution was diluted and used as standard, appropriate amounts being examined under the same geometric conditions as the tissue samples. The results obtained are expressed as percentages of the administered dosis.

RESULTS AND DISCUSSION

The distribution of radioactivity in rat tissues after subcutaneous injection of isotopes of iodide, bromide and chloride, is presented in Table 1. It is noteworthy that there is a marked accumulation of iodide in the contents of the stomach, while the accumulation of bromide and chloride is several times lower. At 60 and 90 min. after the injection the ratio of isotope concentration inside the stomach to that in blood plasma was for iodide, respectively, 78 and 59. The ratio for bromide was about 2 and for chloride about 1. Similar results were obtained in experiments in which the stomach had been surgically separated *in vivo* (Table 2).

The differences in the behaviour of iodide and that of bromide and chloride were further confirmed by the results of experiments on their disappearance from the isolated stomach (Table 3). After 80 min. practically all the iodide administered into the stomach was present there while nearly a half of bromide and chloride has penetrated across the stomach wall and was transported to other tissues.

The presented results indicate that iodide, bromide and chloride ions accumulate in the stomach at higher concentrations than in blood plasma. However, large quantitative differences observed indicate a special mechanism of iodide secretion into the stomach. According to some authors [10, 7] this secretion consists in active transport proceeding against the concentration gradient. It seems that it might be connected

Table 1

Radioactivity in rat tissues after subcutaneous injection of sodium salts of ^{131}I, ^{82}Br or ^{36}Cl

The results are expressed, except for the thyroid, as percentages of the applied dosis per 1 g. of tissue. The small intestine was divided into 12 - 15 segments of equal length (about 5 cm.); the radioactivity of the segments were measured with their contents.

| Tissue | Expt. no. | $^{131}I^-$ (18 μc) | | $^{82}Br^-$ (18 μc) | | $^{36}Cl^-$ (5 μc) | |
| | | Time (min.) | | | | | |
		60	90	60	90	90	90
Whole blood	1	0.47	0.36	0.74	0.82	0.90	0.95
	2	0.52	0.35	0.79	0.78	0.95	0.89
Plasma	1	0.57	0.38	0.84	0.89	1.00	1.20
	2	0.53	0.42	0.83	0.89	1.16	1.19
Stomach wall	1	3.00	4.98	0.60	0.60	0.67	0.50
	2	2.99	4.56	0.66	0.58	0.59	0.70
Stomach contents	1	45.0	20.0	1.50	2.03	1.38	1.31
	2	40.9	27.3	2.00	2.10	1.07	1.50
Small intestine							
Ist segment	1	0.50	0.69	0.30	0.34	0.53	0.43
	2	0.49	0.70	0.29	0.30	0.53	0.51
middle segment	1	0.40	0.59	0.37	0.30	0.40	0.39
	2	0.50	0.63	0.29	0.36	0.42	0.36
Parotid gland	1	0.39	0.38	0.32	0.34	0.40	0.41
	2	0.34	0.38	0.31	0.34	0.39	0.43
Brain	1	—	—	0.09	0.12	—	—
	2	—	—	0.08	0.10	—	—
Kidney	1	0.40	0.38	0.44	0.54	0.37	0.41
	2	0.56	0.41	0.42	0.47	0.41	0.42
Skin	1	1.18	1.37	0.42	0.44	0.47	0.57
	2	1.06	1.27	0.39	0.45	—	0.36
Area of injection	1	0.40	0.41	0.36	0.40	0.60	0.42
	2	0.39	0.40	0.38	0.38	0.62	0.56
Urine	1	25.7	4.0	1.02	0.90	—	0.90
	2	17.6	4.8	0.59	1.25	—	0.44
Saliva	1	—	0.6	0.78	0.71	—	—
	2	0.6	0.6	0.78	0.75	—	—
Thyroid (per whole organ)	1	1.25	2.80	0.12	0.17	0.07	0.06
	2	2.15	2.60	0.17	0.18	0.06	0.08
Mean ratio: concn. in stomach contents to concn. in plasma		78	59	2.1	2.3	1.14	1.17

Table 2

Radioactivity in tissues 60 min. after subcutaneous injection of $^{131}I^-$,
$^{82}Br^-$ or $^{36}Cl^-$ to rats with the surgically separated stomach

The results are expressed as percentages of the applied dosis per 1 g. of tissue.

| Tissue | Expt. no. | $^{131}I^-$ (18 μc) | | $^{82}Br^-$ (18 μc) | | $^{36}Cl^-$ (5 μc) | |
| | | Animals | | | | | |
		with separated stomach	control	with separated stomach	control	with separated stomach	control
Plasma	1	0.17	0.14	1.11	1.04	1.11	1.19
	2	0.22	0.25	1.03	1.03	1.11	1.11
Stomach wall	1	1.22	0.92	0.86	0.66	0.73	0.59
	2	1.18	1.08	0.88	0.69	0.83	0.63
Stomach contents	1	8.80	5.39	2.79	1.92	1.30	1.20
	2	10.2	4.00	2.20	1.88	1.18	1.19
Small intestine Ist segment	1	0.10	0.69	0.43	0.43	0.47	0.51
	2	0.09	0.70	0.49	0.42	0.41	0.58
middle segment	1	0.32	0.47	0.37	0.41	0.31	0.33
	2	0.36	0.40	0.44	0.39	0.29	0.39
Mean ratio: concn. in stomach contents to concn. in plasma		47	23	2.3	1.8	1.12	1.04

Table 3

Absorption of iodide, chloride and bromide from rat stomach surgically
separated in vivo

The results are expressed as percentages of the applied dosis found in the stomach
contents and in whole blood 80 min. after the injection of the isotope into the
stomach.

| Tissue | $^{131}I^-$ (18 μc) | | $^{82}Br^-$ (18 μc) | | $^{36}Cl^-$ (5 μc) | |
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Stomach contents	99.4	99.2	49.5	67.5	61.2	60.9
Whole blood	0.22	0.30	8.9	6.8	9.9	8.7

with the fact that during embryonal development both the thyroid gland
and the glandular epithelium of the stomach arise from a common
entodermal layer. The process of iodide secretion into the stomach
seems to be in many respects similar to the concentration of iodide in
the thyroid gland. A common mechanism of iodide accumulation by the

thyroid and by stomach mucosa was also suggested by Kutzim *et al.* [8] who established that perchlorate inhibiting the accumulation of iodide by the thyroid gland has a similar effect on the gastric concentration of iodide.

The results presented in Table 3 indicate the lack of absorption of iodide from the stomach into the blood. This was also observed by other authors [1, 3, 5, 6] but has not yet been elucidated.

As the half-life of the fluorine radioisotope is very short we were unable to include it in the present experiments. Reports of other authors [2, 4, 11] indicate that in mammals fluoride is rapidly absorbed from the stomach, which suggests a similarity with the behaviour of chloride and bromide.

SUMMARY

The concentration of ^{131}I, ^{82}Br and ^{36}Cl by the stomach was studied in the rat after subcutaneous injection of the respective sodium salts. It was shown that ^{131}I concentration in gastric contents after 90 min. was approximately 60 times higher than that in blood plasma, the respective value for ^{82}Br being 2, and for ^{36}Cl 1.

Iodide introduced into the stomach surgically separated *in vivo* from the digestive tract was not absorbed into the blood. Bromide and chloride were cleared from the gastric contents up to 40% under the same experimental conditions.

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WĘDRÓWKA JODKÓW, BROMKÓW I CHLORKÓW PRZEZ ŚCIANĘ ŻOŁĄDKA U SZCZURÓW

Streszczenie

Określono stopień zagęszczenia w żołądku szczura izotopów ^{131}J, ^{82}Br i ^{36}Cl podanych podskórnie w postaci soli sodowych. Stwierdzono, że po 90 min. stężenie ^{131}J w zawartości żołądka szczura jest około 60 razy większe niż w osoczu. Stężenie ^{82}Br jest 2 razy większe, a stężenie ^{36}Cl takie samo.

Stwierdzono, że jodki wprowadzone do żołądka szczura chirurgicznie izolowanego od przewodu pokarmowego (*in vivo*) prawie zupełnie nie przenikają do krwiobiegu. Bromki i chlorki w podobnych warunkach opuszczają żołądek w ilości około 40%.

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STUDIES ON SOME ASPECTS OF DEPOLYMERIZATION OF F-ACTIN

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Recent studies of Hayashi & Rosenbluth [11], as well as those of Grubhofer & Weber [9], have shown that homogenization of F-actin pellets leads to formation of monomeric G-ADP-actin. The existence of such a compound was also proved by experiments of Szent-Györgyi [19] in which F-actin was depolymerized at a high concentration of potassium iodide.

In the present study another system for depolymerization of F-actin was used. G-ATP-actin, after removal of free ATP, was polymerized with $MgCl_2$ or $CaCl_2$ and, after polymerization, bivalent cations were removed using Dowex 50. It was expected that in these conditions F-actin would depolymerize to monomeric G-ADP-actin. The obtained product, however, seemed to correspond, especially at higher concentrations, rather to an oligomeric than to a monomeric form of actin. Some properties of this product are the subject of the present investigation.

A preliminary report of some of the results appeared earlier [6].

MATERIAL AND METHODS

Actin, free of tropomyosin, was extracted from acetone-dried muscle powder at 0° [5] and purified according to Mommaerts [16] by centrifugation for 3 hr. at 105 000 g in Spinco ultracentrifuge model L. The F-actin pellets obtained after centrifugation were carefully rinsed with 0.2 mM-ATP in 2 mM-tris-HCl buffer, pH 8.0, and homogenized by a Teflon homogenizer in a solution of the same composition. The actin solutions obtained at the concentration of about 4 - 5 mg. per ml. were finally dialysed for 40 hr. against the same solution of ATP and tris buffer. After dialysis G-actin was clarified by centrifugation for 20 min. at 26 000 g; its reduced viscosity was usually 0.2 to 0.4.

Free nucleotides were removed by treatment of the actin solution for 3 min. at room temperature with Dowex 1, chloride form (200 - 400 mesh). Dowex 1 was previously washed successively with 1 N-NaOH, with 1 N-HCl, with several portions of distilled water, and

afterwards with 2 mM-tris - HCl, pH 8.0; it was finally suspended in the same tris buffer in proportion 1:1 (v/v). The resin suspension was added in the amount corresponding to 0.1 mEq. of Dowex per ml. of actin. Free bivalent cations were removed in a similar way by Dowex 50 (sodium form) which was also previously washed successively with 1 N-HCl, 1 N-NaOH and distilled water and afterwards equilibrated with 2 mM-tris - HCl, pH 8.0.

Viscosity was measured in Ostwald viscometers with an outflow time of approximately 80 sec. at 21°.

Protein concentration was determined with the biuret reagent [8]. Calcium chloride (^{45}Ca) was purchased from The Radiochemical Centre, Amersham, England. ATP and ADP (sodium salts) were products of Pabst Laboratories, USA. Radioactivity was measured using a Chicago Nuclear Corporation gas-flow counter, with a "Micromil" window, obtained as a gift from the Rockefeller Foundation.

RESULTS

When F-actin obtained from G-actin by polymerization with 1 mM-MgCl$_2$ or CaCl$_2$ is treated with Dowex 50 (sodium form) the resin is expected to exchange all free bivalent cations present in the solution with its bound sodium ions. NaCl which appears in the solution at 2 mM-concentration in place of bivalent cations is by itself not able to cause polymerization of G-actin; therefore the depolymerization of F-actin should take place in these conditions. If actin used for polymerization had been

Table 1

The influence of Dowex 50 treatment on F-actin polymerized with bivalent cations

G-actin was treated with Dowex 1 and after centrifugation of the resin it was polymerized with 1 mM-CaCl$_2$ and left to stay at 0°. After several hours ^{45}CaCl$_2$ was added to a final concentration of 0.1 mM in order to equilibrate with the free calcium pool. The obtained solution was treated three times with Dowex 50. After each treatment in one sample viscosity and in another one radioactivity were measured.

Actin solution	Reduced viscosity	Counts/min./ml.	Calculated concn. of free Ca^{2+} (10^{-5} M)	Concn. of protein (10^{-5} M)*
Initial	9.0	17 190	110.0	7.8
After first Dowex treatment	3.0	215	1.38	7.2
After second Dowex treatment	2.7	180	1.15	6.7
After third Dowex treatment	2.0	60	0.43	5.3

* The molecular weight of G-actin was accepted in this paper as 60000.

deprived of free ATP by previous treatment with Dowex 1, in order to avoid the exchange with bound ADP during depolymerization, the appearance of monomeric G-ADP-actin might be expected. The experiments showed, however, that this was not the case, since the reduced viscosity of actin obtained in such a way, although lower than that of F-actin, was much higher than that of G-actin. Table 1 (column 1) shows that the viscosity found after the first treatment with Dowex 50 does not change substantially after the second and third treatment.

The question could arise whether Dowex 50 really removed in these conditions all free bivalent cations; and some experiments were therefore performed in order to check this supposition. The results of one of the experiments are shown in the same Table. In this experiment a certain amount of radioactive $^{45}CaCl_2$ was added to actin several hours after polymerization with 1 mM-$CaCl_2$. In these conditions according to Bárány et al. [1] $^{45}Ca^{2+}$ added could equilibrate only with free calcium but not with that bound with F-actin. Hence, the radioactivity remaining after subsequent treatment with Dowex 50 could be a direct measure of the amount of free calcium present in the solution. As can be seen from Table 1 (columns 2 and 3), already a single treatment with the resin removes practically all free calcium. This observation is in good agreement with the results of our previous paper [7] in which G-actin, after removal of free bivalent cations, was polymerized in 1 mM-$^{45}CaCl_2$ and subsequently treated with Dowex 50. Our preliminary chemical determinations have also shown that the sum of bivalent cations remaining bound to actin, previously polymerized with 1 mM-$MgCl_2$, corresponds after treatment with Dowex 50 to about one mole per mole of actin.

Table 2

Ultracentrifugation of depolymerized actin

F-actin polymerized with 1 mM-$MgCl_2$ in the absence of free ATP was treated with Dowex 50. After removal of the resin 1 mM-ATP was added and one part of the solution was centrifuged for 3 hr. in Spinco ultracentrifuge at 0° [16] and the other, serving as control, was kept during the same period of time at 0°. After centrifugation, protein concentration was measured in the supernatant and in the control sample.

Protein concentration (mg./ml.)	
Initial solution	Supernatant
2.8	2.8
3.6	3.6
1.8	1.8
2.2	2.2

Two possibilities might account for the relatively high reduced viscosity of actin depolymerized with Dowex 50. The process of depolymerization could be stopped in those conditions at the stage of shorter polymers of actin; or a mixture of undepolymerized F-actin and monomeric G-actin could exist in the solution. In order to elucidate this question F-actin, after depolymerization with Dowex 50, was centrifuged for 3 hr. in a preparative ultracentrifuge at 105 000 g at 0°. The results presented in Table 2 show that practically no protein has sedimented during centrifugation. This fact suggests the absence of long polymers of the F-actin type in our depolymerized actin.

Actin resulting from the depolymerization of F-actin with Dowex 50 seems to be still active since it repolymerizes after addition of 0.1 M-KCl - 1 mM-MgCl$_2$; during incubation, however, its ability to polymerize
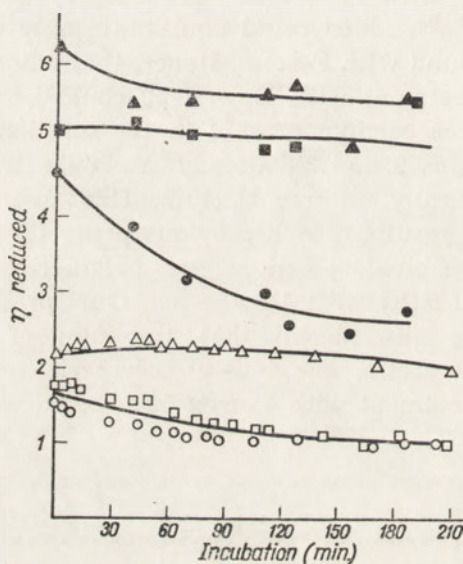
Fig. 1. Viscosity of F-actin depolymerized by the use of Dowex 50. Solution of G-actin, deprived of free ATP, polymerized with 1 mM-MgCl$_2$ and subsequently treated with Dowex 50, was divided into three parts. To the first and the second ATP and ADP, respectively, were added to a final concentration of 1 mM; the third portion served as control. The solutions were incubated at 21°. Three-ml. samples of each solution were placed in the viscometers at the same temperature and at time intervals indicated in the Figure viscosity was measured. (○), Control actin; (△), actin incubated in the presence of 1 mM-ATP; (□), actin incubated in the presence of 1 mM-ADP. From the rest of the incubation mixture at various time intervals 3-ml. samples were taken out and their viscosity was measured several hours after addition of KCl and MgCl$_2$ to a final concentration of 0.1 M and 1 mM, respectively. Viscosity after polymerization: (●), control actin; (▲), actin incubated in the presence of 1 mM-ATP; (■), actin incubated in the presence of 1 mM-ADP. Concentration of actin, 2.1 mg./ml. Zero time in the Figure corresponds to 30 min. after addition of Dowex 50.

diminishes (Fig. 1). At the same time the reduced viscosity of depolyme-rized actin also decreases. When, however, free ATP is added the viscosity of depolymerized actin does not drop. Simultaneously, actin retains its ability to polymerize, and no release of bound nucleotide can be observed. On the other hand, free ADP has no effect on the spontaneous decrease of viscosity of depolymerized actin, although it prevents actin from the loss of polymerizability to almost the same degree as ATP does. However, the question remains to be elucidated whether ADP acts by itself or this effect is due to ATP formed during several hours of incubation by the traces of myokinase, possibly present in actin preparations. Figure 2 indicates that both the decrease in visco-sity of depolymerized actin and the loss of polymerizability seem to
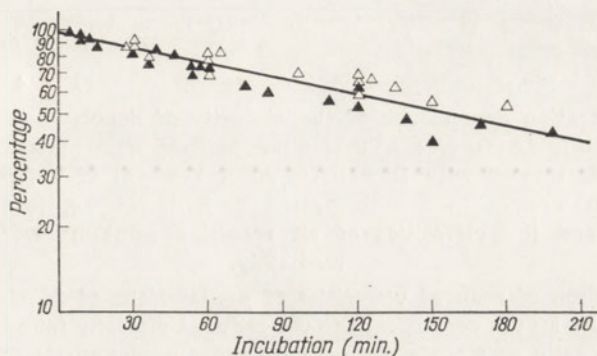

Fig. 2. Half-life time of actin depolymerized with Dowex 50. In several experiments the decrease of viscosity of actin depolymerized with Dowex 50 and its loss of polymerizability were measured during incubation at 21°. For experimental details see Fig. 1. The points in the Figure correspond to the values of the reduced viscosity expressed as the percentage of its initial value in a given experiment. (▲), The decrease of viscosity of actin depolymerized with Dowex 50; (△), the loss of polymerizability of actin depolymerized with Dowex 50.

obey the first order kinetics and to proceed at about the same rate. The calculated half-life time of this form of actin is about 160 min. No appreciable differences were observed in respect to the rates of depoly-merization and inactivation between actin which has been previously polymerized either by $CaCl_2$ or by $MgCl_2$.

Figure 3 indicates that the reduced viscosity of actin depolymerized with Dowex 50 shows a strong concentration dependence. With the decrease of concentration the viscosity drops to the level of that of monomeric actin; hence, the intrinsic viscosity of resin-treated F-actin seems to be the same as that of G-actin. The results presented in Table 3 indicate that the viscosity of actin depolymerized with Dowex 50 is also considerably decreased, almost to the level of the viscosity of G-actin,

3

in the presence of 1.67 -urea [12] or as a result of a short homogenization [9].

It must be pointed out, however, that the reduced viscosity of F-actin treated with Dowex 50 varied highly in the individual experiments.
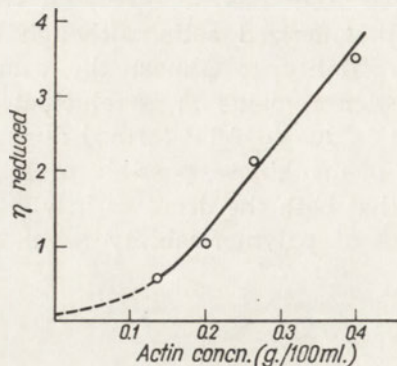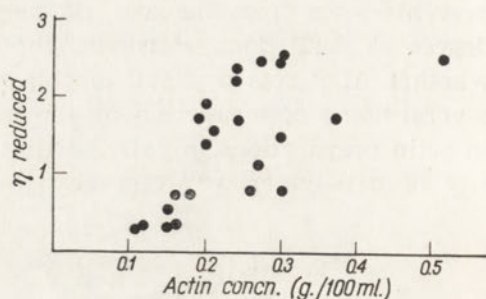

Fig. 3   Fig. 4

Fig. 3. Concentration dependence of the viscosity of depolymerized F-actin. Actin solution containing 0.2 m$_M$ free ATP was polymerized with 1 m$_M$-MgCl$_2$ and several hours later was treated with Dowex 50. After removal of the resin the samples of the solution were diluted with 2 m$_M$-tris - HCl, pH 8.0, to the desired final concentration and the viscosities of all resulting solutions were measured immediately.

Fig. 4. Comparison of reduced viscosities of preparations of depolymerized F-actin. Each point in the Figure corresponds to the reduced viscosity found in the individual experiment for actin previously polymerized either with 1 m$_M$-MgCl$_2$ or 1 m$_M$-CaCl$_2$. Measurements were taken 60 min. after the beginning of depolymerization by addition of Dowex 50.

Table 3

The influence of homogenization or treatment with urea on the viscosity of depolymerized actin

Viscosities of the samples of actin polymerized previously with 1 m$_M$-MgCl$_2$ were measured immediately after treatment with Dowex 50 both after homogenization by Teflon homogenizer or after addition of 1.67 m-urea as well as in the control sample.

Reduced viscosity			Protein concn. (mg./ml.)
Control	After homogenization	After addition of urea	
1.55	0.96	—	2.7
1.52	0.50	—	3.6
1.11	0.32	—	1.8
2.39	0.55	—	2.1
2.20	0.55	—	3.6
1.13	—	0.29	2.9
3.65	—	0.81	3.2
1.52	0.86	0.92	2.6

In order to elucidate whether the concentration of actin used for poly-
merization and depolymerization affects its subsequent viscosity the
reduced viscosity of several actin preparations has been plotted against
the concentration of actin. As it is seen in Fig. 4 at higher actin con-
centrations the values of reduced viscosity considerably differ from
experiment to experiment although they are always higher than those
of G-actin. Only at lower actin concentrations, up to about 1.5 mg.
per ml., the reduced viscosity of depolymerized F-actin is low being
similar to that of G-actin. This may suggest that full depolymerization
takes place in these conditions.

ATP is one of the agents which seem to affect the viscosity of
depolymerized actin. As it was already shown in Fig. 1, the addition
of ATP prevents the progressive decrease of viscosity of depolyme-
rized actin. Preliminary experiments have shown that this effect is
even more pronounced when free ATP had been added to actin before
treatment with Dowex 50. In this case the resulting viscosity both before
and after homogenization, or after treatment with urea, is much higher
than that found in the case when free ATP is added to actin after the
removal of resin. The subsequent content of bivalent cations remaining
in the solution after Dowex 50 treatment seems to be also somewhat
higher if ATP is added to actin before the addition of the resin. Even in
the latter case, however, the concentration of the remaining bivalent
cations is also lower than that which causes a partial polymerization
when added directly to G-actin; these small amounts of Ca^{2+} or Mg^{2+}
might enable the preservation of the shorter polymers.

DISCUSSION

Several authors have occasionally postulated the existence of dimers
[20, 17, 13] or other short polymers [9, 14, 3, 4, 12] of G-actin. Some of
these oligomers seem to be random aggregates [13, 15] formed probably
from inactive G-actin; some, however, are fully active, i.e. they contain
bound nucleotide and polymerize to F-actin in the presence of salts.
The latter oligomers have been recently found in actin preparations
obtained from F-actin with the use of homogenization method [11, 9]. Thus,
in our earlier study [4] it was observed that the reduced viscosity of actin
obtained by homogenization of F-actin pellets was higher than that of
G-actin, and that the reduced viscosity of these actin solutions showed
a strong concentration dependence. It was postulated on the basis of this
observation that in such actin solutions one might be dealing with oligo-
mers of G-actin. It appeared later [5] that the actin preparation used
in the above experiments contained some tropomyosin impurities which
affected the viscosity of actin in a similar manner. Therefore we have
recently repeated such experiments using actin free of tropomyosin

(W. Drabikowski, unpublished). The results obtained so far, in agreement with our supposition mentioned above [4], showed that when the final concentration of protein obtained from F-actin pellets by homogenization was higher than about 3 mg./ml. the reduced viscosity of the resulting solutions was also much higher than that of G-actin. This observation seems to explain why in the experiments of Grubhofer & Weber [9] the viscosity of actin depolymerized by homogenization showed a high concentration dependence, although actin used by these authors was most probably free of tropomyosin. Similarly, actin prepared by Bárány et al. [1] by homogenization seemed to have higher reduced viscosity than that of G-actin. In a recent paper these authors [2] mentioned also that not the whole actin obtained by homogenization was true G-actin.

Evidence for the existence of polymers of actin in the absence of salts has also been reported by Grubhofer & Weber [9]. These authors have observed that the viscosity of F-actin depolymerized by dialysis remains high for a very long period of time, despite the removal of KCl. However, this high viscosity drops to the level of that of G-actin after short homogenization. According to Grubhofer [10], van der Waals forces may be responsible for the high viscosity of actin which remains polymerized in the absence of salts.

Kasai et al. [12] found a certain amount of shorter polymers even in G-actin depolymerized by prolonged dialysis. Later on these authors [18] took into account the possibility of existence of short linear polymers in all concentrated G-actin solutions; they also found that these oligomers dissociated to monomers by dilution or in the presence of 1.5 M-urea.

The solution of actin depolymerized by the method described in this paper seems to consist of oligomers of actin. The molecular weight of these kinds of polymers is too low for sedimenting at 105 000 g, in conditions in which sedimentation of the long F-actin filaments takes place. The reduced viscosity of the solutions of the discussed polymers shows strong concentration dependence. The oligomers dissociate upon dilution and therefore, most probably, their relative amount in diluted actin solutions seems to be negligible. On the other hand, the differences in the reduced viscosities of the solutions of depolymerized actin, shown in Fig. 4, may indicate that the length of the oligomers depends not only on the concentration of actin but also on some other factors. Mechanical treatment (homogenization) as well as addition of 1.6 M-urea also break up the oligomers found in actin depolymerized by Dowex 50.

All observations presented above seem therefore to indicate that the oligomers of actin described in this paper do not correspond to the random aggregates of inactive actin the formation of which was observed by some authors in special, rather drastic, conditions [13, 15]. On the

contrary, the oligomers obtained from F-actin by Dowex 50 treatment seem to be of a similar nature as those obtained either by homogenization of F-actin pellets or by short dialysis of F-actin.

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SUMMARY

F-actin, obtained from G-actin in the absence of free ATP by the addition of $MgCl_2$ or $CaCl_2$, was depolymerized with Dowex 50 and some properties of the resulting product were investigated. Its reduced viscosity was much higher than that of G-actin, but lower than that of F-actin. During incubation the viscosity decreased spontaneously and, simultaneously, the ability to polymerize diminished at about the same rate. Free ATP inhibited both processes.

The solutions of actin depolymerized with Dowex 50 did not contain any protein sedimentable at 105 000 g. Their reduced viscosity showed a strong concentration dependence and decreased rapidly after homogenization or in the presence of 1.6 M-urea.

The results obtained seem to indicate that in the described conditions a formation of oligomers of actin takes place.

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BADANIA NAD DEPOLIMERYZACJĄ F-AKTYNY

Streszczenie

F-aktynę otrzymaną z G-aktyny przez polimeryzację przy pomocy $MgCl_2$ lub $CaCl_2$ w nieobecności wolnego ATP, depolimeryzowano przez działanie Dowex 50 i badano niektóre właściwości otrzymanego produktu.

Jego lepkość zredukowana była dużo wyższa niż lepkość G-aktyny lecz niższa niż F-aktyny. W czasie inkubacji lepkość otrzymanej aktyny spadała i jednocześnie zmniejszała się jej zdolność do polimeryzacji, przy czym wolny ATP wykazywał działanie ochronne wobec obu tych procesów.

Lepkość aktyny otrzymanej przez depolimeryzację przy użyciu Dowex 50 wykazuje silną zależność od stężenia białka i spada szybko po homogenizacji lub pod wpływem 1.6 M-mocznika. Roztwory tej zdepolimeryzowanej formy aktyny nie zawierają białka sedymentującego przy 105 000 g. Wszystkie otrzymane wyniki zdają się wskazywać, że w opisanych warunkach doświadczenia ma miejsce tworzenie oligomerów aktyny.

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INFLUENCE OF TEMPERATURE ON THE STABILITY OF THE ACID AND ALKALINE FORMS OF POLYRIBOADENYLIC ACID

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During the course of an investigation on the radiation chemistry of aqueous solutions of synthetic polynucleotides, use was made of the melting profiles of the polymers to reveal modifications in secondary structure resulting from irradiation. It was hoped in this way to detect minor alterations in secondary structure resulting from the use of doses small enough to minimize chemical modifications of the polymer components. A convenient model for such studies proved to be polyriboadenylic acid (poly-A), which possesses a highly organized twin helical structure below pH 6, the details of which have been fully elucidated by Rich *et al.* [18]. Also well known is the influence of environmental conditions, such as pH, ionic strength and temperature, on the secondary structure of the acid form of poly-A [2, 9, 20, 26].

During the course of initial observations on the effect of γ-irradiation on dilute poly-A solutions, it was found that the melting profiles of the non-irradiated controls were irreversible. When a freshly prepared solution of poly-A was submitted to a cycle of heating to the point where the helix to coil transition was complete, brought back to room temperature, and a second melting profile run, this latter differed considerably from the first. The second profile was typical of that for a polymer with a lower degree of secondary structure. Since we are dealing here with a homopolymer, the failure of the twin strands to fully reform on cooling cannot be due to difficulties in base pairing as is the case for "annealing" of DNA [4, 16, 6]. This phenomenon was all the more puzzling in that a number of observers, who have reported the melting profile of the acid form of poly-A [9, 26] have *implicitly* implied that this profile is fully reversible.

In view of the widespread use of poly-A in physico-chemical, enzymic and structural studies, it was considered advisable to investigate the foregoing in greater detail. In addition we have examined the temperature profile of poly-A in neutral and slightly alkaline medium, as well

as the influence of temperature on this profile. The structure of the alkaline form of poly-A is relatively little known and, during the course of these studies, we have found that its melting profile, as hitherto reported [9] is incomplete, and that it exhibits additional structure below room temperature. This fact is of undoubted importance in relation to attempts to elucidate the structure of the alkaline form of poly-A, and may be of significance in relation to its activity in *in vitro* coding studies [10, 13].

MATERIALS AND METHODS

Most of the experiments were performed with a sample of poly-A prepared with polynucleotide phosphorylase from *Azotobacter vinelandii* [11] and a sample kindly supplied by Dr. R. Beers and prepared with an enzyme from *Micrococcus lysodeicticus* [1]. One comparative experiment was performed with a commercial preparation (Miles Laboratories, Clifton, New Jersey, U.S.A.). Results were essentially similar with all three preparations.

Oligo-A, prepared according to Michelson [17], consisted of a mixture of various chain lengths with up to about 15 residues. The shorter chains were removed by chromatography on Whatman paper no. 1 with the aid of the solvent system *n*-butanol - glacial acetic acid - water (5:2:3, by vol.).

The ApA samples, one with 2′-5′ internucleotide linkages, and the other with 3′-5′ linkages, were gifts of Dr. A. M. Michelson. Yeast adenylic acid, consisting of a mixture of the 2′ and 3′ isomers, was obtained from Fluka (Switzerland). Adenosine-5′-phosphate was a Sigma Chemical Co. (St. Louis, U.S.A.) preparation.

Melting profiles were obtained by use of the thermostatic compartment of the Beckman DU spectrophotometer. Temperatures in the cuvettes were determined by means of a constantan-copper thermocouple to an accuracy of $\pm 0.1°$. When long heating times were employed, solutions were contained in sealed ampoules to prevent evaporation.

All chromatographic controls were run on Whatman paper no. 1 with the solvent system given above.

Inorganic phosphate was determined on paper chromatograms by a modification of the technique of Fletcher & Malpress [8]. The dried chromatogram was sprayed with a 5% ammonium molybdate solution and then with 20% HCl. Following gentle drying, the chromatogram was sprayed with 0.05% benzidine (the benzidine was dissolved in 10 ml. glacial acetic acid and brought to 100 ml. with water). Finally the chromatogram was sprayed with a saturated aqueous solution of sodium acetate. Phosphate then appears as a blue spot against a white background, the colour being quite stable for quantities of P_i of the order of 1 μg. or more.

RESULTS AND DISCUSSION

The influence of extended heating on the temperature profile of the acid form of poly-A is illustrated in Fig. 1. A sample of poly-A in 0.02 M-acetate buffer, pH 5.1, was heated for a period of about 3 hr. at a temperature of 85 - 90°, i.e. at a temperature at which the helix to coil transition is complete. The temperature profile of this sample was compared with one which had not undergone previous heat treatment. Both solutions were then allowed to cool slowly to room temperature, following which a melting curve was again run on each of them.
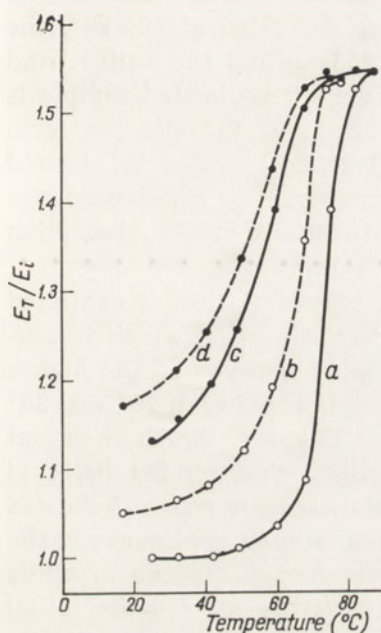
Fig. 1
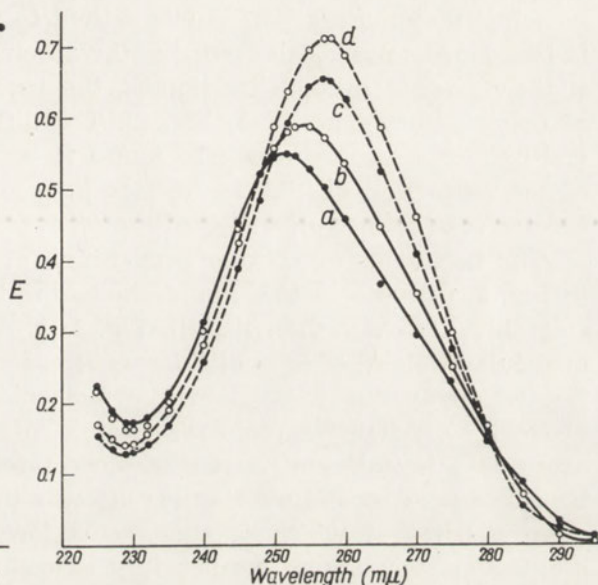
Fig. 2

Fig. 1. Effect of heating on melting curve of acid form of poly-A in 0.02 M-acetate buffer, pH 5.1. In E_T/E_i, E_i is the initial extinction prior to heating and E_T the extinction at temperature T. Extinctions in this and subsequent figures are for a wavelength of 257 mμ. (a), Temperature profile of freshly prepared sample (T_m 73.7°). (b), Sample shown in curve a brought back to room temperature and submitted to second cycle of heating (T_m 65.7°). (c), Temperature profile of sample previously heated for 3 hr. at 85 - 90° (T_m 56.5°). (d), Sample shown in curve c brought back to room temperature and submitted to second cycle of heating (T_m 51.7°).

Fig. 2. Influence of heating on acid form of poly-A as reflected in changes in absorption spectra of acid and alkaline forms. (a), Absorption spectrum of poly-A, unheated, in 0.02 M-acetate buffer, pH 5.1. (b), Following 3 hr. heating at 85 - 90° at pH 5.1. (c), Absorption spectrum of poly-A, unheated, at pH 7.5. (d), Absorption spectrum of poly-A at pH 7.5 following heating of solution at pH 5.1 for 3 hr. at 85 - 90°.

It will be seen that while the initial profile for the unheated sample (curve *a*, Fig. 1) sets in at a temperature of about 50°, the sample previously treated at 85° for 3 hr. (curve *c*) starts melting at about 25°. Furthermore, when both samples are cooled and a second melting curve run on each, the profiles (curves *b* and *d*) undergo additional modifications indicative of further degradation, which must be due to the effect of heating during the running of the initial profiles. It should also be noted that for all samples which have undergone exposure to elevated temperatures, the profiles terminate at a significantly lower temperature.

A comparison of the absorption spectra for the control and heated samples demonstrated that, following heating for 3 hr. at 85 - 90°, the principal maximum at 252 mμ is shifted to 253 mμ and the entire band is broadened towards the red, i.e. the spectrum of the heated sample is slightly altered in the direction of the spectrum of the alkaline form of poly-A (curves *a* and *b*, Fig. 2). The difference between the heated and unheated samples was also found to be reflected in the dependence of the extinction on pH, as well as in modifications of the absorption spectra in alkaline (pH 7.4) medium (curves *c* and *d*, Fig. 2).

The behaviour of another preparation of poly-A (Miles) is exhibited in Fig. 3. Curve *a* in this figure shows the initial profile, which is considerably narrower than that in Fig. 1, probably because of the higher molecular weight. (The small increase in absorption between 20° and 30° is most likely due to random intermolecular linkages, which melt out above 30°). After complete dissociation of the twin-stranded helix at about 90°, the solution was cooled slowly, following the course indicated by curve *b*. A second melting profile was then run to give curve *c*, the melting point of which is almost 19° lower than for curve *a*, while melting starts at room temperature as compared to 70° for the initial profile. Of some interest also is the fact that, on cooling, the partially degraded strands do not at once exhibit maximum reformation (cf. curves *b* and *c*). This hysteresis effect was encountered with all preparations of poly-A examined.

With a view to establishing the lowest temperature at which the melting profile is non-reversible, a sample of poly-A in 0.02 M-acetate buffer, pH 5, was submitted to increasing temperature increments. After each exposure of the solution to a given temperature, it was brought back to room temperature, optical density measurements being made during cooling. It was found in this way that, for a preparation of poly-A for which the T_m was about 75°, heating to this temperature resulted in only a small degree of non-reversibility of the profile up to this temperature. Even heating for 4 hr. at 75° resulted in a minor decrease in extent of reversibility as compared to a short heating period at 85 - 90°.

It would therefore appear that at a temperature below 75° (under

the above conditions) the melting curve for the acid form of poly-A
should be fully reversible. This was, in fact, found to be the case as
is illustrated by Fig. 4, which presents the temperature profile of poly-A
in 0.02 M-acetate buffer plus 0.2 M-NaCl at pH 5.0. The sample shown
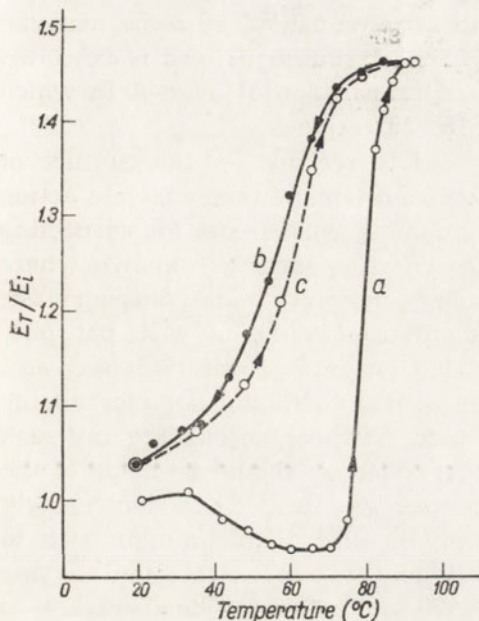
Fig. 3   Fig. 4

Fig. 3. Influence of temperature on melting profile of commercial (Miles)
preparation of poly-A in 0.02 M-acetate buffer, pH 5.05. (a), Initial temperature
profile. (b), Cooling curve for same sample. (c), Second temperature profile. Note
hysteresis on cooling (difference between curves b and c).
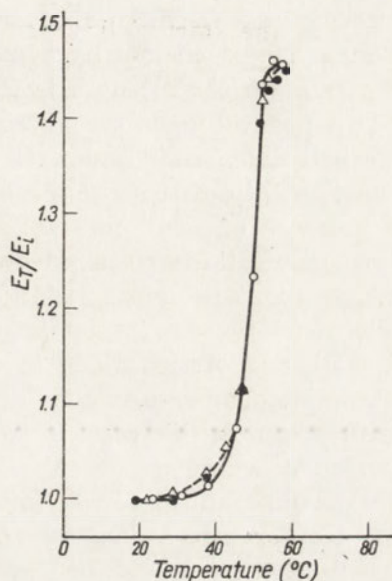
Fig. 4. Temperature profile of poly-A in 0.02 M-acetate buffer - 0.2 M-NaCl, pH 5.05.
(○), Initial profile; (△), cooling curve; (●), second profile.

here was submitted to two cycles of heating and one of cooling, the upper
temperature limit being 60°, at which the twin strands are fully
dissociated. It is clear that under these conditions the profile is fully
reversible; furthermore there is no evidence of a hysteresis effect in the
cooling curve (cf. curves b and c, Fig. 3).

The above results convincingly demonstrate that at elevated temper-
atures (about 75° and higher) in slightly acid medium, some bonds
undergo rupture, thus preventing reformation of the original secondary
structure of poly-A on subsequent cooling. The only plausible candidates
are the phosphodiester bonds in the poly-A chain. This is supported by
the fact that when heated acid poly-A is brought to alkaline pH, where
the double helix does not exist, there is a definite decrease in hypo-
chromicity (see Fig. 2), again suggestive of a shortening in chain length.

More compelling evidence for such an interpretation is forthcoming from a paper chromatographic examination of heated poly-A, using the solvent system n-butanol - acetic acid - water (5:2:3, by vol.) and ascending chromatography. Unheated poly-A shows a single spot which remains at the start; following heating for several hours at 75° at pH about 5.0, the spot is less intense and is accompanied by some material which moves 2 - 3 cm. in an overnight run. A similar pattern is exhibited by an unheated chemically synthesized preparation of oligo-A in which the maximum chain length is about 15 - 20 residues.

Two possible factors may be invoked to account for the rupture of internucleotide bonds under the above conditions: either enzymic action or heat lability of some of the bonds. Some grounds exist for suspecting the presence of some enzyme; and specific instances are known where polyribonucleotides, prepared by means of polynucleotide phosphorylase, undergo spontaneous degradation due to contamination with phosphodiesterase(s) present in the polymerizing enzyme preparation (see, e.g., ref. [23]), and carried along in trace amounts with the polymer during its isolation. The results cited above tend, however, to exclude any such interpretation in this case. In particular, mention should be made of the experiment where a poly-A preparation was heated to increasingly higher temperatures for definite intervals and then brought back to room temperature to test reversibility of the profile. If some enzyme were present, it would be expected at some elevated temperature to act with time. However, below 75° no such action is evident, so that it would have to be an unusual enzyme indeed. A more convincing demonstration against involvement of an enzyme was the following experiment: a preparation of poly-A in 0.02 M-acetate buffer, pH 7.4, was heated for 1 hr. at 100°; this should suffice to inactivate any enzyme. Following cooling to room temperature, the solution was acidified to pH 5.15 and submitted to two heating cycles to obtain the melting profiles. The second profile was again found to exhibit additional degradation, thus excluding enzyme activity as a source of this degradation, and implicating the thermal lability of some of the phosphodiester bonds.

The degree of thermal degradation of poly-A at about pH 5 was also found to be dependent on the ionic strength of the medium. Heating a solution of poly-A in 0.02 M-acetate buffer, pH 5, in the presence of 0.2 M-NaCl (under these conditions the helical structure is fully dissociated at about 60°, see Fig. 4) for 4 hr. at 72° results in a decrease in T_m of 2.2° and only slightly modifies the width of the profile. Five hours heating at 87° decrease T_m by 4.5° with a pronounced broadening of the profile. But these modifications are very much less than those resulting under the same conditions in the absence of NaCl (see Fig. 1) and suggest, furthermore, that thermal degradation of poly-A is inhibited by high salt concentrations. This was confirmed by the following experi-

ment. A solution of poly-A in 0.02 m-acetate, pH 5.05, was heated for 5 hr. at 87° and then brought to room temperature. The melting profile was then compared with that of an unheated sample in order to establish the degree of degradation. The heated and unheated solutions, at room temperature, were then made up to 0.2 m in NaCl and the pH again adjusted to 5.05. The solutions were left at room temperature for 1 hr. and melting profiles then run on each. It was found that addition of salt to the heated sample had little effect on the profile, which differed from the unheated control to the same extent as in the absence of added salt. It follows that high ionic strength protects against heat degradation, but is without sensible effect on the existing secondary structure.

In order to obtain some further insight into the nature of the mechanism of thermal degradation, an examination was now made of the thermal stability of simpler derivatives, in 0.02 m-acetate buffer, pH about 5.0, the results being checked by paper chromatography. Prolonged heating of chemically synthesized oligo-A (containing a mixture of 2'-5' and 3'-5' internucleotide linkages and chain lengths varying up to about 15 residues) at 96 - 100° demonstrated the appearance of only traces of shorter chains. Under similar conditions ApA (with either 2'-5' or 3'-5' linkages) was unaffected. However, even relatively short heating (about 1 hr.) of adenosine-2'(3')-phosphate at about 85° resulted in the formation of adenosine and inorganic phosphate. Prolonged heating of adenosine-5'-phosphate at about 90° also resulted in the formation of traces of adenosine, but to all intents and purposes AMP can for the purposes of the present work be regarded as stable.

Liberation of inorganic phosphate from oligo-A and poly-A. A chemically synthesized preparation of oligo-A, containing chains up to 15 residues, was freed from inorganic phosphate and mononucleotides by paper chromatography. This was then heated in 0.01 m-acetate buffer, pH 5.05, at 85° for about 9 hr. and then chromatographed using the solvent system *n*-butanol - acetic acid - water (5:2:3, by vol.), which clearly resolves inorganic P from the oligonucleotides. Since the oligo-A is synthesized from adenosine-2':3'-phosphate, each chain must be terminated by a (2')3'-phosphate end group. Under the above heating conditions, practically all the phosphate end groups were split off, as for adenosine-2'(3')-phosphate.

Poly-A in 0.02 m-acetate buffer, pH 5.05, was then heated at 85° for 15 hr. and chromatographed in the above solvent system along with an unheated control. The amount of inorganic phosphate was estimated visually, following staining, by comparison with known quantities of inorganic P. The unheated control showed no P_{inorg}, whereas the heated sample exhibited an amount of P_{inorg} corresponding to about 8% of the total poly-A. Bearing in mind that adenosine-5'-phosphate is stable under the above conditions, while adenosine-3'-phosphate liberates P_{inorg}, the

most logical interpretation of the foregoing is that poly-A undergoes chain scission due to splitting of bonds between C_5 and phosphate residues. The resulting fragments, half of which consist of chains terminated in a 3'-phosphate, then liberate this as P_{inorg}.

Complexing of poly-A with poly-U at pH 5. Poly-A normally forms complexes with polyuridylic acid (poly-U) only in alkaline medium. In acid medium such complex formation is prevented by the secondary structure of the poly-A twin helix. It was, however, demonstrated by Cox [3] that, if an equimolar solution of poly-A and poly-U is heated to the point where the acid form of poly-A is fully dissociated, subsequent cooling of the mixture leads to the formation exclusively of poly-(A+U), which is fully stable at room temperature. This observation suggested to us that a mixture of poly-A and poly-U may form a twin--stranded complex in acid medium, even at room temperature, if the poly-A is degraded to shorter chains with reduced stability.

A solution of poly-A at pH 5 was divided into three portions consisting of: (*a*), a control solution; (*b*), a solution heated slowly to 87° and then cooled to room temperature; and (*c*), a sample heated to 85° for 4 hr. The spectra of all three samples were then determined and to each was added an equimolar amount of poly-U. It was found that for solution *a* the resultant absorption spectrum was merely the sum of the two components. Solution *b* likewise showed no change on addition of poly-U, but, after standing 24 hr., there was a 2% decrease in absorption from additivity. Sample *c*, however, showed a 2.5% decrease from additivity immediately after addition of poly-U; following 8 hr. at room temperature the decrease was almost 5%, while after 24 hr. this decrease attained a limiting value of 13% (Fig. 5).

The foregoing is readily interpretable in the light of thermal degradation of poly-A to shorter chains, the stability of which is substantially decreased so that the shorter fragments will have a greater affinity for poly-U than for another poly-A chain. Once a given strand has begun to complex with poly-U, it will continue to do so since the resulting poly-(A+U) complex is more stable.

Referring again to the results of Cox [3] cited above, it is clear that while complex formation between poly-A and poly-U in acid medium during cooling of a heated solution is not due to thermal degradation of poly-A but to the greater affinity of the A-U base pair as compared to the A-A base pair, nonetheless the poly-(A+U) complex formed on cooling such a solution must contain some thermally degraded poly-A strands.

Alkaline form of poly-A. In alkaline medium poly-A exists to a considerable extent in a form commonly referred to as a random coil. Its melting profile, as hitherto reported [9], is very broad from room temperature to just over 80°, at which point melting is complete.

The existence of appreciable temperature hyperchromicity suggests that the alkaline form of poly-A is not merely a random coil, as is undoubtedly the case for poly-U which shows no temperature hyperchromicity above 10° [15, 24], and that it must contain some helical regions (cf. ref. [21]). Furthermore the relatively steep nature of the profile at room temperature [9] is suggestive of the existence of additional structure below room temperature. And, in fact, when the melting profile of poly-A is followed below room temperature, it reaches a plateau only at about 3° (Fig. 6), with almost 30% of the total temperature hyperchromicity below 25°. It should be recalled in this connection that poly-U exhibits some secondary structure only below about 8° [15, 24]. An even more striking example is that of poly-rT, the temperature profile of which places in evidence a high degree of secondary structure
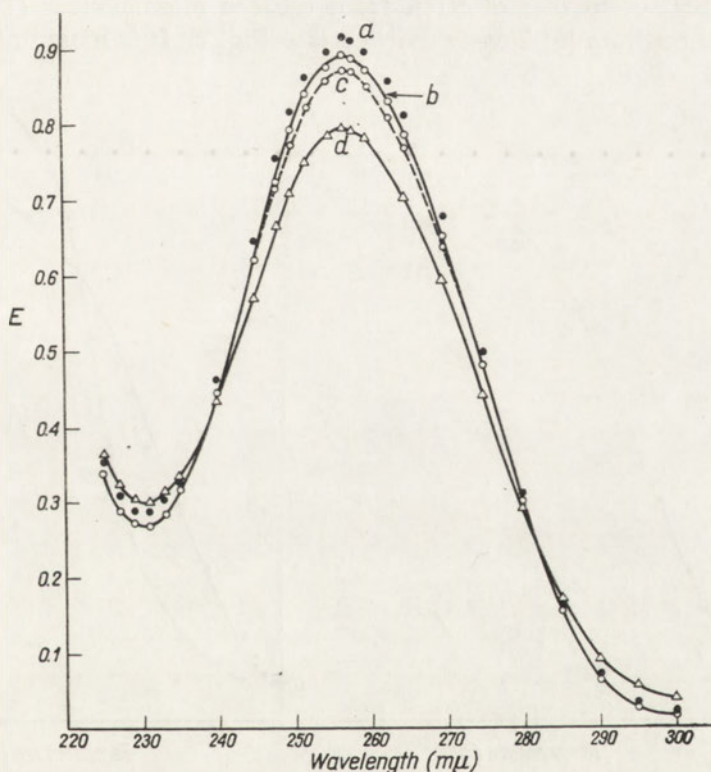
Fig. 5. Complexing of thermally degraded acid form of poly-A with poly-U at room temperature in 0.02 M-acetate buffer, pH 5.02. A solution of poly-A in 0.02 M-acetate, pH 5, was heated at 85° for 4 hr., cooled to room temperature and an equimolar amount of poly-U added. (a), Resultant calculated additive absorption spectrum of the two components. (b), Observed spectrum 30 min. after addition of poly-U, and showing 3% hypochromicity with respect to curve a. (c), Observed spectrum 8 hr. after addition of poly-U, and showing 5% hypochromicity. (d), Observed spectrum 24 hr. after addition of poly-U, showing 13% hypochromicity.

which melts out largely in the room temperature range [25], a property that has been correlated with its activity as a function of temperature in the *in vitro* coding system [22] and most likely due to the temperature dependence of its binding by ribosomes. From Fig. 6 it may be deduced that the coding ability of alkaline poly-A, which stimulates the incorporation of lysine [10], may be similarly dependent on temperature, although perhaps to a lesser extent that poly-rT.

Particularly striking is the fact that the temperature profile of alkaline poly-A, and the corresponding T_m, are relatively independent of the ionic strength of the medium (see Fig. 6). This is, indeed, contrary to the implication from temperature hyperchromicity that a certain degree of secondary structure exists.

On the other hand the resemblance of the temperature profile of alkaline poly-A to that of RNA from tobacco mosaic virus (TMV-RNA) in neutral medium [5] is very marked (see Fig. 7). It is most unlikely that
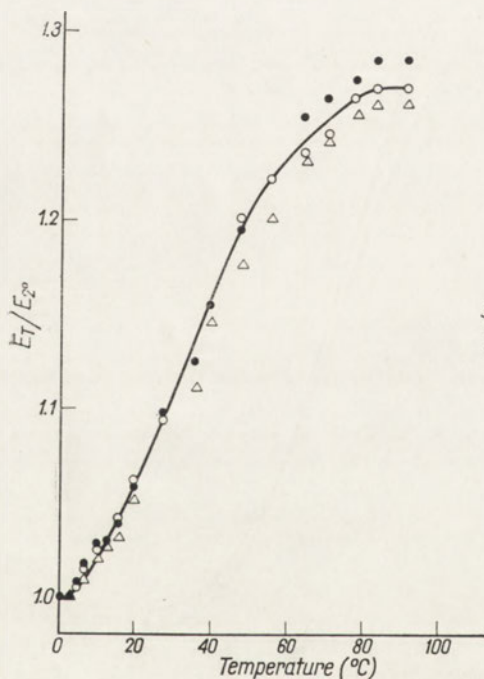
Fig. 6
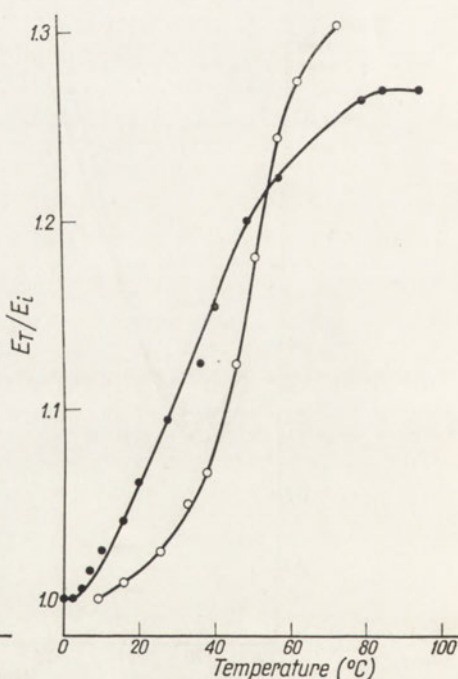
Fig. 7

Fig. 6. Temperature profile of alkaline form of poly-A between 0° and 95° at different ionic strengths in 0.005 M-phosphate buffer, pH 7.2. (O), No salt added, μ 0.01; (●), 0.015 M-NaCl, μ 0.025; (△), 0.15 M-NaCl, μ 0.16.

Fig. 7. Temperature profiles for: (O), TMV-RNA in 0.1 M-phosphate buffer, pH 7, measured at 258 mμ (taken from ref. [5]). (●), Alkaline form of poly-A in 0.005 M-phosphate buffer, pH 7.2, with or without addition of NaCl, measured at 257 mμ (see Fig. 6).
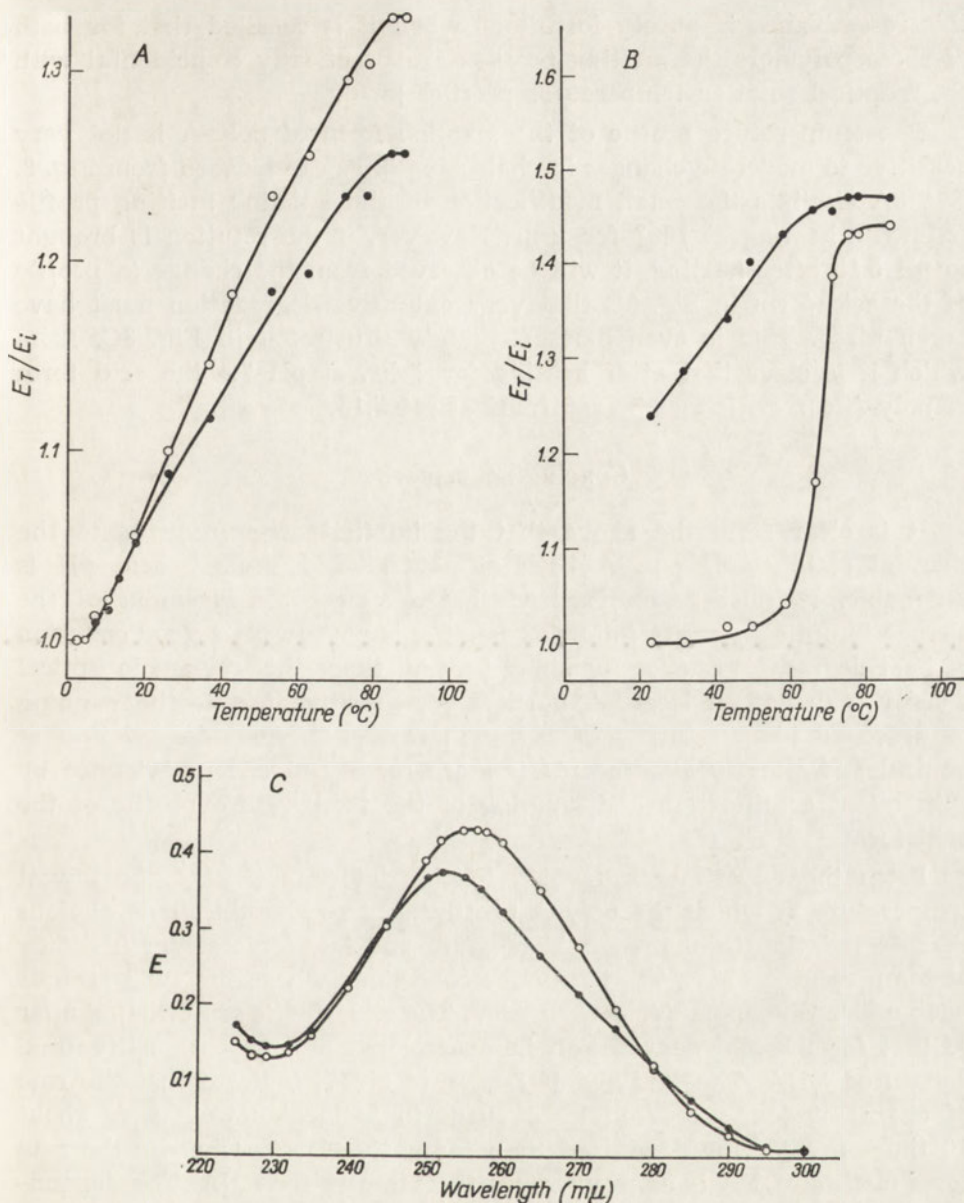
Fig. 8. Demonstrating heat degradation of poly-A following heating for 1 hr. at 100°
in 0.02 M-acetate buffer, pH 7.4. *A*, (○), Temperature profile of unheated, control,
solution at pH 7.4. (●), Temperature profile of heated solution at pH 7.4. *B*, (○),
Temperature profile of unheated, control solution at pH 5.15. (●), Temperature
profile of heated solution brought to pH 5.15 after heating. *C*, (●), Absorption
spectrum of unheated, control solution at pH 5.15. (○), Absorption spectrum of
heated solution brought to pH 5.15 after heating. Note that the absorption spectrum
is almost identical with that for poly-A in neutral or alkaline medium, showing
that degradation has been so extensive that the acid form no longer exists
at pH 5.15.

this resemblance is purely fortuitous when it is recalled that for both of these polymers the melting profiles are practically coincidental with their optical rotation-temperature profiles [5, 9].

The temperature profile of the alkaline form of poly-A is not very sensitive to moderate changes in chain length, as can be seen from Fig. 8. Fig. 8A exhibits the small modification of the alkaline melting profile following heating at 100° for 1 hr. However, if the solution is brought to pH 5.15 after heating, it will be observed from the change in profile of the acid form in Fig. 8B that very extensive degradation must have taken place. This is even more strikingly illustrated in Fig. 8C, from which it is clear that after heating for 1 hr. at pH 7.4 the acid form of poly-A can no longer be reformed at pH 5.15.

Concluding remarks

It is clear from the above that the implicit assumption as to the thermal stability of poly-A both at neutral and slightly acid pH is untenable. It must be emphasized that a simple measurement of the change in optical density following heating is not always a good criterion of heat degradation, since for short heating times this change in optical density may not be large. A much more sensitive test is the running of a second temperature profile. Furthermore, thermal degradation at neutral and slightly alkaline pH is more readily placed in evidence by subsequent acidification and running of the temperature profile of the acid form.

From the experiments devoted to the determination of the lowest temperature at which the poly-A profile is non-reversible, it is obvious that degradation in slightly acid medium and low ionic strength occurs at temperatures above T_m, i.e. under conditions where the twin strands have undergone appreciable separation. The situation is somewhat similar to that for thermal degradation in neutral medium of both native and denatured DNA. The findings of Eigner *et al.* [7] indicate that the rate of chain scission, measured by changes in molecular weight, is about 10 times greater for denatured than for native DNA, whereas the rate for denatured DNA is approximately the same as for RNA. The dependence of the rate of chain scission in DNA and RNA on secondary structure, and the similarity in thermal lability between the acid, helical, form of poly-A to that of native DNA, suggest that the acid, helical, form of poly-A and its alkaline form with only partial secondary structure will undergo chain scission at different rates.

Attention should also be drawn to the implicit assumption that thermally induced chain scission in DNA and RNA is a random process. There is no evidence either for or against this. But it should be noted that the thermal profiles for poly-dAT (which is a twin stranded co-po-

lymer containing alternating sequences of deoxyadenosine and thymidine) and poly-C (polycytidylic acid), which embrace fairly high temperatures, are fully reversible [27], from which it follows that thermal breakage of phosphodiester linkages in natural nucleic acids probably occurs only between specific bases. More extensive studies with synthetic polynucleotides are required to elucidate this matter.

One point which is not clear is the apparent protective effect of higher ionic strength (0.2 M-NaCl) on the thermal degradation of the acid form of poly-A, since under these conditions the twin strands separate at a lower temperature (Fig. 4). It is possible that under these conditions the individual separated strands may assume a more stable configuration because of the neutralization of charges on the chains by the salt ions. At low ionic strengths, where repulsive forces between charged groups result in a marked increase in chain length of the strands, it may be that conditions are somewhat comparable to those in DNA subjected to shear forces. While there is no strict analogy between degradation of DNA by shear forces and thermal degradation of poly-A, there are some similarities between the two processes. It was shown by Levinthal & Davison [14] that the rate of chain scission of DNA by shearing increases rapidly with chain length. A similar situation prevails for the thermal degradation of poly-A, as may be seen by a comparison between curves a and b of Fig. 1 and curves a and c of Fig. 3; the profile of the more highly polymerized preparation is altered considerably more as a result of heating than those of the less polymerized samples. The same conclusion follows from a comparison of the decreases in temperature at which melting is complete from which it is also seen that longer chains are more rapidly degraded. In striking contrast is the stability of shorter chains, viz. oligo-A with chain lengths of up to 15 residues. A number of observers [14, 19, 12] have emphasized the fact that chain scission of DNA by shearing occurs more or less at the middle of the chains. While we have no concrete data as to where thermally induced chain scission occurs in poly-A, it is of some significance that paper chromatography demonstrates the absence of formation of short oligonucleotides; this, together with the thermal stability of oligo-A with a chain length of 15 residues, indicates that thermal degradation in poly-A does not occur near the ends of the chains.

SUMMARY

Under conditions normally employed, the temperature profile of the acid form of polyriboadenylic acid (poly-A) is not reversible. This lack of reversibility is due to thermal rupture of phosphodiester linkages with the resultant shortening of chain length. Rupture occurs at the phosphate linkages to $C_{5'}$ of the sugar residues; the resulting $C_{3'}$ phosphate end groups are split off on prolongation of the heating times.

The rate of thermally induced chain scission increases with chain length. Short chains (up to 15 residues) are thermostable.

Conditions have been defined under which the temperature profile of the acid form of poly-A is fully reversible and it is only under these or comparable conditions that the temperature profile of poly-A may be employed as a criterion for following the effects of various physico-chemical conditions such as the action of chemical mutagens, irradiation, etc.

A comparison with reported results for several other synthetic polynucleotides suggests that thermally induced chain scission in natural nucleic acids does not occur randomly, but most likely preferentially between specific base residues.

Thermally degraded poly-A complexes with poly-U in slightly acid medium.

The neutral or alkaline form of poly-A is likewise susceptible to thermal degradation. The alkaline temperature profile is not a sensitive criterion of such degradation. A more sensitive test, following heating at neutral or alkaline pH, is acidification followed by a running of the acid temperature profile.

The temperature profile of the alkaline form of poly-A has been measured down to $0°$ and it has been shown that an additional 30% hyperchromicity occurs below room temperature $(25°)$. This is likely to be of some significance in relation to the biological activity of poly-A at physiological pH. The complete temperature profile of alkaline poly-A was also found to be very similar to that for RNA from tobacco mosaic virus (TMV-RNA), confirming the suggestion of other observers that alkaline poly-A must contain localized regions with helical structure.

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WPŁYW TEMPERATURY NA TRWAŁOŚĆ KWAŚNEJ I ALKALICZNEJ FORMY KWASU POLIRYBOADENILOWEGO

Streszczenie

Stwierdzono, że w powszechnie stosowanych warunkach profil temperaturowy kwaśnej formy poli-A jest nieodwracalny. Spowodowane to jest pękaniem wiązań fosfodwuestrowych w podwyższonej temperaturze i wynikającym z tego skróceniem łańcuchów polinukleotydu. Pękanie wiązania fosfodwuestrowego zachodzi między grupą fosforanową a C_5, reszty cukrowej; powstająca końcowa grupa fosforanowa przy C_3, ulega odszczepieniu w ciągu dalszego grzania. Szybkość cieplnej fragmentacji łańcuchów wzrasta z długością łańcucha; krótkie łańcuchy (do 15 reszt) są termostabilne.

Określono warunki, w których profil temperaturowy poli-A jest całkowicie odwracalny i tylko w tych, lub podobnych, warunkach profil temperaturowy poli-A może być stosowany jako kryterium zmian polinukleotydu pod wpływem różnych czynników fizyko-chemicznych, takich jak chemiczne czynniki mutagenne, napromienienie itp.

Porównanie opisanych wyników z danymi uzyskanymi dla kilku innych syntetycznych polinukleotydów sugeruje, że cieplna fragmentacja łańcuchów w naturalnych kwasach nukleinowych nie zachodzi w sposób przypadkowy, lecz najprawdopodobniej wybiórczo między specyficznymi resztami zasad.

Cieplnie zdegradowane poli-A tworzy kompleks z poli-U w środowisku słabo kwaśnym. Stopień skompleksowania zależny jest od stopnia degradacji poli-A.

Poli-A ulega także termicznej degradacji w środowisku obojętnym lub alkalicznym, jednak profil temperaturowy formy alkalicznej nie jest czułym wskaźnikiem tych zmian. Degradacja polinukleotydu zachodząca pod wpływem ogrzewania w środowisku obojętnym lub alkalicznym może być ujawniona jedynie po zakwaszeniu środowiska, a następnie oznaczeniu profilu temperaturowego kwaśnej formy poli-A.

Profil temperaturowy alkalicznej formy poli-A oznaczono do 0° i stwierdzono występowanie dodatkowej hyperchromazji w temperaturach niższych niż pokojowa. Może to mieć pewne znaczenie w odniesieniu do biologicznej aktywności poli-A

w fizjologicznych warunkach. Całkowity profil temperaturowy poli-A jest bardzo
podobny do profilu oznaczonego dla RNA z wirusa mozaiki tytoniowej, potwier-
dzając sugestie innych badaczy, że alkaliczna forma poli-A musi zawierać obszary
o heliksowej strukturze.

Received 2 June 1964.

Addendum: Since submission of the foregoing, two publications have
appeared which bear directly on the foregoing findings:

(1) The presence of partial secondary structure in the alkaline form
of poly-A has been demonstrated by J. Brahms (*Nature* **202**, 797, 1964)
from a study of the circular dichroism of this polymer, in agreement
with our results.

(2) In an extensive study by W. Ginoza, C. J. Hoelle, K. B. Vessay
& C. Carmack (*Nature* **203**, 606, 1964) on the mechanism of thermal
inactivation of single-stranded virus nucleic acids, it was shown that
such inactivation is the result of breakage of phosphodiester bonds and
is more rapid with longer molecules. These results are complementary
to our physico-chemical findings and we shall revert to them in
a subsequent publication.

HALINA SIERAKOWSKA*, J.-E. EDSTRÖM** and D. SHUGAR*

INTRACELLULAR LOCALIZATION OF NUCLEASE ENZYMES BY A MICRODISSECTION-MICROELECTRO PHORETIC TECHNIQUE

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Most of the existing methods for the intracellular localization of nuclease enzymes are of rather questionable validity. Cell fractionation techniques, notwithstanding improvements introduced in recent years, by no means exclude difficulties arising from enzyme diffusion and readsorption, with resultant contamination of individual cellular fractions. Cell fractionation in non-aqueous media, with its attendant advantages in the case of some nuclease enzymes, has hitherto been applied on a rather limited scale [e.g. ref. 1], and only with the use of such non-specific substrates as RNA and DNA.

Similar difficulties are to be expected and are, in fact, encountered with cytochemical techniques. The diffusion of RNase [1] from Carnoy-fixed pancreas sections was observed some years ago by Lagerstedt [11] and must be borne in mind in evaluating the validity of the promising method of Marshall [13] for localization of RNase and DNase by means of the fluorescein antibody technique. Subsequent attempts to localize various RNases in fixed tissue sections with the use of specific substrates such as the ribonucleoside cyclic phosphates in conjunction with the Gomori technique [19] were likewise hampered by the excessive diffusion of the enzymes from tissue sections during incubation; similar difficulties

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[1] The following abbreviations will be used in this text: RNase, ribonuclease; DNase, deoxyribonuclease; PDase, phosphod'esterase; Cp!, cytidine-$2':3'$-cyclic phosphate; Cp, cytidine-$2'(3')$-phosphate; C2'P, cytidine-$2'$-phosphate; C3'P, cytidine-$3'$-phosphate; C5'P, cytidine-$5'$-phosphate; C, cytidine; Up!, uridine-$2':3'$-cyclic phosphate; Up, uridine-$2'(3')$-phosphate; U5'P, uridine-$5'$-phosphate; U, uridine; Ap!, adenosine-$2':3'$-cyclic phosphate; Ap, adenosine-$2'(3')$-phosphate; A, adenosine; ApA, adenylyl-$(2',5')$-adenosine; Gp, guanosine-$2'(3')$-phosphate; G, guanosine. The term "nucleases" is used to denote all enzymes participating in the catabolism of nucleic acids; whereas "phosphodiesterases" denotes enzymes hydrolysing phosphodiester bonds.

were encountered with DNases. An extensive discussion on this subject is to be found in ref. [19].

In one instance a fully satisfactory and highly specific cytochemical method has been developed for a phosphodiesterase, viz. PDase I, using an azo-dye coupling technique. The success of this procedure was due in large part to the lack of diffusion of this enzyme from tissue sections during the incubation periods employed [20, 21].

Although the possible extension of the azo-dye method to additional nuclease enzymes is currently under investigation, considerable doubt may be entertained as to whether it will be applicable to such enzymes as pancreatic acid and alkaline RNases, the diffusion of which is so extensive from tissue sections [11, 19]. It was consequently deemed advisable to simultaneously examine other possible approaches to this problem, and one which suggested itself was the microdissection of individual cells in non-aqueous media, followed by the estimation of the enzymic activity of the dissected fragments. Such a procedure is theoretically feasible by making use of the microelectrophoresis technique developed by Edström [7] for the estimation of the nucleic acid composition of individual cells.

MATERIALS AND METHODS

Ovaries from the starfish *Asterias rubens*, obtained in March from the Zoological Station, Kristineberg, Sweden, were fixed for 2 hr. in chilled 80% ethanol, dehydrated overnight in chilled 96% ethanol, followed by methyl benzoate and benzene, and paraffin embedded. In some experiments overnight fixation in chilled absolute acetone, followed by pre-embedding in cellulose acetate and then embedding in paraffin, was employed. Phosphodiesterase activities of the oocytes were practically identical following the use of both these procedures, as contrasted with complete inactivation by fixation in Carnoy's fluid.

Sections 8 μ thick were flattened on cover slips, deparaffinized in chloroform, immediately covered with liquid paraffin, and dissected under the paraffin in a de Fonbrune oil chamber with the help of a de Fonbrune micromanipulator equipped with two glass needles. Micromanipulation was performed as described by Edström *et al.* [8], yielding uncontaminated cytoplasm and nucleoplasm, and nucleoli contaminated with traces of nucleoplasmic material. The amount of adhering nucleoplasm was very small and considered unlikely to markedly affect the results. The isolated units were immersed in 0.25×10^{-3} mm^3 drops of buffer (see below) adhering to the lower surface of a cover slip overlying the oil chamber. Eleven nucleoli, nucleoplasm corresponding to about 10 complete 8 μ sections, and cytoplasm corresponding to one 8 μ section of a small cell, were each immersed in separate buffer drops which were then made up to 0.5×10^{-3} mm^3 with 0.02 M-buffered substrate solution.

Controls consisting of similarly dissected material were incubated in buffer alone and then combined with substrate. Chopped-up oocytes containing all the cellular components incubated in full incubation media served in certain instances as additional controls, corresponding to homogenate macroscale experiments. Evaporation from the incubation drops was eliminated by placing a strip of wet filter paper in the bottom of the oil chamber trough. The chamber was placed in a vapour-saturated Petri dish and incubated for 18 hr., unless otherwise indicated, at 37°. Several control experiments were run to verify that hydrolysis of substrates was approximately linear over this time period. Following incubation one-half the incubation drop was applied to a 30 μ diameter fibre of regenerated cellulose, pre-treated and impregnated as described in the next paragraph.

The fibres were alkali-treated according to Edström [7] and impregnated with buffer solution prepared as follows: 0.2 M-maleic acid - NaOH buffer, pH 2, 10 ml.; 10.5 g. each of glucose, fructose, galactose, sucrose; 16 g. glycerol; 3.6 g. NaCl; 4.5 g. KCl; 0.5 g. $MgCl_2 \cdot 6H_2O$. All the foregoing were combined in a small flask and heated in a boiling water bath with continuous stirring until nearly all the solid matter had dissolved. When the solution turned a light honey colour, it was cooled rapidly. Prolonged heating, resulting in browning and increased absorption of the buffer solution in the ultraviolet, was avoided.

The foregoing buffer solution was a modification of the original Edström buffer [7] adapted as above for the separation of the nucleoside cyclic phosphates, dinucleotides, nucleotides and nucleosides. The magnesium ions were introduced to counter the identical electrophoretic mobility of the cyclic and non-cyclic nucleoside phosphates, the relative mobilities of which were altered as a function of magnesium concentration, although the mechanism of this phenomenon is not clear. Monovalent metal salts increased the sharpness of separations. The use of several sugars in place of glucose alone increased the total sugar solubility. Finally the low pH value of the buffer made it possible to readily differentiate uracil from cytosine derivatives by utilizing the differences in their extinctions at 257 and 275 mμ at acid pH [17].

Fibres were impregnated with the buffer solution either by vigorous shaking for 30 min. or by storage in the refrigerator overnight, following which they may be stored in the refrigerator for several weeks without deterioration. The fibres were drawn and mounted on quartz slides as described by Edström [7]. Drops containing incubation mixtures, controls, and reference substances were applied at spaced intervals on the fibre. Fibre lengths varied from 8 to 12 mm. with a total potential drop along the fibre of 4000 volts. Electrophoresis times varied from 8 min. for an 8-mm. fibre to 12 min. for a 12-mm. fibre for adenine and cytosine derivatives. For uracil derivatives these times varied from 5 to 7 min., respectively.

Following electrophoresis the fibres were photographed with monochromatic light (257 mμ) in the Köhler ultraviolet microscope, using a cadmium spark as light source. Cytosine derivatives were also photographed at 275 mμ in order to note possible shifts in absorption maximum from 275 to 257 mμ, indicating the formation of deaminated cytosine derivatives. Ultraviolet-absorbing material eluted from the tissue, and constituting the blank value, was identified by the lack of electrophoretic mobility and by the presence of maximum absorption at 257 mμ. The plates were scanned with a Joyce Loebl recording microdensitometer. Percentage substrate hydrolysis was calculated from the relative areas under the curves, determined by weighing or planimetry.

Direct measurement of volumes of irregular fragments of dissected material proved tedious and was also subject to considerable error. Recourse was therefore had to a procedure relating the volumes of cellular elements of starfish oocytes to their RNA contents [8], which is quite accurate. Following incubation the dissected tissue was rinsed with water and its RNA content estimated [6].

Nucleotides were commercial Sigma (U.S.A.) and CalBiochem (U.S.A.) preparations. The nucleoside cyclic phosphates were prepared according to procedures elsewhere described [18]. C3'P was prepared quantitatively from Cp! by exhaustive hydrolysis with Worthington (U.S.A.) crystalline pancreatic RNase. C2'P was separated from Cp by column chromatography. ApA was a gift from Dr. A. M. Michelson. Alkaline phosphodiesterases were assayed using as substrates Cp!, Up!, Ap! and ApA (2',5' internucleotide linkage) at a concentration of 0.01 M in 0.02 M-tris - HCl buffer, pH 8.2; 0.02 M-phosphate buffer, pH 8.2, was also used in the case of Cp! with similar results. Acid PDases were assayed with Cp! and Up! at a concentration of 0.01 M in 0.02 M-acetate - HCl buffer, pH 5, or tris - maleate buffer, pH 5.8. Alkaline phosphomonoesterases were assayed with Cp, Up, Gp, C2'P, C5'P and U5'P at 0.01 M in 0.02 M-tris - HCl buffer, pH 8.2 and 9.2, with and without 0.003 M-MgSO$_4$.

Figure 1 illustrates the microelectrophoretic separation patterns of substrates and possible hydrolysis products. The location of the various components was ascertained in all cases by runs of nucleotides in different combinations.

Several experiments were supplemented by macroexperiments on a test tube scale with paper chromatographic analysis of the enzymic hydrolysates. Sections 8 μ thick were collected in a test tube, deparaffinized with benzene and air-dried. The dry sections were weighed, homogenized and combined with the buffered substrate solutions to make a 2.5% (on dry weight basis) homogenate in incubation media identical with those in microscale experiments. Heat-inactivated homogenates were used as controls. Volumes of 40 - 100 μl. with 1 μl. of toluene added to
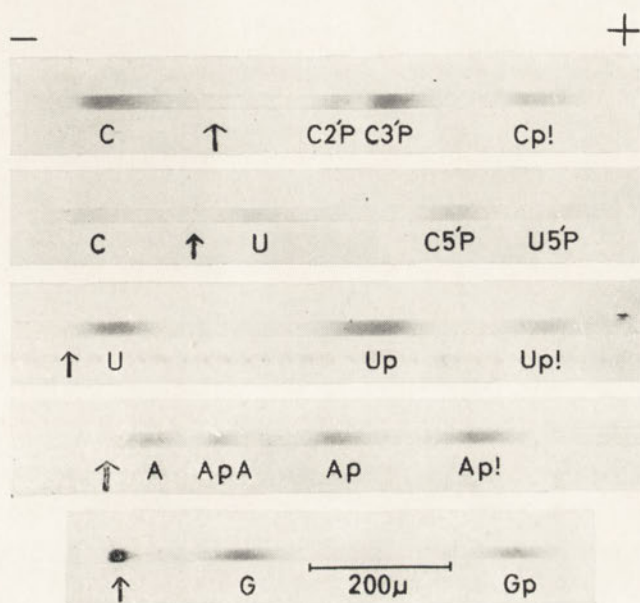
Fig. 1. Microelectrophoretic separation patterns of nucleotides and nucleosides in combinations used in the enzymic determinations, photographed at 275 mμ (two upper separations) or 257 mμ (three lower separations). Arrows indicate origins. Uracil coincides with U in this system and cytosine migrates towards the cathode (left) far ahead of C and is not included in the two upper photographs. Explanation of symbols and separation conditions given in the text.

avoid bacterial contamination were incubated in small stoppered test tubes with continuous agitation for 18 hr. at 37°. Following incubation aliquots of 20 μl. were applied on Whatman no. 1 paper and developed in water-saturated n-butanol or isopropanol - conc. NH₄OH - H₂O (7:1:2, by vol.).

RESULTS

Phosphodiesterase activities

Cp! pH 8.2 *(Table 1).* Each of the three cellular components hydrolysed Cp! to C2′P signifying the presence of a specific phosphodiesterase. By far the largest part of this activity is contained in the cytoplasm and only a very small fraction in the nuclear components (Table 2). The cytoplasm readily hydrolysed the C2′P to C.

Cp! pH 5 and 5.8 *(Table 1).* Each of the cellular components hydrolysed Cp! to C2′P; only the cytoplasmic fraction hydrolysed the C2′P to C.

Table 1

Hydrolysis of cytidine-2′:3′-cyclic phosphate (Cp!) by starfish oocyte components

Symbols used in Tables: (+), positive, product present; (—), negative, product absent; (±), product is found in small amounts; (+ ?), result questionable.

Cell component	pH	Hydrolysis products			No. of expts.
		C2′P	C3′P	C	
Cytoplasm	8.2	+	—	+	20
Nucleoplasm	8.2	+	—	—	12
Nucleoli	8.2	+	—	—	14
Cytoplasm	5.0 and 5.8	+	—	+	8
Nucleoplasm	5.0 and 5.8	+	—	—	6
Nucleoli	5.0 and 5.8	+	—	—	5

Table 2

Specific activity of alkaline phosphodiesterase against cytidine-2′:3′-cyclic phosphate in starfish oocyte components

Cell component	Specific activity (μmoles Cp! ×10¹⁰/hr./μ³ of tissue) mean value ± standard error	No. of expts.
Cytoplasm	0.91 ± 0.33	6
Nucleoplasm	0.096 ± 0.046	5
Nucleoli	0.118 ± 0.035	6

Up! pH 8.2 *(Table 3)*. The cytoplasm and nucleoplasm both hydrolysed Up! to Up; only the cytoplasm hydrolysed it further to U.

The nucleolar phosphodiesterase content requires further experimental evidence.

Table 3

Hydrolysis of uridine-2':3'-cyclic phosphate (Up!) by starfish oocyte components

Symbols as for Table 1.

Cell component	pH	Hydrolysis products		No. of expts.
		Up	U	
Cytoplasm	8.2	+	+	10
Nucleoplasm	8.2	+	—	2
Nucleoli	8.2	+?	—	2
Cytoplasm	5.0 and 5.8	+	+	3
Nucleoplasm	5.0 and 5.8	+	—	2
Nucleoli	5.0 and 5.8	+	—	1

Up! pH 5 *(Table 3)*. Each of the cellular components hydrolysed Up! to Up; only the cytoplasmic fraction hydrolysed it further to U.

Ap! and ApA, pH 8.2. Neither of these substrates was hydrolysed by individual cellular components or by complete cells.

Diffusion of phosphodiesterase

Cytoplasm pooled from several oocytes was preincubated in 0.02 M-tris-HCl buffer, pH 8.2, for 2 hr. at 37°. The buffer was then removed, combined with an equal volume of 0.02 M-Cp! solution and incubated for an additional 18 hr. at 37°. The cytoplasmic fragments were rinsed with buffer and likewise incubated with 0.01 M-Cp! in 0.01 M-tris-HCl buffer, pH 8.2, for an additional 18 hr.

Electrophoretic analysis indicated complete hydrolysis of Cp! by the preincubated cytoplasmic fragments, but only 60% Cp! hydrolysis by the buffer employed for preincubation. It was estimated that less than 30% of the total phosphodiesterase diffused out of ethanol-fixed oocyte cytoplasm during the course of preincubation.

Phosphomonoesterases

Cp hydrolysis (Table 4). Hydrolysis of Cp did not exceed 20% after 48 hr. of incubation with the cytoplasmic fraction and complete cells. There was no hydrolysis with the nucleoli or the nucleoplasm. Decrease of substrate concentration to 0.001 M, change of buffer, or extension of incubation time to 48 hr. brought no further hydrolysis.

Table 4

Hydrolysis of cytidine nucleotides by starfish oocyte components

Symbols as for Table 1.

Substrate	Cell component	pH	Hydrolysis product C	No. of expts.
Cp	Cytoplasm	8.2	±	14
Cp	Nucleoplasm	8.2	—	4
Cp	Nucleoli	8.2	—	4
C2′P	Cytoplasm	8.2	±	4
C3′P	Cytoplasm	8.2	±	4
C5′P	Cytoplasm	8.2	+	5
C5′P	Nucleoplasm	8.2	—	2
C5′P	Nucleoli	8.2	—	2

C2′P and C3′P, pH 8.2 (Table 4). Hydrolysis with the cytoplasmic fraction did not exceed 20% for either substrate. No hydrolysis was observed with the nucleoli or nucleoplasm.

In view of insignificant dephosphorylation of C2′P and C3′P as contrasted with complete dephosphorylation of C2′P released from Cp! by the cytoplasm, the possible inhibitory effect of C3′P on the degradation of Cp! by the oocyte cytoplasm was investigated. Addition of C3′P to a regular Cp! incubation medium resulted in degradation of Cp! to C, as in control experiments, with the added C3′P remaining essentially unaffected. Similarly addition of Cp to a Cp! incubation medium did not influence the degradation of Cp! to C, with Cp unaffected.

C5′P (Table 4). 50% hydrolysis to C occurred only with the cytoplasm. No hydrolysis took place with the nucleoplasm or the nucleoli.

Up (Table 5). No dephosphorylation was obtained with the cytoplasm or complete cells.

U5′P (Table 5). There was no hydrolysis with the nucleoli and nucleoplasm. U5′P hydrolysis by the cytoplasmic fraction was not always reproducible.

Table 5

Hydrolysis of uridine nucleotides by starfish oocyte components

Symbols as for Table 1.

Substrate	Cell component	pH	Hydrolysis product U	No. of expts.
Up	Cytoplasm	8.2	—	7
U5′P	Cytoplasm	8.2	+?	4
U5′P	Nucleoplasm	8.2	—	1
U5′P	Nucleoli	8.2	—	1

Macroexperiments

Cp! was 90% hydrolysed to Cp and traces of cytidine. Up! was completely hydrolysed to Up and traces of uridine. Cp and Up were hydrolysed to the extent of only 10 - 20% to their respective nucleosides. C5′P was 30 - 40% hydrolysed to cytidine. No further degradation of the nucleosides occurred under bacteriostatically controlled conditions.

DISCUSSION

A comparison of the results obtained in the micro- and macroscale experiments does not indicate any loss of enzymic activity due to exposure of tissue to microvolume operations. It may therefore be assumed that the intracellular distribution data obtained with microdissected material are fully valid.

The occurrence of phosphodiesterase in the nucleoplasm, nucleoli and, of course, the cytoplasm seems unquestionable. The microelectrophoretic results show that the enzyme hydrolyses Cp! to the 2′-nucleotide, resembling in this respect a PDase found in the spleen [14] and the pancreas [3]. It differs from it, however, in being active only against the pyrimidine cyclic phosphates. It can be distinguished from the pancreatic RNase by the product formed (C2′P as compared to C3′P for pancreatic RNase), the relatively insignificant diffusion from fixed tissues, and inactivation by Carnoy-fixation [10].

A comparison of our results with the intracellular localization of phosphodiesterases investigated by cell fractionation techniques is somewhat difficult due, among others, to the use in fractionation studies of high molecular weight substrates susceptible to hydrolysis by a number of phosphodiesterases of varying specificities. The use of low molecular, relatively specific, substrates like the nucleoside cyclic phosphates limits the number of enzymes participating in their degradation and allows for a closer characterization of the enzymes involved. The available cell fractionation data on intracellular phosphodiesterases employ RNA as substrate, and give values resulting from the combined action of RNases and PDases. Nuclear-bound liver and pancreas RNase activity has been reported both for the alkaline [15, 12, 4] and acid pH range [16, 5, 24, 12, 4]. Roth & Vincent (quoted by Vincent, [23]) found no RNase activity in the nucleoli of starfish oocytes. Baltus [2], on the other hand, has recently reported the presence of acid and alkaline nucleolar RNases in the same material.

Phosphomonoesterase activity is evident mainly in the case of nucleotides derived from the hydrolysis of the cyclic nucleotides and in the case of the 5′-nucleotides. The dephosphorylation of the 2′-nucleotides derived from the cyclic nucleotides, but not of the directly introduced

2′-nucleotides, is rather difficult to interpret. A possible explanation may be the formation, following opening of the cyclic phosphate ring, of an activated cytidine-2′-phosphoryl group more sensitive to phosphomono-esterase hydrolysis. The possibility of such an activated nucleoside phos-phoryl group retaining the energy of the phosphodiester bond has been postulated by Heppel & Whitfield [9] in phosphodiesterase-catalysed transfer reactions.

The dephosphorylation of nucleotides formed by incubation of the cyclic nucleotides with the cytoplasmic fraction, as contrasted with lack of dephosphorylation of nucleotides formed by incubation of the cyclic nucleotides with the nucleoli and nucleoplasm, indicates the presence of phosphomonoesterase activity only in the cytoplasm. Dephosphoryla-tion of the nucleoside-5′-phosphates solely by the cytoplasmic component constitutes additional evidence for the absence of phosphomonoesterases in the nucleoli and nucleoplasm.

The phosphatase activity demonstrable in the present macroscale experiments appears lower than for the microscale results particularly in the case of nucleoside phosphates derived from hydrolysis of the cyclic phosphates, and in the case of the 5′-nucleotides.

The localization of phosphomonoesterases exclusively in the cytoplasm is in accord with the findings furnished by non-aqueous cell fractiona-tion techniques attributing no significant alkaline phosphatase and nucleo-tide phosphomonoesterase activity to the nuclei [22]. Vincent [23] studied starfish nucleoli isolated in aqueous media and found them to contain no alkaline phosphatase, but some acid phosphatase of uncertain origin.

The microdissection-microelectrophoresis approach has also proven useful in checking nuclease localization at the histochemical level, i.e. results obtained by the Daoust technique, in which case histological units were isolated under non-aqueous conditions and their enzymic content assayed. Its application and extension seems worthwhile in those cases where the validity of nuclear and cytoplasmic content of nucleolytic enzymes, established by cytochemical or cell fractionation techniques, requires confirmation.

For this purpose, however, it is necessary to ameliorate two disadvan-tages which at present exist: (1) the rather indirect and cumbersome estimation of tissue mass, and (2) difficulties in microdissection of average size cells. Starfish oocytes are particularily suitable for micro-dissection because of their large size. Microdissection of clean nucleoli from most mammalian nuclei is at present not feasible. Preliminary experiments with rat kidney cells indicated the possibility of isolation of uncontaminated nuclei subject to further refinement of microdissection techniques.

SUMMARY

A procedure is described for the intracellular estimation of nucleolytic enzymes, based on the microdissection of individual cells in non-aqueous media, incubation of the isolated cell fragments with specific substrates, and microelectrophoresis of the enzymic hydrolysis products. The method is relatively free from artifacts due to enzyme diffusion.

Application of the foregoing procedure to starfish oocytes demonstrated the presence of acid and alkaline nucleases which hydrolyse cytidine-2':3'-cyclic phosphate and uridine-2':3'-cyclic phosphate to cytidine-2'-phosphate and uridine-2'(3')-phosphate, respectively. The relative activities of the alkaline enzyme in cytoplasm, nucleoplasm and nucleoli were determined. The oocytes were inactive against adenosine-2':3'-cyclic phosphate and adenylyl-(2',5')-adenosine. Phosphomonoesterase activity was found only in the cytoplasm.

The overall potentialities of the method, as well as its limitations, are outlined and discussed.

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WEWNĄTRZKOMÓRKOWA LOKALIZACJA ENZYMÓW NUKLEOLITYCZNYCH METODĄ MIKRODYSEKCJI-MIKROELEKTROFOREZY

Streszczenie

Opracowano metodę wewnątrzkomórkowej lokalizacji enzymów nukleolitycznych polegającą na mikrodysekcji poszczególnych komórek w środowisku bezwodnym, inkubacji wyizolowanych fragmentów w obecności specyficznych substratów, a następnie mikroelektroforetycznej analizie produktów hydrolizy enzymatycznej. Metoda eliminuje artefakty wynikające z dyfuzji enzymów.

Stosując powyższą technikę, stwierdzono występowanie w oocytach rozgwiazdy nukleazy kwaśnej oraz alkalicznej, hydrolizujących cykliczny 2':3'-fosforan cytydyny do 2'-fosforanu cytydyny oraz cykliczny 2':3'-fosforan urydyny do 2'(3')-fosforanu urydyny. Określono względne aktywności alkalicznego enzymu w cytoplazmie, nukleoplazmie oraz jąderkach. Oocyty rozgwiazdy nie hydrolizowały cyklicznego 2':3'-fosforanu adenozyny oraz adenylo-2',5'-adenozyny. Obecność fosfomonoesterazy stwierdzono wyłącznie w cytoplazmie.

Omówiono możliwości rozszerzenia zastosowania tej metody.

BARBARA ŻMUDZKA and D. SHUGAR

PREPARATION AND CHEMICAL AND ENZYMIC PROPERTIES OF CYCLIC PHOSPHATES OF D-GLUCOPYRANOSE AND SYNTHESIS OF DERIVATIVES OF N-(D-GLUCOPYRANOSYL) PYRIDINE

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The conception of the existence of cyclic phosphate esters was first advanced in connection with the phenomenon of phosphoryl group migration [18] encountered during the synthesis of phosphate esters. Subsequently the subject took on added significance when it was found that compounds of this type are of some importance in biological processes. Particular interest attaches to the ribonucleoside-2′:3′-cyclic phosphates, which are intermediates in the alkaline and enzymic hydrolysis of RNA; and the 3′:5′-cyclic phosphate of adenosine, which is involved in glycogenolysis [23, 16] and most likely in other biological functions [28]. Cyclic phosphates have also been isolated from the alkaline hydrolysis products of various biologically active substances, their formation being, in fact, considered to account for the alkaline lability of these compounds. These include, e.g. pantothenic acid-2′:4′-cyclic phosphate from coenzyme A [2], G-1:2-P[1] from uridine diphosphate glucose and other analogous derivatives [21, 37], 5-phosphoryl-D-ribose--1,2-cyclic phosphate from 5-phosphoryl-D-ribose-α-1-pyrophosphate [25], riboflavin-4:5-cyclic phosphate from flavin adenine nucleotide [10].

More systematic studies on the properties of the cyclic diesters of phosphoric acid became possible following the development of several methods for the chemical synthesis of these compounds [13, 4, 6, 33]. In addition to the ribonucleoside 2′:3′- [6, 32] and 3′:5′- [26] cyclic phosphates, procedures have been described for the isolation of 6- and 7-membered cyclic phosphate rings of aliphatic alkyls [15], as well as

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[1] The following abbreviations are used in this text: G-1-P, glucose-1-phosphate with similar connotations for G-2-P, G-3-P, G-4-P, G-6-P; G-1:2-P is glucose-1:2--cyclic phosphate, and similarly for G-1:3-P, G-2:4-P, G-3:6-P and G-4:6-P; DCC, dicyclohexylcarbodiimide.

various cyclic phosphates (3:5, 3:6, 5:6) of glucofuranose [31], its derivatives such as methyl glycoside or its 1,2-isopropylidene derivative [31], glucopyranose (4:6) [1, 29, 30], and 1-(β-D-glucopyranosyl)uracil (3':6', 4':6', 2':4') [37]. During the course of these investigations it was observed that, in contrast to the marked lability of 5-membered cyclic phosphates, 6- and 7-membered cyclic phosphate rings are relatively stable to acid and alkali [15, 36, 37] and, in fact, exhibit a stability which is of the order of that for non-cyclic diesters of phosphoric acid, such as, e.g. dimethylphosphate [14].

Enzymes generally employed for the hydrolysis of the ribonucleoside-2':3'-cyclic phosphates are inactive or only weakly active against ribonucleoside-3':5'-cyclic phosphates [26], the latter in turn being susceptible to a diesterase isolated from beef heart by Butcher & Sutherland [5], which is completely inactive against ribonucleoside-2':3'-phosphates. Enzymes attacking adenosine-3':5'-phosphate were found in many mammalian tissues, its content in the brain being particularly high [27].

A brain extract prepared according to Drummond & Perrott-Yee [7] was previously employed by us [36, 37] for the decyclization of the 3':6'- and 4':6'-cyclic phosphates of 1-(β-D-glucopyranosyl)uracil, as well as the product of cyclization of G-6-P, which we considered at that time to be G-4:6-P but was subsequently found to be a mixture of G-3:6-P and G-4:6-P. While the rates of enzymic decyclization of these cyclic phosphates were considerably lower than for adenosine-3':5'-phosphate, the products of the reactions were in all cases the 6'-monophosphates in complete analogy to that for adenosine-3':5'-phosphate, which is adenosine-5'-phosphate [7]. The foregoing findings were of particular interest in relation to the glucose cyclic phosphates because of the possibility of their being involved in intermediary metabolism. It consequently appeared of interest to us to examine in greater detail the nature of the enzymes capable of attacking glucose cyclic phosphates. This in turn made it desirable to prepare a number of glucopyranose cyclic phosphates as potential enzymic substrates and to examine their mode of formation as well as some of their properties.

We have consequently subjected the 1-, 3-, 4- and 6-monophosphates of glucose to cyclization treatment with a view to obtaining 5-, 6- and 7-membered cyclic phosphates with ring closure via the various glucose carbons, including the primary C_6, the secondary C_2, C_3 and C_4, and finally the C_1. Particularly interesting was the possibility of preparing G-1:2-P and G-1:3-P with a view to determining whether ring size is a factor in the stability of cyclic phosphates containing a C_1 carbon in the ring since it is known that G-1:2-P (previously isolated as a hydrolysis product of uridine diphosphate glucose [21]) and synthetic ribose-1:2-P [34, 33] are very labile compounds which readily decompose to yield the free 2-monophosphates and free sugar [15].

RESULTS AND DISCUSSION

Glucose-4:6-phosphate was first prepared by Baddiley et al. [1] by treatment of phenyl-β-D-glucoside with phenyl phosphodichloridate in pyridine, followed by reductive removal of phenyl groups. The product was considered to be homogeneous since (a) it consumed two moles of periodate, and (b) it gave a single spot on paper chromatography in two solvent systems: butanol - acetic acid - water (4:1:5, by vol.) and n-propanol - ammonia - water (6:3:1, by vol.). Subsequently Khorana et al. [15] prepared what they believed to be the same compound by treatment of glucose-6-phosphate with DCC in pyridine; the product was reported to consume two moles of periodate and exhibit a single spot with the two foregoing solvents. This was considered [15] to be in accord with the formulation of G-6-P in the stable C1 conformation, thus favouring cyclization with the equatorial hydroxyl on C_4, as shown in Scheme 1.
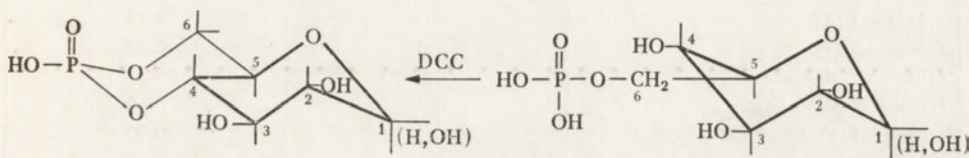
Scheme 1

Cyclization of G-6-P was initially carried out by us as described by Khorana et al. [15], i.e. by reaction of G-6-P with DCC in 75% pyridine for 72 hr. at room temperature, modified slightly with regard to isolation of the final product. The course of the reaction was essentially as described by the foregoing authors, the reaction yield being practically 100% (in practice there always remains several per cent of unreacted monophosphate in the reaction mixture), while a chromatographic control in solvent A (Table 1) and in butanol - acetic acid - water demonstrated the appearance of only one spot corresponding to a cyclic phosphate [2]. This product was therefore at first considered to be exclusively G-4:6-P as suggested by Khorana et al. [15] and Baddiley et al. [1]. However, when it was chromatographed in solvent B, it gave two well-resolved spots with R_F values of 0.65 (I) and 0.60 (II), see Tables 1 and 2, with relative yields of 60% and 40%, and which must be G-4:6-P and

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[2] The butanol - acetic acid - water solvent employed by Baddiley et al. [1] and Khorana et al. [15] is a two-phase solvent system. Neither of the foregoing authors is explicit as to the form in which chromatography was carried out with this solvent. To eliminate any doubts, therefore, chromatography of the reaction mixture was carried out with each phase separately as well as with various combinations of the two phases. Only one reaction product was observed in all cases.

G-3:6-P, the two theoretically possible products of cyclization of G-6-P (see below for identification of these two products).

In our opinion the foregoing establishes unequivocally the formation of two cyclic phosphate products by the reaction of G-6-P with DCC in 75% pyridine. As pointed out by Baddiley et al. [1], the formation of a (more strained) 7-membered 3:6-cyclic phosphate of glucose is sterically feasible, since the 3- and 6-hydroxyl groups are cis to each other.

Table 1

Ascending paper chromatography on Whatman paper no. 1

Solvent systems: (A), propan-2-ol - ammonia (d 0.88) - water (7:1:2, by vol); (B), methyl cellosolve - methyl ethyl ketone - 3_N-NH_4OH (7:2:3, by vol.).

Compound	R_F in solvent	
	A	B
Glucose	0.45	0.75
Glucose-1-phosphate	0.05	0.17
Glucose-3-phosphate	0.05	0.20
Glucose-4-phosphate	0.05	0.20
Glucose-6-phosphate	0.05	0.15
Glucose-1:2-phosphate	0.26	0.70
Glucose-1:3-phosphate	0.26	0.65
Glucose-3:6-phosphate	0.26	0.60
Glucose-4:6-phosphate	0.26	0.65
Compound X	0.90	0.98
Compound Y	0.05	—
Compound Z	0.15	—

It remains to establish whether the glucose cyclic phosphate obtained by Baddiley et al. [1] was indeed exclusively G-4:6-P, as they claimed, or consisted of two component cyclic phosphates. It should be noted in this connection that Baddiley et al. [1] actually prepared the cyclic phosphate of phenyl-β-D-glucoside; reductive removal of the phenyl group then gave the free glucose cyclic phosphate. It is consequently of some significance that, whereas the reaction of G-6-P with DCC in pyridine gives two cyclic phosphates, the reaction of 1-(β-D-glucopyranosyl)uracil--6′-phosphate leads to the formation of only one cyclic phosphate, the 4:6 [36]. From this it follows that substitution of C_1 influences the course of the cyclization reaction, either because of steric factors or as a result of additional strain in the ring. We are therefore led to conclude that the glucose cyclic phosphate obtained by Baddiley et al. [1] was indeed exclusively G-4:6-P; and this is further supported by the fact that phosphorylation and cyclization of methyl-α-D-glucoside was convincingly demonstrated by Szabó & Szabó [30] to lead to the unambiguous formation of only one cyclic phosphate, viz. the 4:6.

Table 2

Synthesis of glucose cyclic phosphates as a function of temperature and time of reaction

G-3:6-P and G-4:6-P were obtained by cyclization of G-6-P. For method of separation see text. The percentages of reaction products are only approximate and are based on a visual examination of the relative intensities of the spots due to the reaction products. "Trace" means less than 10% and these values were not taken into account in estimating the other spots.

Starting compound	Reaction conditions			Reaction products (%)			
	Solvent	Reaction temp.	Reaction time (hr.)	Mono-phosphate	Cyclic phosphate	Com-pound X	Glucose
G-1-P	75% pyridine	0°	2	50	50	0	0
			48	trace	100	0	0
			72	0	80	20	0
		18°	2	40	60	0	0
			24	40	40	20	0
			72	trace	0—40	60—100	0
	75% methanol	0°	48	20	80	0	0
		18°	24	40	60	0	0
			48	20	80	0	0
G-3-P	75% pyridine	0°	4	20	80	0	0
			48	trace	100	0	0
		18°	4	10	90	0	0
			24	10	45	45	0
			48	trace	30	70	0
			72	trace	0—30	70—100	0
G-4-P	75% pyridine	18°	72	trace	100	0	0
G-6-P	75% pyridine	18°	5	100	trace	0	0
			48	20	80	0	0
			72	trace	100	0	0
	75% methanol	18°	5	30	70	0	0
			18	0	100	0	0
Acid hydrolysis products of G-3:6-P	75% pyridine	18°	24	trace	50	trace	50
			72	trace	25	25	50
Acid hydrolysis products of G-4:6-P	75% pyridine	18°	48	trace	100	0	trace
			72	trace	100	0	trace

With a view to identifying the two isomeric reaction products of G-6-P with DCC in pyridine, several milligrams of each was isolated by paper chromatography on Whatman no. 3 MM paper. Each product was then hydrolysed in acid to the corresponding 3- or 4-monophosphate (as described below, see Table 3, for the acid hydrolysis of cyclic phosphates leading to the formation of monophosphates linked to the secondary carbon) and the two hydrolysis products, *I* and *II*, then submitted to cyclization with DCC at room temperature for 72 hr. Cyclization of each product followed a different course, the difference being analogous to that between authentic G-3-P and G-4-P. The hydrolysis product of *I* yielded a cyclic phosphate similar to that from G-4-P, so that *I* was assigned to G-4:6-P. On the other hand the course of cyclization for the hydrolysis product of *II* was very similar to that for G-3-P, with formation of a cyclic phosphate which, on further reaction, was transformed into a new compound which we shall denote by *X* (see below). Since compound *X* was found to be formed from G-3-P, and not at all from G-4-P or G-6-P, it was concluded that *II* with R_F 0.60 is G-3:6-P.

Independently of the above, a simpler method was devised for the cyclization of G-6-P. The free acid glucose phosphate was treated with DCC in 75% methanol for 18 hr. (overnight) at room temperature to give the cyclic phosphate(s) in quantitative yield. Following removal of

Table 3

Chemical and enzymic hydrolysis products of glucose cyclic phosphates

The percentages of reaction products are only approximate and are based on a visual examination of the relative intensities of the spots due to the reaction products. "Trace" means less than 10% and these values were not taken into account in estimating the other spots.

Cyclic phosphate	Hydrolysis conditions	Hydrolysis products (%)						
		Non-hydro-lysed	G-1-P	G-2-P	G-3-P	G-4-P	G-6-P	Glu-cose
G-1:2-P	0.1 N-HCl, 100°, 5 min.	0	0	50	—	—	—	50
G-1:3-P	0.1 N-HCl, 100°, 5 min.	trace	0	—	90	—	0	10
G-3:6-P	0.1 N-HCl, 100°, 5 min.	100	—	—	trace	—	0	0
	1 N-HCl, 100°, 60 min.	trace	—	—	70	—	30	trace
	Brain extract, 37°, 30 hr.	70	—	—	—	—	15	15
G-4:6-P	0.1 N-HCl, 100°, 5 min.	100	—	—	—	trace	0	0
	1 N-HCl, 100°, 60 min.	trace	—	—	—	70	30	trace
	Brain extract, 37°, 30 hr.	70	—	—	—	—	15	15

cyclohexylurea, the cyclic phosphate product was isolated in the form of a salt with cyclohexylurea such as that described by Khorana [13]. The cyclic phosphate was separated from this cation on a Dowex 50 column and isolated as the barium salt. Chromatography showed that the product consisted of 90% G-4:6-P and 10% G-3:6-P. This procedure therefore possesses the advantages that it not only gives a fully quantitative yield of the cyclic phosphates, but is simpler in operation and gives mainly the 4:6-cyclic phosphate.

Analogous formation of a mixture of 6- and 7-membered cyclic phosphates of glucose was reported by Szabó & Szabó [31] for the cyclization of 1,2-isopropylideneglucofuranose-3'-phosphate with DCC in 75% pyridine. The initial product was the 3:5-cyclic phosphate, which subsequently disappeared with time to be replaced by the 3:6-cyclic phosphate. The latter was the only product after a reaction time of 72 hr. It should, however, be pointed out that the conditions leading to formation of cyclic phosphates of glucofuranose differ from those for glucopyranose. This is illustrated by the fact that for the furanose form cyclization in 75% pyridine is quantitative in 5 hr.; with the pyranose form the reaction is only initiated during this time period, and even after 72 hr. is not fully terminated.

In 75% pyridine the course of cyclization of G-4-P is closely similar to that for G-6-P, with almost complete conversion to cyclic phosphates. The product is homogeneous in solvents A and B. No attempt was made to determine whether the product consisted of one or both of the theoretically possible cyclic phosphates (2:4 and 4:6). It was confirmed only that prolongation of the reaction time does not lead to formation of additional products as in the case of G-1-P and G-3-P.

The reactions with DCC of G-1-P and G-3-P differ in certain respects from those of G-4-P and G-6-P. When reacted with DCC in 75% pyridine at 0° for 48 hr., both give quantitative yields of cyclic phosphates. When the reaction time was prolonged, the cyclic phosphates gradually disappeared and were replaced by a new compound with a high R_F in solvents A and B (see Table 1) and referred to above as compound X. After 72 hr. this was the principal reaction product. Consequently for the isolation of the cyclic phosphates the reaction was conducted uniquely at 0° and terminated prior to any conversion of the cyclic phosphates to X, even when the yield of cyclic phosphates was not quite 100%. Such a procedure was also indicated by the fact that preparations of G-1:2-P were always contaminated with traces of monophosphate due to the very labile nature of G-1:2-P.

All the cyclic phosphates described above exhibited an R_F of 0.25 in solvent A, gave a positive phosphate reaction on the paper chromatograms, and were completely resistant to prostate monophosphatase. Their stability and hydrolysis products in acid were studied by submitting

them to the action of 0.1 N-HCl at 100° for 5 min. and to 1 N-HCl at 100° for 60 min. G-3:6-P and G-4:6-P proved to be relatively stable, the cyclic phosphate rings opening only after 1 hr. at 100° in 1 N-HCl, the products being predominantly G-3-P and G-4-P respectively. This is in agreement with the findings of others [15] as well as with our previous results for glucosyluracil cyclic phosphates [37, 36] concerning the stability of 6- and 7-membered cyclic phosphates and their tendency to hydrolyse in acid to products with the phosphate group linked to a secondary carbon.

By contrast the cyclic phosphates from G-1-P and G-3-P vere very labile and underwent complete hydrolysis in 5 min. in 0.1 N-HCl at 100°. In the case of G-3-P, this substantiates our contention that its cyclization product is G-1:3-P since if G-3:6-P was formed in whole or in part as a reaction product, it would be fully resistant to hydrolysis under these conditions. The lability of G-1:3-P, notwithstanding that it contains a 6-membered cyclic phosphate ring, is undoubtedly related to the presence in the ring of the acid-labile bond to C_1.

The lability of the cyclic phosphate(s) formed from G-1-P is not unexpected since both the theoretically expected cyclic phosphates in this case, viz. G-1:2-P [15] and G-1:3-P, are acid labile. Theoretically it is to be expected that the cyclization product of G-1-P would be G-1:2-P, in accordance with the observed rule [15, 31] that, in the event of the possible formation of a 5- or 6-membered ring, the formation of the 5-membered ring will be favoured. Periodate titration of the cyclization product confirmed this, since G-1:3-P should be resistant to periodate, whereas G-1:2-P should consume one mole as was in fact observed.

Acid hydrolysis of G-1:2-P resulted in the liberation, in addition to G-2-P, of free glucose which is undoubtedly formed from the labile G-1-P forming one of the hydrolysis products.

Attempts were also made to examine the susceptibility to enzymic hydrolysis of the various cyclic phosphates, using a brain extract prepared according to Drummond & Perrott-Yee [7], and which is active against adenosine-3:5-phosphate and which we had shown to be capable of decyclizing 1-(β-D-glucopyranosyl)uracil cyclic phosphates [36]. It was, in fact, found that the 4:6- and 3:6-cyclic phosphates of glucose were very slowly transformed by such an extract to glucose-6-phosphate (in practice part of the enzymic hydrolysis product was glucose, due to the presence of some monophosphatase in the brain extract). Rather lengthy incubation periods of up to 30 hr. were required for this hydrolysis, and aseptic conditions were maintained during this time by addition of thymol. Under the same conditions, however, it was not found possible to test the susceptibility of G-1:2-P or G-1:3-P since prolonged incubation at 37° led to spontaneous hydrolysis of these cyclic phosphates in the control samples. A search is therefore under way for enzymic extracts from other sources capable of decyclization of these compounds.

It remains to discuss the nature of the compound X, to which reference was made above in connection with the cyclization of G-1-P and G-3-P; and two additional compounds derived from X which we shall denote by Y and Z.

Compound X is formed during the cyclization in pyridine, in the presence of DCC, of G-1-P and G-3-P, i.e. those glucose monophosphates the cyclic phosphate rings of which include carbon C_1. X is apparently formed directly from G-1:2-P and G-1:3-P for, when G-1-P or G-3-P are treated with DDC in pyridine, only the cyclic phosphates are formed initially. Subsequently these disappear and are replaced by compound X with an R_F of 0.9 in solvent A. X is readily visible on the chromatogram under a dark UV lamp, and this is also reflected by the fact that it exhibits a characteristic absorption spectrum with a maximum at 260 mμ. This is readily explicable on the assumption that X contains one or more molecules of pyridine and, in fact, compound X is not formed when G-1-P and G-3-P are submitted to cyclization in methanol. X does not exhibit reducing properties when tested by Nelson's method or the Fehling reagent. It contains phosphate, but is not a monoester since it is resistant to prostate monophosphatase. It is relatively unstable and, on heating in 0.1 N-HCl at 100° for several minutes, it decomposes to give a relatively stable product, with R_F of 0.5 in solvent A, with the simultaneous precipitation of a substance insoluble in water and soluble in acetone. The m.p. of this precipitate, 229°, corresponds to cyclohexylurea.

This compound with R_F 0.5 in solvent A, and denoted by us as Y, also exhibits a characteristic absorption spectrum in the UV with a maximum at 260 mμ, possesses no reducing properties, and contains a phosphate group which is readily removed on incubation with prostate and intestinal monophosphatases. The dephosphorylated product, Z, with R_F 0.15 in solvent A, is rather labile, contains no phosphate, and likewise possesses a characteristic maximum at 260 mμ in the ultraviolet. When heated for 10 min. in 1 N-HCl at 100°, compound Z undergoes about 50% hydrolysis.

Compound Y is relatively stable and undergoes only 10% decomposition on heating for 1 hr. in 0.1 N or 1 N-HCl at 100°. The decomposition product is probably identical with the enzymic dephosphorylation product of Y, since it exhibits the same R_F, 0.15, in solvent A, contains no phosphate, and possesses a characteristic maximum at 260 mμ. The decomposition products also include some free glucose.

Compound Y is also resistant to 1 hr. exposure to 0.1 N-NaOH at 100°. On heating for 1 hr. in 1 N-NaOH at 100° in a sealed ampoule, it decomposes to give several products, including free pyridine which can be detected by its odour on opening the ampoule. Furthermore, when

the hydrolysate is examined in the ultraviolet, it exhibits the pH-depen-
dent modifications in absorption spectrum characteristic of free pyridine.
On the other hand the absorption spectrum of X (as well as Y and Z)
is independent of pH. The presence of pyridine in Y and Z (and hence
also in X) was confirmed independently by elementary microanalysis.

Compound Z proved to be a chloride salt and elementary micro-
analysis, plus analysis for chloride, corresponded to a molecule contain-
ing one mole pyridine, one mole glucose and one atom chlorine. These
results, plus its lability in alkali and relative stability in acid, as well as
solubility in water as contrasted with its low solubility in absolute
alcohol, suggest that compound Z is similar to the chloride salt of
glucopyranosylpyridine reported by Karrer *et al.* [12], and to an ana-
logous acetylated derivative prepared by Fischer & Raske [8]. However,
the sharp melting point of our compound, 159 - 160°, as compared to

Compound X

Compound Y

Compound Z

Scheme 2
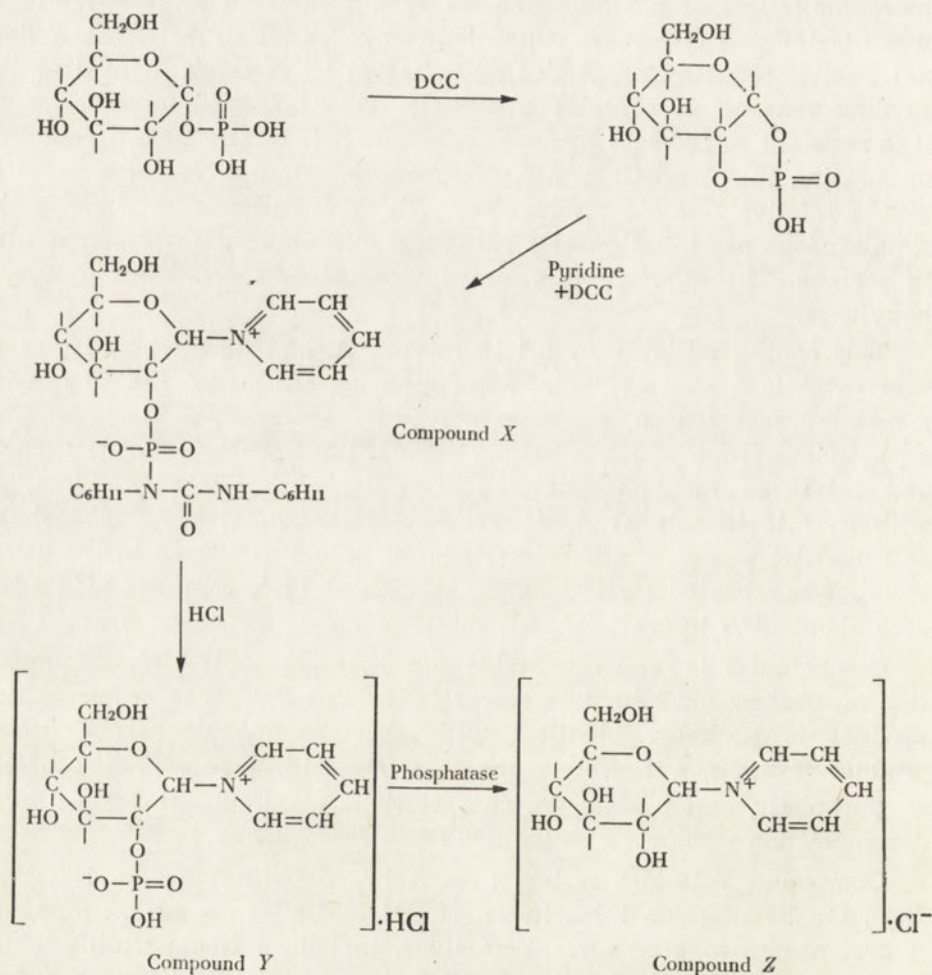
177° reported by Karrer *et al.* [12] for theirs, suggests that the two compounds may be different anomers.

The foregoing derives additional support from an analysis of the hydrochloride salt of Y, corresponding to one mole HCl, one mole pyridine, one mole glucose and one residue of phosphate. The greater stability of Y, as compared to Z, is undoubtedly related to the presence in the former of the phosphate group.

Compounds X, Y and Z may consequently be represented as shown in Scheme 2.

It remains to clarify whether Z is the α or β *anomer* of N-(D)-gluco-pyranosyl)pyridine if, in fact, there is not formed during the initial condensation a mixture of the anomers, of which only one crystallizes from 96⁰/₀ ethanol under the conditions herein employed. The formation of both anomeric pyridine glycosides during the condensation of pyridine with an acetohalogenosugar has been observed by a number of workers.

The foregoing synthesis of a pyridine nucleotide represents an interesting departure from the standard procedures normally applied to the synthesis of pyridine nucleosides and is undoubtedly deserving of further study. There is little doubt that the initial condensation proceeds as a result of the addition of DCC to the cyclic phosphate with the resultant opening of the cyclic phosphate ring and activation of the C_1 carbon, followed by the addition of pyridine. In all probability this procedure may be modified to permit of the synthesis of a variety of other pyridine nucleotide analogues.

EXPERIMENTAL

The course of the reactions described below was followed by means of paper chromatography, ascending, with Whatman no. 1 paper, using the solvents given in Table 1. Localization of the individual products, depending on their properties, was achieved by one or more of the following methods: (a) spraying with aniline phthalate [22]; (b) spraying with the molybdenum reagent for phosphate [3]; (c) use of a "dark" ultraviolet lamp.

In several instances, milligram quantities of reaction products were separated on Whatman no. 3 MM paper, their location determined on a small strip by one of the above methods, and the remainder then eluted and concentrated under vacuum.

Quantitative determinations of reducing sugar were made according to Nelson [19]; phosphate according to Fiske & Subbarow [9]; and protein by spectrophotometry [11].

Establishment of conditions for synthesis of G-1:2-P and compound X: *(1) In pyridine*: 100 mg. of the $K_2 \cdot 2H_2O$ salt of G-1-P was converted to the pyridine salt and then submitted to cyclization under conditions as

described for the preparation of G-1:2-P (see below). The reaction mixture was divided into two portions, one of which was shaken continuously at room temperature, the other left at 0°. At intervals of 2, 24, 48 and 72 hr., 100 µl. aliquots were withdrawn, 100 µl. water added, and the mixtures were extracted three times with ether. The aqueous phases were then chromatographed in solvent A and the reaction products placed in evidence by methods a, b and c. The results are set forth in Table 2 and demonstrate that for synthesis of G-1:2-P the reaction mixture should be left at 0° for 48 hr. (100% yield); whereas for the preparation of compound X, the reaction mixture should be shaken at room temperature for 72 hr. (60 - 100% yield).

(2) In methanol. To 30 mg. of the $K_2 \cdot 2H_2O$ salt of G-1-P, suspended in 10 ml. of 75% methanol, was added 50 mg. DCC, and the reaction mixture shaken at room temperature or at 0°. Aliquots were removed for chromatography as in the preceding section at intervals of 24 and 48 hr. The results are set forth in Table 2 and show that the maximum yield of cyclic products under these conditions is about 80% after 48 hr. with no formation of compound X. The reaction was followed for up to 72 hr. and then examined spectrophotometrically, but there was no evidence for formation of UV-absorbing products, such as typical for compound X.

Preparation of G-1:2-P. 1 g. of the $K_2 \cdot 2H_2O$ salt of G-1-P was suspended in 5 ml. water and converted to the free salt by addition of Dowex 50 (H+). The resin was filtered off, washed with water and the total filtrate neutralized with pyridine and concentrated under vacuum below 30° to 10 ml., followed by the addition of 30 ml. pyridine and 3 g. DCC. The reaction mixture was left at 0° for 48 hr., with shaking from time to time, following which chromatography demonstrated the presence of only one compound with R_F 0.26 in solvent A (spot revealed by method b). To the reaction mixture was added 10 ml. water and, after standing for 30 min., the precipitated cyclohexylurea was filtered off. The filtrate was extracted three times with ether and concentrated under vacuum below 30° to a volume of several milliliters. The very fine turbidity which formed during concentration was filtered off. The filtrate was passed through a column of Dowex 50 (H+) and rapidly neutralized with saturated $Ba(OH)_2$ to pH 8. The solution was concentrated to about 1 ml., freed from cloudiness by centrifugation, and 3 ml. acetone added to the clear supernatant to precipitate an oil. The supernatant was removed by careful pipetting and the oil triturated with ethanol and acetone to formation of a white powder, yield 980 mg. Occasionally the final product contained traces of inorganic phosphate and glucose monophosphate; these were removed by solution of the product in water and addition of 2 volumes of ethanol to give a precipi-

tate which was removed by centrifugation, following which the supernatant was evaporated to dryness under vacuum.

Cyclic phosphate of G-3-P. 10 mg. of G-3-P (free acid, from the Levene collection in the Rockefeller Institute, New York) [17] was reacted with DCC under the conditions employed for the preparation of G-1:2-P with the exception that part of the reaction mixture was left at 0° and part shaken at room temperature (18°). After 48 hr. the main reaction product at 0° was G-1:3-P (R_F 0.26 in solvent *A*), and after 72 hr. at room temperature compound *X* (R_F 0.9 in solvent *A*). See Table 2 for details.

To the reaction mixture at 0° was added 200 μl. water, the resulting precipitate removed by centrifugation, the supernatant extracted three times with ether, and the aqueous phase then concentrated under vacuum and chromatographed on Whatman no. 3 MM paper in solvent *A*. The main spot (R_F 0.3) was eluted, concentrated under vacuum, and in this form employed for further experiments. The compound contained trace amounts of monophosphates formed from the cyclic phosphate during elution and concentration.

Cyclic phosphate of G-4-P. 5 mg. of the pyridine salt of G-4-P (from the Levene collection at the Rockefeller Institute) [24] was treated with DCC as for G-1:2-P, above. Following shaking at 18° for 72 hr., conversion of G-4-P (R_F 0.05) to the cyclic phosphate was complete (R_F 0.26). No formation of compound *X* could be observed. To the reaction mixture was added 100 μl. water, the precipitate centrifuged off and the supernatant extracted three times with ether. Pyridine was then removed on Dowex 50 (H+), the percolate neutralized with NaOH and then concentrated for use in further experiments.

Preparation of G-4:6(3:6)-P. 0.5 g. of the Ba·7H₂O salt of G-6-P was converted to the free acid by passing through Dowex 50(H+), the solution concentrated to a volume of 2.5 ml., and 10 ml. pyridine plus 1.5 g. DCC added. The reaction mixture was shaken for 72 hr. at 18°, following which chromatography exhibited the presence of 10% of starting product, which did not disappear even on prolongation of the reaction time. Addition of 10 ml. water gave a precipitate which was filtered off and the filtrate evaporated to dryness. The residue was dissolved in water and passed through a Dowex 50 (H+) column. The acid percolate was neutralized with Ba(OH)₂ to pH 8 and a slight precipitate filtered off, following which the solution was concentrated to 1 ml. Addition of 2 vol. of ethanol gave a precipitate consisting of barium phosphate, the barium salt of G-6-P and a portion of the cyclic phosphate product. This was centrifuged off, the supernatant again concentrated and 6 vol. ethanol added to precipitate the G-4:6(3:6)-P(1/2Ba), yield 251 mg., which gave only one spot in solvent *A*, and separated into two components in solvent *B* with R_F values of 0.65 and 0.60.

Preparation of G-4:6-P. 1 g. of the $Ba \cdot 7H_2O$ salt of G-6-P was decationized on a Dowex 50 (H^+) column, the percolate concentrated to 5 ml. To this solution was added 10 ml. methanol and 3 g. DCC in 5 ml. methanol. The reaction mixture was shaken continuously for 18 hr. at room temperature, following which chromatography showed the presence of only one compound with R_F 0.26 in solvent A. 20 ml. water was added, the precipitated cyclohexylurea filtered off and the filtrate concentrated to dryness. The oily residue was dissolved in water and the neutral solution passed through Dowex 50 (H^+). The acid percolate was neutralized with $Ba(OH)_2$ to pH 8, a slight turbidity filtered off and the filtrate concentrated to 1 ml. Addition of 1 ml. acetone precipitated an oil, from which the supernatant was pipetted off. Trituration of the oil with ethanol converted it to a white powder, 590 mg., ratio of P to Ba 2:1, and containing $90^0/_0$ or more G-4:6-P and $10^0/_0$ or less G-3:6-P.

Preparation and isolation of compounds X, Y *and* Z. 980 mg. G-1-P $(K_2 \cdot 2H_2O)$ was converted to the pyridine salt and shaken with 3 g. DCC in $75^0/_0$ pyridine at room temperature for 72 hr., following which chromatography in solvent A demonstrated the presence of two products: the cyclic phosphate with R_F 0.26 and compound X with R_F 0.9. To the reaction mixture was added 20 ml. water and after 30 min. the cyclohexylurea precipitate was filtered off and the filtrate brought to dryness under reduced pressure. The resulting residue was washed with several milliliters of water on a fritted glass filter to give 415 mg. of compound X, free of G-1:2-P. The filtrate was freed from G-1:2-P by chromatography on Whatman no. 3 MM paper and a further 413 mg. X was obtained by elution from the paper with methanol. Compound X was then crystallized from methanol to give a product with an ill defined m.p. of 205-212°.

Compound X (380 mg.) was dissolved in 1 ml. 0.1 N-HCl and heated for 5 min. at 100°. The resulting precipitate was isolated by filtration, 150 mg., m.p. 229°, soluble in acetone, insoluble in water, and must therefore be cyclohexylurea. The filtrate was brought to dryness, triturated with ethanol and extracted with ether to remove traces of HCl, then dissolved in hot methanol and precipitated by addition of ether. The resulting product Y (210 mg.) was chromatographically homogeneous in solvent A $(R_F$ 0.05); B (0.12); water-saturated butanol (0.0); ethanol - 1 M-ammonium acetate, 5:2 v/v (0.31); $1^0/_0$ ammonium sulphate - propan- -2-ol, 2:6 v/v (0.37). Spots were revealed on the chromatograms by methods *a*, *b* and *c*. Product Y melted at 160 - 167° (decomp.) and elementary analysis gave: C, $37.64^0/_0$; H, $5.88^0/_0$; N, $3.67^0/_0$. Calculated for HCl·1-(2'-phosphate-D-glucopyranosyl)pyridine; $C_{11}H_{17}O_8NPCl$: C, $37.00^0/_0$; H, $4.80^0/_0$; N, $3.9^0/_0$.

Compound Y (200 mg.) was dissolved in 20 ml. 0.05 M-phosphate buffer, pH 9, 20 mg. Worthington intestinal phosphatase added and the

mixture incubated for 18 hr. Paper chromatography demonstrated the quantitative formation of one product, compound Z, with R_F 0.15 in solvent A, and absorbing in the ultraviolet but containing no phosphate (method b). The incubation mixture was brought to dryness and the residue extracted with anhydrous ethanol. The ethanol extract was concentrated to a small volume from which Z crystallized, m.p. 159 - 160°. Elementary analysis gave (following correction for 1.5% ash): C, 48.23%; H, 5.87%; N, 4.99%; Cl, 11%. Calculated for the chloride salt of 1-(D-glucopyranosyl)pyridine, $C_{11}H_{16}O_5NCl$: C, 47.6%; H, 5.75%; N, 5.04%; Cl, 12.75%.

Acid hydrolysis of glucose cyclic phosphate. Samples of cyclic phosphates were heated in sealed ampoules on a water bath either for 5 min. in 0.1 N-HCl or for 1 hr. in 1 N-HCl. The course of hydrolysis in each case was followed by paper chromatography with solvent B, spots being revealed on the chromatograms by the methods referred to above. Detailed results are presented in Table 3.

Enzymic trials. (1) With brain extract. The brain extract was prepared according to Drummond & Perrott-Yee [7]. Incubation conditions were as follows: 1 mg. substrate in 20 µl. water, 200 µl. brain extract (15 mg. protein per ml. determined according to Kalckar [11]) in 0.02 M-tris buffer, pH 7.5, and 1 µl. 0.1 M-$MgCl_2$ were mixed and incubated at 37° for 30 hr., a crystal of thymol being added to prevent bacterial contamination. Aliquots were removed at predetermined time intervals and chromatographed, using solvents A and B. Spots were revealed by use of methods a and b. Results are set forth in Table 3. The lability of G-1:2-P and G-1:3-P made it impossible to obtain any conclusive results for these compounds.

(2) With prostate monophosphatase [20]. Incubation was carried out in 0.02 M-acetate buffer, pH 5.2, or phosphate buffer, pH 5.35, at 37°. Hydrolysis products were chromatographed in solvent A. Methods a and b were employed for placing in evidence spots due to cyclic phosphates and methods a, b and c for compounds X and Y.

All the cyclic phosphates were resistant even after 4 hr. incubation.

Compound X (Na^+ salt) was fully resistant during 4 hr. incubation. Compound Y (Na^+ salt) was completely dephosphorylated in 2 hr. in acetate buffer to give one product, Z.

SUMMARY

A study has been made of the course of formation of cyclic phosphate esters from D-glucopyranose-1-, 3-, 4- and 6-phosphates, by the reaction of these latter with dicyclohexylcarbodiimide (DCC) in aqueous pyridine. Under these conditions glucose-6-phosphate is converted in high yield to a mixture of glucose-3:6-phosphate and glucose-4:6-phosphate. In methanol as solvent the yield is fully quantitative and 90% or more is glucose-4:6-phosphate. The course of the cyclization reaction is influenced by substitution on the C_1 carbon and this point is discussed in detail. The products of cyclization of glucose-1 and 3-phosphates are, respectively, glucose-1:2-phosphate and glucose-1:3-phosphate. All the cyclic phosphates were submitted to acid and enzymic hydrolysis and the products described.

Glucose-1:2-phosphate and glucose-1:3-phosphate, i.e. the two compounds which include C_1 in the cyclic phosphate rings, react with DCC in pyridine to give N-glycoside derivatives. Starting with glucose-1-phosphate a product, X, was isolated; this was transformed on mild acid hydrolysis to a product, Y, with the release of cyclohexylurea. Product Y, in turn, was enzymically dephosphorylated to yield Z, which was identified as N-(D-glucopyranosyl)pyridine chloride. Hence Y is the $2'$-phosphate of Z and X is the cyclohexylurea derivative of Y. Some of the properties of these compounds are described.

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SYNTEZA, CHEMICZNE I ENZYMATYCZNE WŁAŚCIWOŚCI CYKLICZNYCH
FOSFORANÓW D-GLIKOPIRANOZY ORAZ POCHODNYCH N-(D-GLIKO-
PIRANOZO)PIRYDYNY

Streszczenie

Badano przebieg cyklizacji glikozo-1-, 3-, 4- i 6-fosforanów pod wpływem dwu-cykloheksylokarbodwuimidu (DCC) w 75% pirydynie. W tych warunkach cyklizacja glikozo-6-fosforanu zachodziła z dużą wydajnością a produktem reakcji była mieszanina glikozo-3:6 i 4:6-fosforanów. Gdy reakcję prowadzono w metanolu cyklizacja zachodziła ilościowo a w produkcie było 90% glikozo-4:6-fosforanu. W pracy omówiono wpływ podstawienia węgla C_1 na przebieg cyklizacji glikozo-6-fosforanu. Produkty cyklizacji glikozo-1 i 3-fosforanu identyfikowano jako glikozo-1:2 i 1:3--fosforany. Wszystkie cykliczne fosforany poddano hydrolizie kwasowej i enzymatycznej oraz opisano produkty tych reakcji.

Glikozo-1:2 i 1:3-fosforany, tzn. te cykliczne związki, które w pierścieniu fosforanowym zawierają węgiel C_1 reagują z DCC i pirydyną dając N-glikozydowe pochodne. Wydzielono taki związek, X, otrzymany z glikozo-1-fosforanu i na drodze hydrolizy kwasowej przekształcono go w związek Y z wydzieleniem cykloheksylomocznika. Produkt Y enzymatycznie defosforylowano do związku Z, który zidentyfikowano jako chlorek N-(D-glikopiranozo)pirydyny. Wobec tego produkt Y uznano za 2′-fosforan Z, a produkt X za cykloheksylomocznikową pochodną Y. Podano niektóre właściwości związków X, Y, Z.

B. LIPIŃSKI, A. Z. BUDZYŃSKI, Z. S. LATAŁŁO and E. KOWALSKI

ISOLATION AND CHARACTERIZATION OF COLD-INSOLUBLE FIBRINOGEN COMPLEX FROM BOVINE PLASMA

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This paper is dedicated to the memory of the late Professor
Dr. Bolesław Skarżyński

A cold-insoluble precipitate separated from plasma of endotoxin-treated rabbits [15] as well as from plasma of patients with certain neoplastic or inflammatory diseases has been recently shown to be composed of fibrinogen and of a thrombin-altered fibrin intermediate [7, 16, 13]. It is also known that some of the fibrinogen degradation products [1], formed by proteolysis of native fibrinogen with plasmin, interfere with the fibrinogen - fibrin conversion [12, 5, 8, 17, 1, 10]. Moreover some of the products were found to be incorporated into the fibrin clot [2].

During preparation of highly purified fibrinogen from normal bovine citrate plasma we have always observed a considerable amount of protein fractions which were reversibly precipitated in cold and which contained a high percentage of thrombin-clottable protein.

In the present paper evidence is given that the cold-insoluble fraction isolated from normal plasma is a complex of fibrin with both fibrinogen and some of the fibrinogen degradation products.

MATERIAL AND METHODS

Purified fibrinogen ($98\% \pm 2\%$ clottable) was prepared according to the method of Kekwick *et al.* [6] from bovine citrate plasma (1 part of 3.8% citrate and 9 parts of blood). In this paper the conventional term "fibrinogen" will be used for this preparation. Fibrinogen degradation products were prepared by digestion of fibrinogen with plasmin as described previously [4, 9].

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[1] Abbreviations: FDP, fibrinogen degradation products; CIF, cold-insoluble fraction.

Thrombin was a commercial preparation, Hoffman-La Roche Co. (Switzerland). Ether *pro narcosi* was purchased from Chemical Works Pronit in Pionki (Poland). All other reagents were of analytical purity grade.

NaCl - citrate buffer, pH 6.3, μ 0.45, was composed of 85 parts of 0.45 M-NaCl and 15 parts of 0.075 M-citrate buffer, pH 6.3. 0.05 M-tris buffer, pH 7.4, was prepared in 0.15 M-NaCl.

The clottable protein content in fibrinogen preparations was determined by the method described by Blombäck & Blombäck [3].

Protein concentration was determined by the spectrophotometric method. A sample was dissolved in a solution of 40% urea in 0.2 M-NaOH, and absorbancy read at 280 mμ in Unicam SP-500 spectrophotometer. A standard curve was prepared using dry ash-free bovine fibrinogen.

Viscosity measurements were carried out in Ostwald type viscometer at pH 6.3 and at $25° \pm 0.05°$. Limiting viscosity number was calculated from a plot of reduced specific viscosity, η_{sp}, against protein concentration in g./ml. Kinematic corrections were neglected in this calculation.

Starch-gel electrophoresis was conducted in 0.05 M-formate buffer, pH 3.8, containing 40% urea, at the voltage gradient 7 V/cm. for 20 hr.

RESULTS

Fractionation of bovine citrate plasma in order to obtain purified fibrinogen is schematically presented in Fig. 1. The method was virtually the same as described by Kekwick *et al.* [6], the only difference being that the first ether precipitate was extracted at $25°$ and that no ether was used for precipitation of the cold-insoluble fraction. This fraction usually represented about 50 - 70% of total clottable proteins contained in the plasma. The content of the thrombin-clottable component in CIF varied from 90% to 95% $\pm$ 2%.

CIF dissolved at $25°$ in a small quantity of 0.05 M-phosphate buffer, pH 7.4, in 0.15 M-NaCl, then diluted with the same buffer to 0.3% of protein concentration and cooled down to $4°$, yielded about 15% of a protein in the form of a flocculous precipitate. This precipitate contained 96% $\pm$ 2% of thrombin-clottable protein, and was termed by us cryofibrinogen. The supernatant from cryofibrinogen was shown to contain still a considerable amount of fibrinogen which could be recovered by precipitation after addition of 11% of ether (Fig. 1).

Solubilities of fibrinogen, CIF, and cryofibrinogen were studied as follows: about 2% solutions of these proteins in NaCl - citrate buffer were dialysed against three changes of the same buffer for 48 hr. Series of 3 ml. samples of dialysed fibrinogen, CIF, and cryofibrinogen at various concentrations (from 0.5 to 10 mg./ml.) were prepared in the same buffer at room temperature and then left for 24 hr. at $4°$. Precipitates
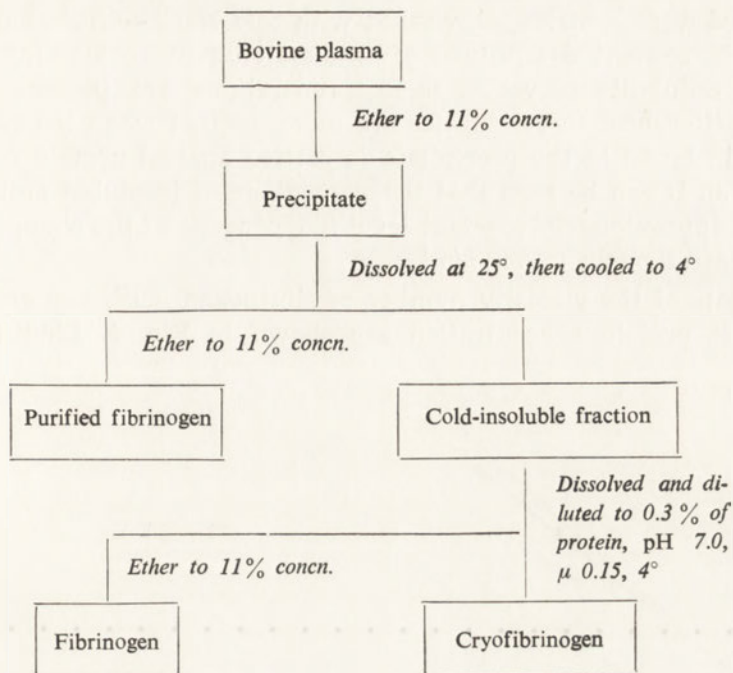
Fig. 1. Scheme of fractionation of bovine citrate plasma according to the method of Kekwick *et al.* [6] for preparation of purified fibrinogen.
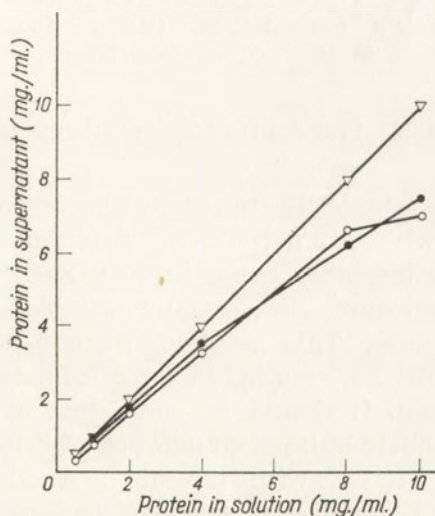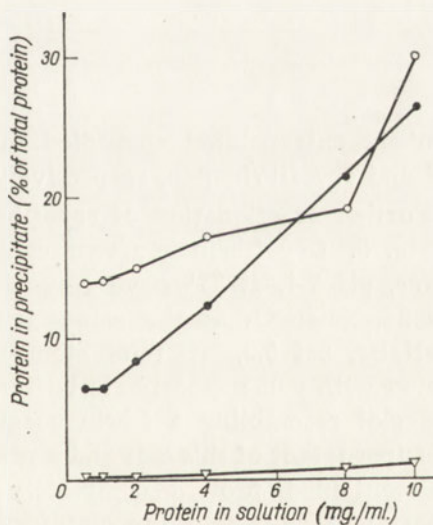
Fig. 2   Fig. 3

Fig. 2. Solubility curves in NaCl - citrate buffer, pH 6.3, μ 0.45 at 4°. (∇), Fibrinogen; (⬤), CIF: (○), cryofibrinogen.

Fig. 3. Plot of percentage of protein found in precipitate against protein concentration. NaCl - citrate buffer, pH 6.3, μ 0.45, 4°. (∇), Fibrinogen; (⬤), CIF; (○), cryofibrinogen.

formed were centrifuged, washed with cold NaCl - citrate buffer and the protein content determined after dissolving in an alkaline solution of urea. Solubility curves of these preparations are presented in Fig. 2. The differences in solubilities are more distinct when the percentage of protein found in the precipitate is plotted against protein concentration (Fig. 3). It can be seen that the percentage of insoluble protein remains at an approximately constant level independent of the changes in protein concentration up to 8 mg./ml.

Plots of the viscosity number of fibrinogen, CIF, and cryofibrinogen against protein concentration are shown in Fig. 4. Limiting viscosity
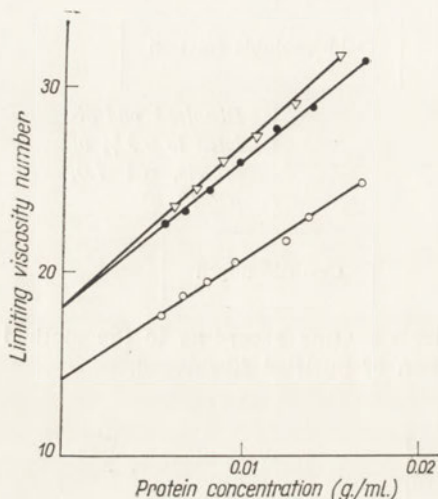
Fig. 4. Viscosity numbers as a function of protein concentration. (∇), Fibrinogen; ($\bullet$), CIF; ($\bigcirc$), cryofibrinogen.

numbers extrapolated amounted to 18.3, 17.8 and 14.3 for fibrinogen, CIF and cryofibrinogen, respectively.

Further fractionation of cryofibrinogen is illustrated by the scheme in Fig. 5. Cryofibrinogen extracted with 0.15 M-NaCl in a phosphate buffer, pH 7.4, at 37° gave a residue insoluble either in 0.3 M-NaCl or in 0.2 M-Na_2HPO_4 at the same temperature, but readily soluble in 2 M-NaBr, pH 5.2, at room temperature. This solution when 5-fold diluted with 0.05 M-phosphate buffer, pH 6.5, resulted in rapid formation of a clot resembling a fibrin coagulum. It should be noted that both the supernatant of this clot and a phosphate buffer extract of cryofibrinogen contained protein completely clottable with thrombin. Another important fact is that the clot formed from cryofibrinogen was more opaque and friable than the clot from fibrinogen.

The clots were thoroughly washed with 0.05 M-phosphate buffer, pH 6.5, dissolved in 40% urea, pH 3.8, and subjected to starch-gel electrophoresis in the same medium. It may be seen from the electrophorogram presented in Fig. 6 that the clot obtained from cryofibrinogen is com-

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                        ┌─────────────────────┐
                        │   Cryofibrinogen    │
                        └─────────────────────┘
                                  │  Extraction with 0.15 M-NaCl,
                                  │  pH 7.4, 37°
                ┌─────────────────┴─────────────────┐
      ┌───────────────────┐              ┌───────────────────────┐
      │    Supernatant     │              │   Insoluble residue   │
      └───────────────────┘              └───────────────────────┘
                │  Thrombin                         │  2 M-NaBr, pH 5.2
        ┌───────────────┐                   ┌───────────────┐
        │     Clot      │                   │   Solution    │
        └───────────────┘                   └───────────────┘
                                                    │  5 vol. of 0.05 M-
                                                    │  phosphate buffer,
                                                    │  pH 6.5
                                             ┌───────────────┐
                                             │     Clot      │
                                             └───────────────┘
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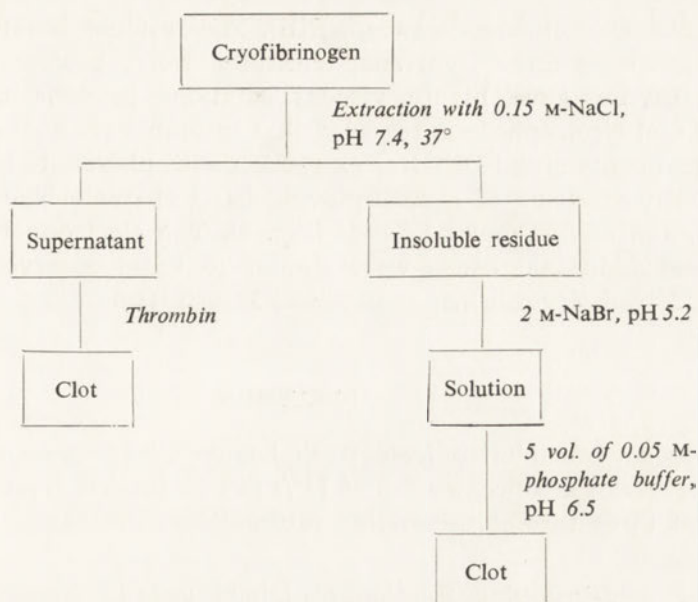
Fig. 5. Scheme of fractionation of cryofibrinogen isolated from bovine plasma.

posed of at least three fractions, only one corresponding to the fibrinogen and/or fibrin band. Electrophoretic mobilities of the fast-moving bands of cryofibrinogen are very similar to some of those present in FDP (Fig. 7). This implied that the clot obtained from cryofibrinogen is composed not only of fibrinogen and fibrin but also of fragments formed by the action of plasmin on fibrinogen.

Consequently attempts were made to restore the complex of fibrin with FDP in experiments *in vitro*. A 2% fibrinogen solution in tris buffer, pH 7.4, was mixed with FDP at a protein ratio 10:1 and a thrombin solution, which did not clot such a mixture within 1 hr., was added. The mixture was then diluted with the same buffer to a final protein concentration of 0.3%, incubated for 3 min. at 37° and then left for 24 hr. at 4°. The flocculous precipitate formed was then treated similarly as cryofibrinogen according to the scheme presented in Fig. 5. The clot obtained after dilution was dissolved in formate buffer, pH 3.8, containing 40% of urea and subjected to starch-gel electrophoresis. The photograph of the gel presented in Fig. 7d shows that the clot isolated from a mixture of fibrinogen, FDP, and thrombin possesses besides the main fraction additional fast-moving bands absent from the clot obtained from fibrinogen (see Fig. 6b).

In order to create conditions analogous to those prevailing *in vivo*, to the system containing fibrinogen and FDP, calcium acetate to a final concentration of 2 mM and fresh bovine plasma were added. The ratio of fibrinogen, FDP and plasma proteins was 10:1:0.2. The control mixture

contained all components except FDP. The residues isolated from cryo-fibrinogens obtained from such mixtures were, however, insoluble in 2 M-NaBr, but gave highly viscous solutions in 40% urea at pH 3.8. Starch-gel electrophoresis revealed that insoluble residues obtained from a system containing FDP after extraction with phosphate buffer and with 2 M-NaBr, consisted of a slow-moving band characteristic for stabilized fibrin, and of fast-moving bands (Fig. 8). The electrophoretic mobilities of these additional bands were similar to those observed in the clot isolated from cryofibrinogen obtained from plasma during ether fractionation.

DISCUSSION

Proteins precipitated from fresh bovine citrate plasma by means of ethyl ether at a concentration of 11% can be further fractionated to give at least three thrombin-clottable subfractions: fibrinogen, CIF and cryo-fibrinogen.

The cold-insoluble fraction contained usually from 90 to 95% of clottable protein and in this respect resembles fraction I-3 described by Blombäck & Blombäck [3]. The term cryofibrinogen was applied for thrombin-clottable protein precipitating from plasma of certain patients at 4° [14, 11]. It seemed justified to apply this term also to the fraction precipitating in the cold during preparation of fibrinogen (Fig. 1).

Examination of solubilities of CIF and cryofibrinogen revealed that in diluted solutions a dissociation took place resulting in a soluble and an insoluble fraction, what was not observed in the case of fibrinogen (Figs. 2 and 3).

Fractionation of cryofibrinogen yielded a soluble thrombin-clottable protein and an insoluble residue which could be transformed into a clot without addition of thrombin (Fig. 5). This indicates that cryofibrinogen represents a complex of fibrinogen and fibrin. This fact is in accordance with the data of other authors [7, 15, 16, 13] who, however, studied cryofibrinogen isolated from pathological plasma.

Electrophorograms of clots obtained from cryofibrinogen preparations (Figs. 6c, 7a and 8c) show, in addition to the band characteristic for fibrinogen and/or fibrin, at least two fast-moving components. It is noteworthy that the components of such a clot are very strongly bound together, because it is impossible to separate them either by extraction with 0.2 M-Na$_2$HPO$_4$ or by precipitation from NaBr solution. The electrophoretic mobilities of the fast-moving bands of the cryofibrinogen clot were similar to some fractions of fibrinogen degradation products obtained by digestion of fibrinogen with plasmin (Fig. 7b and c). All this indicates that the clot isolated from cryofibrinogen may represent a complex of fibrinogen, fibrin and certain components of FDP.
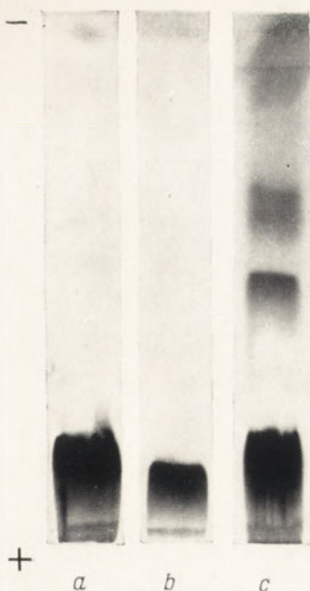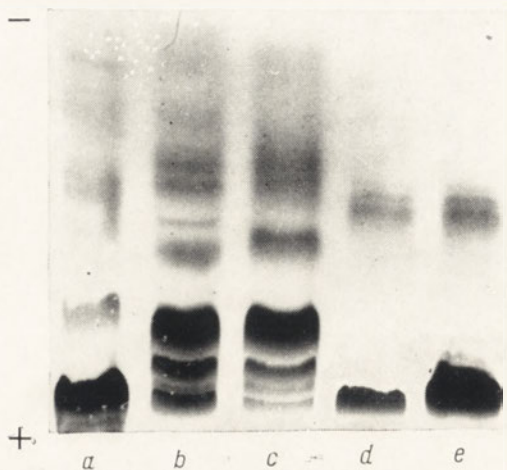
Fig. 6. Starch-gel electrophoretic patterns in 0.05 M-formate buffer, pH 3.8, containing 40% urea. (a), Fibrinogen; (b) fibrin; (c), clot obtained from cryofibrinogen.

Fig. 7. Starch-gel electrophoretic patterns in 0.05 M-formate buffer, pH 3.8, containing 40% urea. (a), Clot obtained from cryofibrinogen isolated from plasma; (b), and (c), fibrinogen degradation products formed due to the action of plasmin on fibrinogen, after 20 and 30 min. of digestion, resp.; (d), clot obtained from cryofibrinogen isolated after incubation from a mixture of fibrinogen, FDP and thrombin; (e), double amount of a sample as in (d).
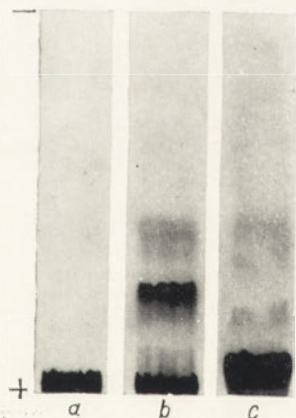
Fig. 8. Starch-gel electrophoretic patterns in 0.05 M-formate buffer, pH 3.8, containing 40% urea. (a), Insoluble residue isolated from cryofibrinogen obtained from fibrinogen incubated with plasma and Ca^{2+}; (b), insoluble residue isolated from cryofibrinogen obtained from a mixture of fibrinogen and FDP incubated in the presence of plasma and Ca^{2+}; (c), clot obtained from cryofibrinogen isolated from plasma.

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It was possible to show that a similar complex is formed during incubation of an excess of fibrinogen with FDP and with a small amount of plasma as a source of thrombin and fibrin-stabilizing factor in the presence of Ca^{2+} ions. The insoluble residue isolated from cryofibrinogen precipitating in cold from the incubation mixture was shown to be composed of fibrin stabilized under such conditions and of additional fractions (Fig. 8b). No protein fragments were incorporated in the insoluble residue isolated from a control mixture containing fibrinogen, plasma, Ca^{2+} ions but not FDP (Fig. 8a). It must be mentioned that clots obtained from a mixture of fibrinogen, FDP and thrombin in the absence of plasma and Ca^{2+} ions formed complexes similar to those obtained in the presence of plasma and Ca^{2+} ions but did not possess the slower moving major fraction (Fig. 7d).

It follows from the results of these experiments that certain fragments of the fibrinogen molecule degraded with plasmin are capable to co-polymerize with fibrin monomers. It is supposed that these fragments must possess donor and/or acceptor groups of hydrogen bonds which fit to the corresponding groups in fibrin monomers or intermediate polymers. The co-polymers described in the present paper might be regarded as "defective clots" formed in whole blood plasma, as reported by Bang et al. [2].

Formation of complexes of fibrin with certain fibrinogen degradation products results from the action of two plasma enzymes on fibrinogen, namely plasmin and thrombin. We are fully aware that this action may take place after withdrawal of blood; nevertheless it is very probable that a similar process occurs inside the blood vessels.

SUMMARY

In the course of preparation of bovine fibrinogen by a modified Kekwick method, two protein preparations were obtained which clotted under the action of thrombin. They differed, however, from fibrinogen by insolubility in the cold.

It was shown that these preparations are complexes composed of fibrinogen, fibrin and fibrinogen degradation products.

It is suggested that similar complexes may be formed *in vivo* both in physiological and pathological conditions.

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IZOLOWANIE I CHARAKTERYSTYKA NIEROZPUSZCZALNEGO NA ZIMNO
KOMPLEKSU FIBRYNOGENU Z OSOCZA WOŁOWEGO

Streszczenie

Podczas izolowania fibrynogenu wołowego zmodyfikowaną metodą Kekwicka, otrzymano dwa preparaty białkowe, które krzepną pod wpływem trombiny, ale różnią się od fibrynogenu nierozpuszczalnością na zimno.

Wykazano, że te preparaty stanowią kompleksy składające się z fibrynogenu, fibryny i produktów proteolitycznej degradacji fibrynogenu. Sugeruje się, że podobne kompleksy mogą powstać również *in vivo* w warunkach fizjologicznych i patologicznych.

RECENZJE KSIĄŻEK

PROGRESS IN BIOPHYSICS AND MOLECULAR BIOLOGY (J. A. V. Butler
and H. E. Huxley, eds.), Vol. XIII, Pergamon Press, Oxford, England, 1963;
str. VII + 328, cena 90 s.

In taking up this volume, the reader will undoubtedly be struck by the fact
that the series title has been modified from PROGRESS IN BIOPHYSICS AND
BIOPHYSICAL CHEMISTRY. While one may well argue as to the merits of such
a change in title, it must ultimately be regarded as a sign of the times. The
general tendency throughout all fields of biology today is to extend the level
of investigation to that of molecules and molecular processes. The editors point
out in the Introduction that the change in title is not meant to imply a change
in policy and that "it has always been difficult to draw any clear lines of demar-
cation between physiology, biophysics and the related parts of biochemistry".
One cannot but agree with this and reflect, as did Shakespeare, on "What's
in a name".

While the subject matter of this volume is extremely varied, even a cursory
perusal demonstrates that the authors of the various chapters have succeeded
in presenting reasonably up-to-date outlines of their specialty in such a manner
as to be readily followed by a non-specialist in that particular field. The publishers
are also to be complimented on having put out this volume in a form which
is very well set and readable while each chapter is subdivided into sections and
sub-sections which are listed in the title page. It is to be regretted, however, that
the previous policy of frequently including the titles of reference papers, has been
entirely discontinued. In a review article the reader may frequently more easily
assess the value of a given reference from the title.

A good deal of the data on the effects of ionizing radiations on biological
systems has been reasonably well interpreted on the assumptions that they were
due either to the reactions of radiation-produced water radicals or the production
of an ionization directly in some key cellular constituent. It was likewise usually
assumed that these events were entirely random in nature. There is, however,
considerable evidence that in a number of biological systems this is not the case,
and that there may be some preferential localization of the effects of radiation.
The author of Chapter 1, L. G. Augenstein, has examined this latter data to produce
a broad review of what is known about the transfer of energy from ionizing
radiations to biological material; an evaluation of the energy requirements for
enzyme inactivation as well as the molecular modifications accompanying such
inactivation; a discussion of the mechanisms of excitation energy transfer; and
an examination of the possible processes which might explain specific, as compared
to random, radiation effects. There is a useful section on the effect of solute
concentration on radiochemical yields. It is to be regretted that there is
almost no mention of the recent developments on the hydrated electron,
in particular the very fine work of Hart & Boag and their collaborators;
while only brief reference is made to Platzman's valuable contribution on the

subject of super excited states of molecules which are formed in the field of an ionizing particle. In the section on possible exciton migration in water aggregates, mention should at least have been made of the newer theoretical contributions of Scherega & Nemethy to the problem of the structure of water. Nonetheless this review is undoubtedly a valuable contribution in a difficult and complex field of work, and should be of interest not only to those engaged in radiobiological research, but also to photochemists, radiation chemists and others.

The review by G. Scholes of the radiation chemistry of aqueous solutions of nucleic acids and nucleoproteins in Chapter 2 is particularly timely and follows logically on Chapter 1. For a number of years now numerous research workers have been struggling to bring some order into a field which has posed many difficulties in interpretation. While the radiation chemistry of nucleic acids is of wide interest to radiation chemists, one cannot overlook the fact that what we want to learn from a biological standpoint are the primary chemical modifications resulting from the action of ionizing radiations on the various components, including quantitative data on radiochemical yields. Such information is essential for any interpretation of mutagenesis and other biological effects of radiation. In the past 3 - 4 years some small but definite progress has been made in this direction e.g. the identification of the hydroperoxide products formed on irradiation of thymine in the presence of oxygen; and the relevant information is concisely reviewed here. Particularly interesting is the section devoted to recent work demonstrating that in nucleoproteins the initial action of radiation appears to be largely on the protein component, although the biological significance of this phenomenon remains to be evaluated.

The passage of an X-ray beam through a medium is accompanied by diffraction at small angles from inhomogeneities in the electron density at colloidal orders of magnitude. Such inhomogeneities exist, for example, in a "solution" of proteins or nucleic acids. Theoretical considerations show how to calculate, from the degree and extent of small-angle scattering of an X-ray beam, useful information about the size, shape and mass of the scattering particles. In a chapter which is much more than just a review article (Chapter 3), O. Kratky provides the interested reader with an excellent account of the theoretical background of the techniques employed as well as an account of their applications to the measurement of some of the basic physical size data for proteins, nucleic acids and nucleoproteins (including viruses), as well as the nature and degree of hydration. Numerous critical comparisons are made with results obtained by other methods. The author is to be commended on the lucid and readable manner in which he has presented a subject which should be of interest to all those engaged in research on biological macromolecules and their properties, be they biologist or physical chemist.

Marianne Grunberg-Manago, co-discoverer with Ochoa of polynucleotide phosphorylase, and an active worker in this field, presents in Chapter 4 a review of the various enzyme systems associated with the synthesis of nucleic acids, their mode of action and some of the principal properties of the polymers synthesized. It is perhaps not surprising that almost two thirds of this chapter are devoted to the problem of ribonucleic acid synthesis, since this problem is much less clear at the moment than that of DNA synthesis. The table of contents of this chapter is particularly detailed so that it enables the reader at a glance to look up any particular point of interest in the text.

In Chapter 5 on "Ultrastructure and kinetic aspects of solute translocation in the stems of plants", R. D. Preston has made a novel departure from the usual type of review article in volumes of this kind. The problem of phloem

transport in stems of flowering plants was discussed at a symposium organized by the British Biophysical Society at Nottingham in July, 1961. In this chapter Preston provides a brief résumé of the views in this domain presented by the various participants, together with a critical discussion of his own as to the present status of the subject, and the problems that remain to be resolved.

The mechanism of the elementary processes by means of which chemical energy is converted into mechanical work during muscle contraction is one of the oldest, and still most interesting, problems in biochemistry. At the molecular level it is now generally established that the contractile system embraces two proteins, myosin and actin, which interact with one another and with ATP so as to hydrolyse the latter and release energy which is converted into work by the actinomyosin system, in a manner which is as yet not elucidated. The primary source of energy in this system is ATP, while the secondary source is phosphocreatine which is coupled to ATP *via* creatine phosphokinase. Chapter 6, by F. D. Carlson, comprises a critical evaluation of *in vivo* investigations of the past 20 years on the mechanism of muscular contraction at the molecular level. While the article presupposes some knowledge on the part of the reader of the chemical and structural properties of the contractile proteins, and of the energetics of muscle contraction, this is to some extent made up for by Section II, which provides a general historical review of the classical concept of the mechanochemical system of the muscle. Particular emphasis is placed on those aspects of muscular contraction which require further study, such as the role of free and bound nucleotides, the chemical basis of the heat production properties of muscle, and the "forging" of more links between the cellular and molecular events. The organized and interesting manner in which the subject matter is outlined should certainly stimulate many to new efforts in this important yield.

David Shugar

DEOXYRIBONUCLEIC ACID - STRUCTURE, SYNTHESIS and FUNCTION - Proceedings of the 11th Annual Reunion of the Société de Chimie Physique, June 1961. Symposium Publications Division, Pergamon Press, Oxford, England, 1962, str. IX + 235, cena 60 s.

This volume reproduces the communications presented at the 11th annual meeting of the French Société de Chimie Physique, devoted to the chemistry, physical chemistry and biological functions of deoxyribonucleic acid, which took place in the French Alps in June 1961. The volume was published at the end of 1962, and one may well question the utility of a review at this late date. For this reason no attempt will be made to assess in detail the many and varied communications contained therein. But there are several points of interest in it which even now are worth commenting upon.

The Conference itself, while international in scope, was limited to about 100 participants, including only those engaged in active work in this field. It was consequently truly a working conference and this is amply reflected in the extensive discussions appended to most of the communications. There is little doubt but that large general Congresses are now outdated and that specialized small Conferences such as this are the order of the day, and the ones most likely to provide a medium for fruitful and detailed discussions of a particular sphere of research.

The organization of such a conference by a Physical Chemistry Society underlines the significance which chemists and physicists attach to fundamental problems

in biology. As the President of the Society, Dr. Raymond Latarjet, states in the Introduction to this volume: "The goal of the Society was to encourage discussions among physicists, chemists and biologists interested in DNA". A perusal of the contents of the volume will suffice to convince the reader that "this goal has been attained".

Of the 32 communications contained in this volume, 5 are devoted to the structure of DNA, two of them quite extensive by Sadron and Wilkins. No less than 6 communications are devoted in whole or in part to the photochemistry and radiation chemistry of DNA and bacteriophages; and these are supplemented by an extensive article by Kasha and his collaborators on the excited states of nitrogen base-pairs and polynucleotides, which is also of considerable theoretical importance in relation to DNA structure. The increased interest in the photochemistry of DNA is quite understandable in view of the recent progress in this field, including especially the identification of specific photoproducts such as thymine dimers which have been related to modifications in biological activity. Chemical mutagenesis forms the subject of 5 papers. There are also a number of communications on DNA biosynthesis, the role of DNA in RNA biosynthesis, and the properties of transforming DNA. Stahl's article on "A Chain Model of Chromosomes" and that of Jacob, Gros & Monod on "La Regulation et la Mode d'Action des Genes" provide thoughtful treatments of the structure and mode of action of genetic elements.

Particular reference should be made to the extensive communication of A. and B. Pullman on the applications of quantum chemistry to the calculation of various properties of purine and pyrimidine bases such as resonance energies, distribution of electronic charge in the aromatic rings, basicity of the ring nitrogens, the relative reactivity of various functional groups, base-pair interactions, etc. There is no question as to the utility of applying quantum mechanical methods to the evaluation of the chemical and physico-chemical properties of proteins and nucleic acids and the many contributions of the Pullmans in this domain are well known. Indeed, the rapid progress at present being made in this field, especially with reference to such problems as the structure of polypeptides and polynucleotides, energy transfer in polymer chains, etc. is most impressive and a very useful guide to the experimentalist. Unfortunately the language of quantum mechanics is not simple to the uninitiated, nor is it clear to most biologists. Furthermore it must be recognized that many quantum chemists do not make any serious attempt to clarify the nature of the calculations involved in such procedures; it would not be an exaggeration to state that some apparently take pride in making what they consider impressive predictions on the basis of theoretical calculations which are a complete mystification to the biologist. To many, therefore, it will be like a breath of fresh air to read the short Commentary on the Scheme of the Semi-Empirical Molecular Orbital Calculation ("Commentary to Pullman's Communication") by Michael Kasha, who presents a very concise outline of the specific steps involved in molecular orbital calculations, the assumptions on which they are based and, what is most important, an assessment of the validity and limitations of the final calculated data. It is to be hoped that quantum chemists venturing into the field of biology will not only read this short commentary of Kasha's, but will also appreciate the fact that it was presented at the request of the biologists present at this conference, and for their enlightenment.

The organizers of the Conference and the participants are to be commended on the promptness with which the proceedings appeared in print. The conference itself was held in the latter part of June, 1961 and the full communications,

together with the appended discussions, appeared in the November and December issues of the official organ of the Society, the Journale de Chimie Physique. Since this journal is widely distributed, one may well ask whether reprinting of these two numbers in special book form was justified. This is a question which frequently poses itself and, in these days when rapid publication and reduced costs are of prime importance, obviously calls for some reflection. In this particular instance, one convincing argument may be adduced against the *reprinting* of the proceedings. The original texts contain a multitude of grammatical and spelling errors, most of them fortunately not too serious (for those who know English well), and due largely to the rapid setting of English type by French typesetters. One might have expected that when a large English publishing firm undertook the reprinting of these proceedings, they would at least have eliminated the numerous errors. Unfortunately this is not so. The book contains all of the spelling and typographical errors of the original text! This should however not seriously detract from the usefulness of a volume which gives a well rounded out picture of the structure and function of DNA up to the middle of 1961.

David Shugar

H. McIlwain: CHEMICAL EXPLORATION OF THE BRAIN, A Study of Cerebral Excitability and Ion Movement. Elsevier Publishing Co., Amsterdam, London, New York, 1963; str. 207.

Monografia ta jest próbą przedstawienia w zwartej formie wyników współczesnych badań biochemicznych nad pobudliwością tkanki nerwowej i zachodzącymi w niej przemieszczeniami jonów. McIlwain jest autorem techniki badania wpływu drażnienia prądem elektrycznym tkanki na zachodzące w niej procesy biochemiczne. McIlwain jest Kierownikiem Zakładu Biochemii w Instytucie Psychiatrii w Londynie. Problemy przedstawiane w książce są odbiciem tematyki prac biochemicznych prowadzonych przez Autora i współpracowników od kilku lub kilkunastu lat. Książka składa się z sześciu rozdziałów, zaopatrzonych w wykazy cytowanej literatury, oraz indeksu rzeczowego. Pierwsze dwa rozdziały dotyczą wpływu drażnienia prądem elektrycznym na oddychanie izolowanej tkanki mózgowej. Podano opisy doświadczeń prowadzonych w celu zidentyfikowania substratów i koenzymów odpowiedzialnych za wystąpienie charakterystycznego wzrostu natężenia oddychania. Zwrócono uwagę na rolę związków zawierających kwas N-acetyloneuraminowy (sjalomukoproteidy, sjalomukopolisacharydy, a głównie gangliozydy) w przywracaniu utraconej w pewnych warunkach zdolności reagowania tkanki na bodziec elektryczny. Antagonistycznie w stosunku do gangliozydów działają białka zasadowe. O mechanizmie działania tych endogennych stymulatorów i inhibitorów reakcji oddechowej tkanki na bodziec elektryczny oraz o mechanizmie działania niektórych leków jest mowa również w dalszych rozdziałach. Rozdział trzeci dotyczy zawartości wody, sodu, potasu i chlorków w tkance mózgowej i przedstawia wyniki badań nad szybkością przenikania tych składników do komórek mózgu oraz nad zmianami ich zawartości w zależności od warunków inkubowania tkanki. W dwu dalszych rozdziałach przedstawione są badania nad frakcją mikrosomalną komórek mózgu i jej podfrakcjami, rozmieszczeniem w niej enzymów i jej morfologią. W rozdziale tym Autor omawia budowę chemiczną błon struktur komórkowych oraz wprowadza szereg dłuższych dygresji o własnościach fizycznych komponent lipidowych tych błon. Szczególnie interesujący jest ostatni ustęp rozdziału piątego oraz rozdział szósty. Autor omawia w nich mechanizmy

biernego przenikania jonów przez błony komórek nerwowych oraz postulowane
na podstawie badań własnych i badań Skou'a prowadzonych w latach 1961 i 1962
mechanizmy czynnego transportu jonów. Pierwsze sformułowania tych koncepcji
ukazały się w drukowanych materiałach Sympozjum Brytyjskiego Towarzystwa
Biofizycznego („Enzyme Mechanisms in Active Transport Systems", Londyn,
10.III.1962). W książce Autor omawia szerzej te zagadnienia. Jednocześnie McIlwain
poddaje krytyce wyniki badań nad rolą cyklu kwasu fosfatydowego jako mecha-
nizmu transportu sodu, lansowanego w latach ostatnich przez L. E. Hokina
i M. R. Hokin, oraz wyniki prac Healda postulujące udział fosfoproteidów w czyn-
nym transporcie jonów.

Książka napisana jest w sposób bardzo zwięzły i dlatego tok rozumowania
Autora trzeba śledzić z dużą koncentracją uwagi. Książkę tę należy polecić bio-
chemikom oraz lekarzom neurologom posiadającym przygotowanie biochemiczne.

Tadeusz Chojnacki

BIOCHEMICAL PROBLEMS OF LIPIDS. Proceedings of the Seventh International
Conference on Biochemistry of Lipids, held in Birmingham, July 1962 (A. C. F r a -
z e r, ed.). Elsevier Publishing Company, Amsterdam, London, New York, 1963;
str. 474, cena 130s.

Omawiany tom jest pierwszym z serii zatytułowanej „BBA Library". Wydawcy
Biochimica et Biophysica Acta zamierzają publikować monografie odnoszące się do
ważnych i obejmujących szeroki zakres tematów biochemii i biofizyki. Następne
trzy tomy tej serii będą poświęcone porfirynom, fosfolipidom i glikoproteidom.

Konferencja lipidowa w Birmingham była poświęcona głównie resorpcji tłu-
szczów z jelita. W sprawozdaniu znajduje się 56 prac, z czego 31 pochodzi ze Sta-
nów Zjednoczonych i Anglii. Sprawozdanie jest wydane starannie, zawiera indeksy
członków konferencji, autorów i indeks rzeczowy. Przy każdej pracy zamieszczono
wypowiedzi dyskutantów, niekiedy dość obszerne.

Całość została podzielona na 6 rozdziałów, z których cztery pierwsze obejmują
następujące tematy: 1, resorpcja tłuszczów z jelita; 2, zagadnienia strukturalne
komórek jelitowych w związku z resorpcją tłuszczów; 3, enzymologia przemian
lipidowych w jelitach: 4, chylomikrony i lipoproteidy. Rozdziały 5 i 6 obejmują
prace o szerszym zakresie tematyki przemiany lipidowej. Wśród autorów prac
znajduje się wielu znanych badaczy lipidów. Polscy autorzy (Dominas, Doroszew-
ski, Niemierko, Włodawer, Wojtczak, Chojnacki) przygotowali trzy referaty z róż-
nych dziedzin badań nad lipidami.

Strona edytorska, wzorowana na Biochimica et Biophysica Acta, jest na wy-
sokim poziomie, zdjęcia mikroskopowe, wykresy i tabele wyraźne i jasno skon-
struowane. Jedyną ujemną stroną sprawozdania jest nieco długi okres czasu, jaki
upłynął od konferencji do chwili ukazania się sprawozdania w druku (1 rok).

Tadeusz Korzybski

B. H o l m s t e d t and G. L i l j e s t r a n d. READINGS IN PHARMACOLOGY.
Pergamon Press. Oxford, London, New York, Paris, 1963; str. 395, rys. 96; cena 50s.

Rozwój nauk doświadczalnych jest w naszych czasach tak żywy, zdobycze są
tak wielkie, że często nie docenia się działalności tych ludzi, którzy fundamenty
tych nauk budowali. A przecież ta praca, która tworzyła pierwsze zręby gmachu

nauki, była szczególnie trudna; musiała być wykonana przez pojedynczych ludzi, bardzo często wbrew ogólnej opinii badaczy; badania zespołowe, starannie zaplanowane, nie leżą przecież u podłoża powstającej nauki, lecz są wyrazem jej zorganizowania i daleko już posuniętego rozwoju.

Badacz wykreślający nowe drogi musi, jeżeli ma uzyskać uznanie dla swoich poglądów, uzasadnić je bardzo starannie, przedstawić dostateczną liczbę dowodów i zwalczyć przeszkody, które stoją na drodze pomysłom i wynalazkom „początkującym nowe koło rzeczywistości". Dlatego zasługują one na to, by nie tylko były wymieniane z tytułu i roku wydania, ale także czytane i brane jako wzór.

Recenzowana książka przedstawia wypisy z prac budujących dzisiejszy gmach farmakologii. Rozdział pierwszy ujmuje rozwój farmakologii od egipskich czasów aż do końca XVIII wieku; drugi jest poświęcony początkom farmakologii doświadczalnej; dalsze rozdziały to anaestica, hypnotica i teoria narkozy; lokalne anaestica; farmakologia autonomicznego układu nerwowego; psychofarmakologia. Następne rozdziały dotyczą postępów technicznych; chemoterapii; witamin i hormonów; toksykologii i innych. Wyjątki z prac są poprzedzone odpowiednim wprowadzeniem dotyczącym istoty zagadnienia oraz danymi o osobie badacza. Czytelnik uzyskuje pogląd na całość historycznego rozwoju farmakologii, z tym jednak ograniczeniem, że Autorzy pomijają tych badaczy, których prace były ujęte w dawniejszych wydawnictwach tego rodzaju. Dlatego przy omawianiu witamin i hormonów brak prac Hopkinsa i Bantinga, chociaż dla całości obrazu umieszczenie nawet krótkich wyjątków byłoby bardzo celowe. Całość jest ujęta zajmująco, ozdobiona licznymi portretami i rysunkami, papier i druk znakomite. Biochemicy mogą zazdrościć farmakologom, że ich nauka otrzymała taki dar.

Włodzimierz Mozołowski

A. F u r s t, CHEMISTRY OF CHELATION IN CANCER., Charles C Thomas Publ., Springfield, Illinois. 1963; str. 143 + XIV, cena $ 7.50.

Książka A. Fursta jest jednym z wydawnictw serii „American lectures in living chemistry", publikowanym pod ogólną redakcją I. Newton Kugelmass'a. Dotyczy ona nie tyle ugruntowanych i znanych faktów z dziedziny biochemii nowotworów, ile koncepcji, która dąży do sprowadzenia różnorodnych i często sprzecznych danych o kancerogenezie i kanceroterapii do jednego wspólnego zjawiska: do tworzenia wiązań chelatowych z metalami.

W pierwszej części książki, po krótkim omówieniu znaczenia terminu „cancer", oraz teorii tworzenia chelatów, Autor przedstawia związek pomiędzy pierwiastkami śladowymi a nowotworami, a następnie dokonuje krótkiego przeglądu związków kancerogennych i związków stosowanych w chemoterapii nowotworów. Przegląd ten, udokumentowany ponad pół tysiącem odsyłaczy do literatury, ma na celu wykazanie, że wszystkie omawiane związki są albo związkami chelatującymi, albo też, pod wpływem przemian zachodzących w żywym ustroju, przechodzą w związki o takich własnościach. Dowody na to nie zawsze są zupełnie przekonywające, ale próba znalezienia wspólnej cechy dla tak różnorodnych związków jakimi jest cała kolekcja związków rakotwórczych jest na pewno godna uwagi, robi duże wrażenie i może być bodźcem do podjęcia wielu doświadczeń dążących do potwierdzenia lub obalenia przedstawianej hipotezy.

W dalszym rozdziale Autor przedstawia biologiczne funkcje pierwiastków śladowych jako tych, które tworzą połączenia chelatowe z witaminami, białkami,

enzymami i kwasami nukleinowymi. Ostatni rozdział jest poświęcony spekulacjom
i wnioskom wynikającym z przedstawionej hipotezy tworzenia związków chelato-
wych jako podstawy powstawania i wzrostu nowotworów. Jakkolwiek wiele sugestii
i analogii wysuniętych w tym rozdziale jest bardzo odległych (np. sugestia, że wi-
rusy wywołujące nowotwory mogą działać dzięki temu, że wprowadzają do tkanki
i do komórki „nienormalny" metal w nich zawarty), to jednak jest to rozdział
napisany bardzo ciekawie. Szczególna wartość tego rozdziału, jak i całej książki
polega na tym, że postawiono w niej szereg teoretycznych hipotez i przypuszczeń,
które należy doświadczalnie sprawdzić. Posługując się teorią chelatową, Autor sta-
wia nawet propozycje nowych związków chemicznych, które powinny mieć, jego
zdaniem, wyraźniejsze działanie kancerostatyczne.

Na końcu książki umieszczono tablicę układu okresowego pierwiastków, w któ-
rej zaznaczono pierwiastki mające jakiekolwiek działanie kancerogenne; można
ich naliczyć aż piętnaście. Cennym niewątpliwie uzupełnieniem książki jest wykaz
ponad 600 tytułów cytowanych w tekście prac, oraz alfabetyczny skorowidz
rzeczowy.

Można oczywiście mieć wątpliwości lub wierzyć w to, że istota wzrostu nowo-
tworowego leży rzeczywiście w tworzeniu związków chelatowych; ale zasługą
Autora jest, że przedstawił teorię, która dąży do integracji bardzo rozproszonego
zagadnienia biochemii nowotworów. Jest to rzeczą cenną nie tylko ze względu na
wyrobienie sobie ogólnego poglądu na zagadnienie, ale także i ze względu na
przyszłą działalność doświadczalną w tej dziedzinie.

Mariusz Żydowo