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ACTA BIOCHIMICA POLONICA

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EFFECT OF MALEIC ACID ON THE KIDNEY

II. HYDROGEN DONORS IN THE REDUCTIVE AMINATION OF α-KETOGLUTARATE IN RAT KIDNEY AND LIVER

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In experiments on the synthesis of amino acids by washed cell particles of rat kidney and liver [6] certain differences between those tissues were observed. The yield of amino acids synthesis was different, and the ratio of α-ketoglutarate utilization to the increase of amino nitrogen was in liver about 1.8 and in kidney 5.0. Besides, in kidney an addition of ATP was necessary for a good yield of amino acids synthesis, and a different effect of maleate on each of these tissues was also observed. In kidney, 50% inhibition of ketoglutarate consumption and of amino acids synthesis occurred at 5×10^{-4} M-maleate concentration.

The experiments presented in the first part of this series [2] indicated that in maleate-treated rats the inhibition of the oxidation of Krebs cycle intermediates occurred only in kidney. One of the main symptoms of maleate intoxication is aminoaciduria [see ref. 1], and to explain this phenomenon the possible ways of amino acids synthesis in the kidney had to be considered. Therefore the experiments *in vitro* were undertaken in which the amination of ketoglutarate was coupled with various reactions providing hydrogen. The differences between kidney and liver were also found especially in the reaction in which citrate was the donor of hydrogen and precursor of α-ketoglutarate as well.

To verify the suggestion made previously [6] that maleate inhibits the oxidative decarboxylation of ketoglutarate and has no effect on the reductive amination, the effect of maleate on amino acids synthesis coupled with dehydrogenation of citrate, malate and glucose-6-phosphate (G-6-P) was studied. It was found that maleate indeed did not inhibit the reductive amination since amination did occur if the reaction which provided hydrogen was not sensitive to maleate. It also appeared that of the reactions tested only ketoglutarate oxidation was inhibited by maleate.

EXPERIMENTAL

Materials and methods

Washed cell particles. Liver and kidney of white rats were used. After cooling, the tissues were homogenized with 6 volumes of tris buffer in a glass homogenizer, and suspensions of washed cell particles were prepared according to Bartley *et al.* [3]. For the homogenization 0.05 M-tris-HCl buffer, pH 7.2, was used which contained additionally 0.1 M-KCl, 0.001 M-$MgSO_4$ and 0.001 M-EDTA. The details were described in the previous paper [6].

Incubation. The synthesis of amino acids in the liver was examined in a system containing 75 μmoles tris buffer, pH 7.2; 100 μmoles KCl; 1.5 μmoles $MgSO_4$; 1.5 μmoles EDTA, and 90 μmoles NH_4Cl. The amounts of substrates and of maleate are given in the Tables. Maleic acid was added after neutralization. In the experiments with kidney 5 μmoles ATP was added. The final pH of the incubation mixture was 7.2, the volume 3 ml. The reaction was initiated by adding washed cell particles in the amount corresponding to 200 mg. of wet weight of tissues. The incubation was carried out for 60 min. in the atmosphere of air, at 38° with constant shaking.

Analytical methods. In the filtrates deproteinized with trichloroacetic acid, citrate was determined according to Beutler & Yeh [4], and ketoacids by the Friedmann & Haugen method [5]. Amino nitrogen was estimated in the ethanol filtrates by the Yemm & Cocking method [7]. The control test for the increase of amino groups was the whole system without NH_4Cl. Thus the net increase of $N\text{-}NH_2$ represented the synthesis of amino acids from exogenous ammonia. The mean values from 16 determinations for endogenous $N\text{-}NH_2$ in liver were 22.0 ± 5.6 and in kidney 35.6 ± 6.2 μmoles per gram of tissue.

Reagents. Citric acid A.R., NH_4Cl A.R., and malonic acid (Biuro Odczynników Chemicznych, Gliwice, Poland). Maleic acid (USSR). ATP, sodium fluoroacetate, and NADP (CalBiochem). Malic acid and tris (Light). Glucose-6-phosphate barium salt was a gift from Dr. M. Orłowski.

Citrate as a hydrogen donor in the reductive amination of α-ketoglutarate

After the incubation of washed cell particles of liver or kidney with citrate and ammonium chloride, there was an increase in $N\text{-}NH_2$ (Table 1). The synthesis could proceed through two chains of reactions. In the first one (reaction 3) from two molecules of citrate two ketoglutarate are formed and then the dismutation reaction leads to the synthesis of one molecule of glutamate (reaction 2).

Table 1

Effect of maleate on the synthesis of amino acids from citrate and ammonia by washed cell particles of rat kidney and liver

The composition and the conditions of incubation are given in Methods. The amount of citrate added was 60 μmoles, corresponding to 300 μmoles per g. of wet tissue weight. In the experiments with kidney 5 μmoles of ATP were additionally added. The average values of endogenous N-NH_2 are for the liver 22.0 ± 5.6 and for the kidney 35.6 ± 6.2 μmoles per g. of tissue. The values given for N-NH_2 represent the net increase. The mean values, ±S.D., from 7-8 experiments are given

Tissue	Maleate (M)	Consumption of citrate	Ketoglutarate found	Calculated consumption of ketoglutarate	Increase of N-NH_2	Consumption of citrate / Increase of N-NH_2	Consumption of ketoglutarate / Increase of N-NH_2
		(μmoles/g. tissue/hr.)					
Liver	—	111.0 ±19.3	18.0 ±5.3	93.0 ±17.7	55.5 ±14.4	2.0	1.7
	8×10^{-2}	159.5 ±38.3	35.5 ±7.8	124.0 ±35.7	112.0 ±36.1	1.4	1.1
Kidney	—	226.0 ±21.0	66.5 ±8.9	160.0 ±27.9	81.5 ±17.2	2.8	2.0
	10^{-2}	218.5 ±21.7	128.5 ±15.4	90.0 ±19.0	79.5 ±19.6	2.7	1.1
	2×10^{-2}	214.0 ±19.5	122.0 ±42.2	92.0 ±16.3	80.5 ±24.9	2.7	1.1

$$2 \text{ citrate} \rightarrow 2 \text{ ketoglutarate} + 2CO_2 + 4H \quad (1)$$

$$2 \text{ ketoglutarate} + NH_3 \rightarrow \text{glutamate} + \text{succinate} + CO_2 \quad (2)$$

$$\text{Sum: } 2 \text{ citrate} + NH_3 \rightarrow \text{glutamate} + \text{succinate} + 3CO_2 + 4H \quad (3)$$

The ratio of the consumption of ketoglutarate formed from citrate to the synthesis of N-NH_2 should be the same as when ketoglutarate is directly used, that is 2 or more. In liver this ratio was found to be 2.0, whereas in kidney 5.0 (Table 3). It follows that in kidney to the formation of one molecule of glutamate corresponded the consumption of as much as five molecules of ketoglutarate; four of them became oxidized to succinate.

Alternatively (reaction 6) a coupling of the reaction catalysed by isocitrate dehydrogenase with the reductive amination can take place:

$$\text{citrate} \rightarrow \text{isocitrate} \rightarrow \text{ketoglutarate} + CO_2 + 2H \quad (4)$$

$$\text{ketoglutarate} + 2H + NH_3 \rightarrow \text{glutamate} \quad (5)$$

$$\text{Sum: citrate} + NH_3 \rightarrow \text{glutamate} + CO_2 \quad (6)$$

When there is a full utilization of the hydrogen liberated from isocitrate for the reductive amination, then the ratio of consumption of ketoglutarate formed from the citrate to N-NH_2 should be 1.0.

Table 2

Effect of maleate on citrate consumption by washed cell particles of rat liver and kidney

The incubation mixture did not contain any ammonium chloride. Apart from this, the composition of medium and the conditions of incubation as in Methods and in Table 1. The mean values, ±S.D., from 6-7 experiments are given

Tissue	Maleate (M)	Citrate consumption		Calculated consumption of ketoglutarate
		Citrate disappearing	Ketoglutarate found	
		(μmoles/g. tissue/hr.)		(μmoles/g. tissue/hr.)
Liver	—	122.5 ± 23.5	85.0 ± 20.0	37.5 ± 3.1
	8×10^{-2}	111.5 ± 16.7	99.5 ± 14.6	12.0 ± 9.3
Kidney	—	188.0 ± 17.8	93.5 ± 17.1	94.5 ± 18.9
	10^{-2}	193.5 ± 21.0	183.0 ± 23.3	10.5 ± 7.0
	2×10^{-2}	202.0 ± 16.0	197.5 ± 17.5	5.5 ± 5.6

It is of course possible that the reactions (3) and (6) proceed simultaneously and then the ratio for ketoglutarate would have the values between 2.0 and 1.0 in liver and between 5.0 and 1.0 in kidney.

As can be seen from Table 1 the ratio of citrate consumption to $N\text{-}NH_2$ synthesis was higher than the ratio of ketoglutarate formed

Table 3

Malate as hydrogen donor for the reductive amination of ketoglutarate in rat kidney and liver homogenates

To the incubation mixture composed as given in Methods 60 μmoles of ketoglutarate was added, and in the experiments with kidney additionally 5 μmoles ATP. Other additions were: malate, 30 μmoles; fluoroacetate, 36 μmoles; maleate, 30 μmoles in experiments with kidney and 200 μmoles in experiments with liver. The results of one typical experiment are given

Addition	Kidney			Liver		
	Consumption of ketoglutarate	Increase of $N\text{-}NH_2$	Consumption of ketoglutarate / Increase of $N\text{-}NH_2$	Consumption of ketoglutarate	Increase of $N\text{-}NH_2$	Consumption of ketoglutarate / Increase of $N\text{-}NH_2$
	(μmoles/g. tissue/hr.)			(μmoles/g. tissue/hr.)		
None	266.0	49.0	5.4	290.0	144.0	2.0
Malate	251.5	46.0	5.5	282.5	139.0	2.0
Fluoroacetate	—	—	—	286.0	141.0	2.0
Fluoroacetate, malate	260.0	47.0	5.5	284.0	132.0	2.1
Maleate	53.0	1.0	53.0	120.0	30.0	4.0
Fluoroacetate, malate, maleate	136.0	50.0	2.7	158.0	114.5	1.4

from citrate to N-NH_2. This was due to the rate of the reaction (4) being greater than the rate of the reaction (5).

In liver this ratio for citrate was 2.0 whereas for ketoglutarate formed from citrate it was 1.7. This seems to indicate that the synthesis of amino acids from citrate and ammonia in rat liver occurred as a result of the dismutation, of ketoglutarate formed. From the data presented in Table 1 as compared with those of Table 2 (without ammonia added) it can be seen that in the liver a marked decrease of ketoglutarate accumulation was found, due to its amination to glutamate. The increase of N-NH_2 was 55 μmoles per gram of tissue per hour.

In kidney the addition of ammonia also decreased the accumulation of ketoglutarate formed from citrate (Tables 1 and 2). This decrease is a result of ketoglutarate amination to glutamate, which gives 81 μmoles N-NH_2 per gram and per hour. The ratio for citrate was 2.8 and for ketoglutarate 2.0. This indicated that in kidney, otherwise than in liver, a part of hydrogen liberated in the reaction catalysed by isocitrate dehydrogenase was utilized for the reductive amination. This assumption was also supported by the fact that the increase of N-NH_2 in the system of citrate and ammonia was markedly greater than in the system of ketoglutarate and ammonia (Table 3). It can be therefore assumed that the reactions which provide hydrogen are the limiting factor in the synthesis of amino acids in kidney.

Effect of maleate on the amino acids synthesis from citrate and ammonia

It has been previously shown [6] that maleate has a marked inhibitory effect on the oxidation of α-ketoglutarate in kidney, and to a smaller degree in liver. From the experiments presented in Table 2 it follows that maleate did not inhibit the conversion of citrate to ketoglutarate. Therefore the synthesis of amino acids from citrate and ammonia in the presence of maleate may take place only as a result of the reaction (6). In liver (Table 1) there was a greater citrate consumption in the presence of maleate and ammonia, and over 75% of the formed ketoglutarate underwent reductive amination. The increase of N-NH_2 was twice as big as without maleate. The ratio of the citrate consumption to N-NH_2 formation was 1.4, and the ratio for ketoglutarate utilization to N-NH_2 was 1.1. This indicates that the hydrogen liberated in the reaction of isocitrate dehydrogenase may be used for the reductive amination of ketoglutarate.

In kidney the utilization of citrate did not change in the presence of maleate (Table 2). The synthesis of amino acids was also unaffected (Table 1) being about 80 μmoles per gram and per hour. As a result of the inhibition of the oxidative decarboxylation, ketoglutarate accumulated and in the presence of ammonia was converted to glutamate. The

ratio of citrate consumption to N-NH_2 was unchanged and amounted to 2.7, indicating that maleate did not affect the activity of isocitrate dehydrogenase. However, the ratio of the consumption of ketoglutarate formed from citrate to the increase of N-NH_2 was 1.1. This proved that in the presence of maleate, the isocitrate dehydrogenase reaction may provide all the hydrogen for the reductive amination. The yield of amino acids synthesis under such conditions was very high. The synthesis of one molecule of glutamate corresponded to the consumption of only one molecule of ketoglutarate formed from citrate.

Malate and glucose-6-phosphate as hydrogen donors for the reductive amination in kidney

The addition of malate did not increase the synthesis of amino acids (Table 3); this means that under normal conditions the malate dehydrogenation is not coupled with reductive amination. However, in the presence of maleate, when the amino acids synthesis resulting from α-ketoglutarate oxidation was inhibited, malate dehydrogenation was the reaction which could provide hydrogen for reductive amination.

Table 4

Glucose-6-phosphate as hydrogen donor for the reductive amination of α-ketoglutarate in rat kidney homogenates

To the incubation mixture composed as given in Methods 60 μmoles of ketoglutarate and 5 μmoles of ATP were added. Other additions were: glucose-6-phosphate, 30 μmoles; NADP, 1 μmole; maleate, 30 μmoles. The results of one typical experiment are given

Addition	Consumption of ketoglutarate (μmoles/g. tissue/hr.)	Increase of N-NH_2 (μmoles/g. tissue/hr.)	Consumption of ketoglutarate / Increase of N-NH_2
None	272.0	60.0	4.5
G-6-P	244.0	57.0	4.3
NADP	271.5	102.0	2.7
G-6-P, NADP	262.0	131.0	2.0
G-6-P, NADP, maleate	62.5	46.0	1.4
Maleate, NADP	45.5	0.9	50.0
Maleate	42.0	0.8	53.0

The experiments in which the reductive amination was coupled with the reaction of dehydrogenation of G-6-P (Table 4) indicated that hydrogen may be provided by G-6-P. The ratio of ketoglutarate consumption to N-NH_2 formation decreased significantly after the addition of G-6-P and NADP. In the presence of maleate the hydrogen

for reductive amination may be derived only from G-6-P. The ratio of ketoglutarate utilization to N-NH_2 was nearly equal to 1.0. It should be pointed out that the addition of NADP alone also considerably increased the synthesis of amino acids. It had, however, no effect in the presence of maleate.

DISCUSSION

The participation of two different pyridine nucleotides in the various reactions of dehydrogenation is of great importance in the energy balance of the organism, determining the fate of liberated hydrogen. It is generally assumed that hydrogen from $NADH_2$ is transferred first of all to the Keilin-Warburg system, with the accumulation of ATP resulting from the transfer of electrons to oxygen. $NADPH_2$, on the other hand, appears to be a major source of hydrogen for biosynthetic reductions. The distribution of hydrogen between two pyridine nucleotides, i.e. the destination for cellular ATP production or for the biosynthesis, is controlled in some degree by transhydrogenases.

The fluctuations in the hydrogen pool depend on many factors differing in various organs, and are greatly influenced by the conditions under which the whole organism or the given organ exist. Also the morphological structure with its compartmentation of the enzymic systems within the cells are of great significance.

When interpreting the results of the experiments in which various oxido-reduction reactions are involved we should take into consideration the equilibrium of the reactions and their structural arrangement. Therefore it appears that the most convenient mechanism for the amino acids synthesis *in vivo* is the dismutation of two molecules of α-ketoglutarate or the oxidation of citrate. In both reactions the equilibrium is shifted towards the dehydrogenation. In kidney the second reaction seems first of all to participate in amino acid synthesis. The rate of citrate oxidation is greater in kidney than in other tissues and in the reaction of isocitrate dehydrogenation two substrates for the reductive amination are formed, i.e. ketoglutarate and $NADPH_2$. As the oxidation rate of this nucleotide in the respiratory chain is low it is predestined to participate in the reductive amination. It can be seen that in kidney the yield of amino acids synthesis from citrate added is greater than from added ketoglutarate. In liver the former reaction is less significant, since the rate of citrate metabolism is lower.

The contribution of hydrogen from malate to the reductive amination of ketoglutarate is not very probable under normal conditions because of the unfavourable equilibrium of malic dehydrogenase reaction, towards the reduction of NAD. The contribution of hydrogen from G-6-P *in vivo* seems rather unlikely since oxidation of G-6-P takes

place in the cytoplasm whereas the reductive amination of ketoglutarate is located in mitochondria.

Among the reactions studied only the oxidation of ketoglutarate is inhibited by maleate. Since the hydrogen for reductive amination may be provided by other reactions it seems unlikely that maleate inhibits the amino acids synthesis *in vivo*. It can be even supposed that in the kidney of maleate-treated rats there are favourable conditions for amino acids synthesis because of the accumulation of ketoglutarate.

SUMMARY

1. In rat liver, hydrogen for the reductive amination of ketoglutarate is provided chiefly by the oxidation of the second molecule of ketoglutarate, whereas in kidney the preferred hydrogen donor is citrate.

2. Maleate does not affect the reductive amination of ketoglutarate if the reaction which provides hydrogen is not sensitive to its action. It seems therefore that in the kidney of maleate-treated rats there is no inhibition of amino acid synthesis.

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DZIAŁANIE KWASU MALEINOWEGO NA NERKĘ

II. DONATORY WODORU W REDUKTYWNEJ AMINACJI KETOGLUTARANU W NERCE I WĄTROBIE SZCZURA

Streszczenie

1. W wątrobie wodór dla reduktywnej aminacji ketoglutaranu jest dostarczany głównie przez utlenienie drugiej cząsteczki ketoglutaranu, w nerce natomiast głównym donatorem wodoru jest cytrynian.

2. Maleinian nie hamuje reduktywnej aminacji, o ile reakcja dostarczająca wodoru jest niewrażliwa na maleinian. Wydaje się wobec tego, że w nerkach szczurów zatrutych maleinianem nie ma zahamowania syntezy aminokwasów.

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BROMINATION OF THYMINE AND PHOTOCHEMISTRY OF 5-BROMO-6-HYDROXYHYDROTHYMINE ANALOGUES

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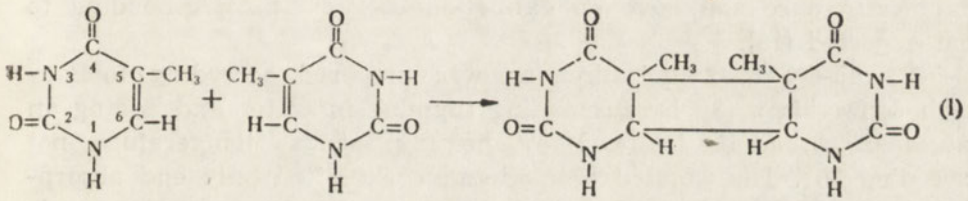
The following study arose out of attempts to demonstrate whether thymine dimer is one of the products resulting from submission of thymine to ionizing radiations in aqueous solution. It is now well established that ultraviolet irradiation of thymine in aqueous frozen medium or of thymine polynucleotides leads to the formation of the dimer (I). Such dimer photoproducts exhibit only end absorption in the



(I)

quartz ultraviolet [20]. We had previously noted that these dimers are dissociated by ionizing radiations, although in low yield (Barszcz & Shugar, unpublished data). In view of this fact, our attempts to examine the possible formation of dimers with ionizing radiations involved the use of relatively low doses, not exceeding 10 kr. Under these circumstances it is necessary to detect small quantities of dimer in the presence of a large excess of thymine, a procedure somewhat difficult to apply with the usual spectral and chromatographic techniques [20, 22]. Since thymine dimer is resistant to bromination, the irradiated solutions were treated with bromine to saturate the 5,6 double bond of unchanged thymine with the resultant elimination of its absorption spectrum; it was then expected that ultraviolet (253.7 mμ) irradiation would dissociate any dimer present with reformation of the absorption spectrum of thymine, exhibiting a characteristic maximum at 260 mμ. This was, in fact, found to occur in preliminary experiments, but, since the quantum yield for the reaction was much lower than that expected for the photodissociation of thymine dimer [20], a control

experiment was performed with brominated thymine alone. It was then observed that the product(s) of bromination of thymine, under the influence of ultraviolet irradiation, was transformed to a product(s) exhibiting selective absorption in the quartz ultraviolet, with a principal maximum at about 260 mμ, indicating reformation of the double-bond character of the 5,6 liaison of the pyrimidine ring.

In view of the general interest attaching to the photochemistry of pyrimidine derivatives, particularly in relation to the photochemistry of nucleic acids, it was considered of interest to examine the foregoing photochemical reaction in greater detail.

This, in turn, raised the problem of the composition and structure of brominated thymine. While the products of bromination of uracil, as well as the course of the bromination reaction itself, have been reasonably well established [16], the same is not true for thymine, as we soon discovered.

MATERIALS AND METHODS

Thymine was a product of Fluka. In contrast to some other commercial preparations of thymine, which were found to contain up to 20% uracil, the product used in this investigation was chromatographically pure and gave an extinction coefficient corresponding to that expected [18].

5-Bromo-6-hydroxyhydrothymine was prepared according to Baudisch & Davidson [3], by suspending thymine in water and adding an excess of bromine, followed by heating at a temperature not exceeding 70°. The isolated bromothymine exhibited only end absorption in the quartz ultraviolet. A similar preparation was also made without the heating step, the isolated bromothymine being washed with cold water until all bromine and unreacted thymine had been removed.

5-Bromo-6-hydroxyhydrothymidine was prepared by suspending 100 mg. thymidine (Fluka) in 1 ml. water, heating until dissolved, and adding 30 μl. bromine with stirring. The resulting solution was left for 30 min. and then evaporated in a stream of air; during this procedure the solution became colourless. The final, oily, residue could not be crystallized. An aqueous solution of this derivative showed no selective absorption in the ultraviolet.

5,5-Dibromo-6-hydroxyhydrouracil, 1,3-dimethyl-5-bromo-6-hydroxyhydrothymine and 6-methyl-5-bromo-6-hydroxythymine were prepared by bromination of 10^{-4} M solutions of the pure parent substances directly in quartz spectrophotometer cuvettes and removal of excess bromine in a stream of air. All of these exhibited only end absorption in the ultraviolet.

Glycol thymine was prepared according to the procedure of Baudisch & Davidson [3]; 5-methylbarbituric acid as described by

Gerngross [10]; and 5-nitro-6-hydroxyhydrothymine according to Johns [13].

Ultraviolet irradiation. Most of the irradiations were performed with a British Thermal Syndicate mercury resonance lamp, the solutions being exposed at a distance of 4 cm. from one limb of the lamp either in 10-mm. spectrophotometer quartz cuvettes at a concentration of about 10^{-4} M or, when it was desired to concentrate the irradiated solution for chromatography, in 1-mm cuvettes at a concentration of 10^{-3} M. In addition, for some experiments a 1:1 aqueous acetic acid filter (thickness 5 mm.) was interposed between the lamp and the cuvettes to eliminate wavelengths below 240 mμ. Larger quantities of material were irradiated with a germicidal lamp as described elsewhere [17].

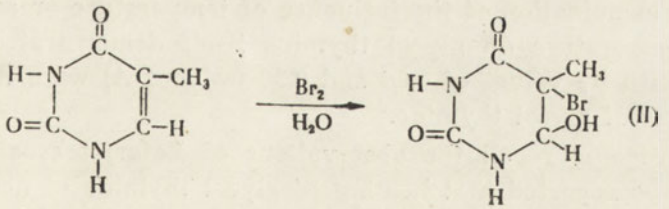
Paper chromatography. Ascending chromatography was employed throughout with Whatman no. 1 paper and, when necessary for small scale preparative purposes, with Whatman no. 3. The solvent systems used were as follows: *A*, water-saturated butanol; *B*, upper phase of *n*-butanol - glacial acetic acid - water, 2:1:1 (by vol.); *C*, isopropanol--conc. NH_4OH - water, 7:1:2 (by vol.); *D*, *n*-propanol - water, 10:3 (v/v).

Ultraviolet absorbing spots were detected by means of a dark UV lamp. Pyrimidine rings with saturated 5,6 bonds were revealed by spraying the chromatograms with alkali to open the rings, and then with *p*-dimethylaminobenzaldehyde [8, 12]. Urea was detected by spraying directly with the benzaldehyde reagent. Compounds with *cis*-glycol groups were located by means of metaperiodate as described by Cifonelli & Smith [4].

RESULTS

Bromination of thymine

According to Baudisch & Davidson [3], bromination of thymine in aqueous medium at a temperature not exceeding 70°, gives quantitatively 5-bromo-6-hydroxyhydrothymine (II). The structure of this compound has been verified by Dimitrow & Kurtew [6]. The brominated product, being a diketopyrimidine with a saturated 5,6 bond, should be unstable in alkali, the 3,4 bond opening under these conditions to give



a ureido acid derivative which should react with p-dimethylaminobenzaldehyde to give a coloured product [8, 12].

When, however, bromothymine was prepared according to Baudisch & Davidson [3], as well as by an analogous milder method (without heating), the presence of four products could be demonstrated chromatographically. Since the spots on the chromatogram reacted with p-dimethylaminobenzaldehyde *only after prior spraying with alkali*, it follows that all four products are pyrimidine rings with saturated 5,6 bonds. Numerous trials demonstrated that it was, in fact, impossible under any conditions to obtain chromatographically pure bromothymine; and it was rather the relative intensities of the various spots on the chromatograms which suggested that, of the four products reacting with p-dimethylaminobenzaldehyde, the one corresponding to bromothymine was that with R_F values of 0.72 and 0.89 in solvents *A* and *B* respectively. The intensity of these spots was decreased considerably in a sample prepared according to Baudisch & Davidson, which involves heating to 70°.

The question then posed itself as to whether the additional products resulted from side reactions during bromination or from the further transformation of bromothymine. To answer this, several mg. of bromothymine preparation was chromatographed on Whatman no. 3 paper, using solvent *B*. The spot corresponding to bromothymine was eluted with water; an aliquot of this was stored in the cold-room for two days at 0°, and another aliquot heated at 100° for 1 hr., following which both were again chromatographed in solvent *A*. The results demonstrated that the other three products, with R_F values of 0.06, 0.21 and 0.30 are derived from the spontaneous transformation of bromothymine in slightly acid medium (pH 5), which proceeds at a measurable rate at 0° and is considerably accelerated at 100°.

The spots with R_F values of 0.06 and 0.21 gave positive reactions with metaperiodate [4], indicating the presence of glycol groups; while that with R_F 0.21 travelled with an authentic sample of glycol thymine in several solvent systems.

Further examination of the influence of temperature or acidification at room temperature on glycol thymine itself demonstrated that the two spots with R_F values of 0.06 and 0.30 (solvent *A*) were transformation products of glycol thymine.

The foregoing recall the observations of Latarjet *et al.* [15] and Ekert [7] who reported that heating of glycol thymine in neutral solution resulted in partial conversion to an isomeric form with R_F of 0.54 in solvent *D*, as compared to 0.45 for the initial glycol thymine. The latter was first assigned the *trans*, and the former the *cis*, form [15]; subsequently this assignment was reversed [7], but without presentation of

the supporting evidence. It was therefore deemed advisable to examine this further.

Glycol thymine was heated in aqueous medium for 4 hr. at 100° and run on Whatman no. 3 paper in solvent *A*. The various spots were eluted and their homogeneity tested by chromatography in solvents *A* and *D*, the spots being revealed with *p*-dimethylaminobenzaldehyde. The various substances were also subjected to a periodate test on paper [4] and in solution [1], with the results shown in Table 1. It may be concluded that the spot with R_F 0.21 in solvent *A*, corresponding to synthetic glycol thymine [3], is a *cis*-glycol, while that with R_F 0.30 may be a *trans* isomer, which does not react even on long exposure to periodate because of steric hindrance due to the methyl group. This was in fact confirmed by elution of the spot with R_F 0.30 and heating it in aqueous medium at 100°, resulting in partial transformation to only one other product, namely that with R_F 0.21 or *cis*-glycol thymine. The identity of the spot with R_F 0.06 is not certain; however, an eluate of this spot, after standing for several days at room temperature, gave two spots with R_F 0.06 and 0.21 in solvent *A*. Since that with R_F 0.21 is partially transformed to that with R_F 0.06 on heating, and both react rapidly with periodate, it appears most likely that the spot with R_F 0.06 is the second possible *cis* isomer of glycol thymine. The compound with R_F 0.63 in solvent *A* (2nd *trans*-glycol ?) was found only after a long heating of *cis*-glycol thymine (R_F 0.21 in solvent *A*). The former appears in traces and this may explain why it could not be observed in spontaneous transformations of bromothymine.

Table 1

R_F *values and periodate reaction of products of heating of glycol thymine in aqueous medium for 4 hr. at 100°*

R_F		% of total	Periodate reaction		Identification
Solvent *A*	Solvent *D*		on paper	in solution	
0.06	absent	1*	+	+ (1 hr.)***	*cis*-glycol
0.21	0.45	76**	+	+ (2 hr.)***	*cis*-glycol
0.30	0.54	12**	—	— (25 hr.)	*trans*-glycol
0.63	0.73	traces	—	not tested	*trans*-glycol?

*Estimated from a determination of the optical density of the total eluate at 230 mμ at pH 12 and assuming an extinction coefficient equal to that for synthetic glycol thymine.

**Determined by direct weighing of the lyophilized eluate.

***Consumption of periodate, 1 mole.

Aside from the foregoing, it was found that bromothymine undergoes an additional transformation in acid medium to a compound which, on alkalization to pH 12, is instantaneously transformed to 5-methylbarbi-

turic acid. This was identified by its ultraviolet absorption spectrum and by a comparison of its chromatographic behaviour in several solvent systems with authentic 5-methylbarbituric acid. From Fig. 1 it will be seen that bromothymine is not transformed directly to 5-methylbarbituric acid in acid medium for, if this were so, then the absorption spectrum of the latter ($pK_a \sim 4.7$) should appear on neutralization of the solution to pH 7 [9]. In fact, the solution must be brought to pH 12 to place in evidence the spectrum of 5-methylbarbituric acid. It is therefore clear that bromothymine undergoes a time-dependent transformation to some intermediate in acid medium and this is, in turn, instantaneously

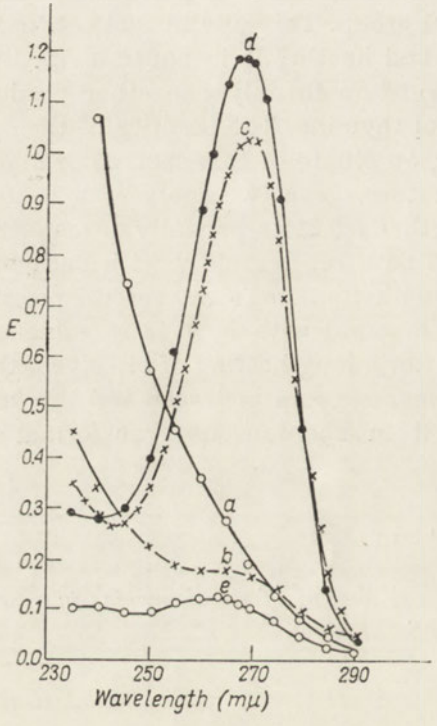


Fig. 1

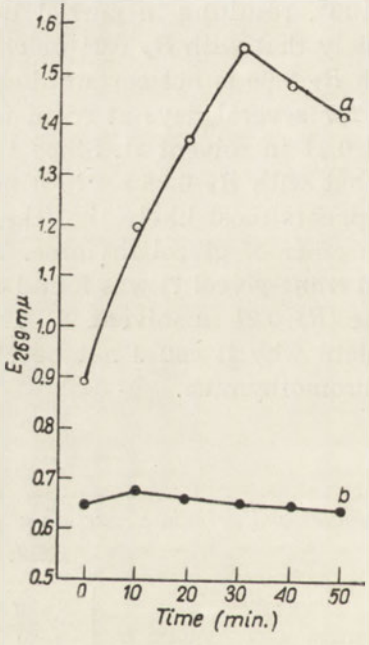


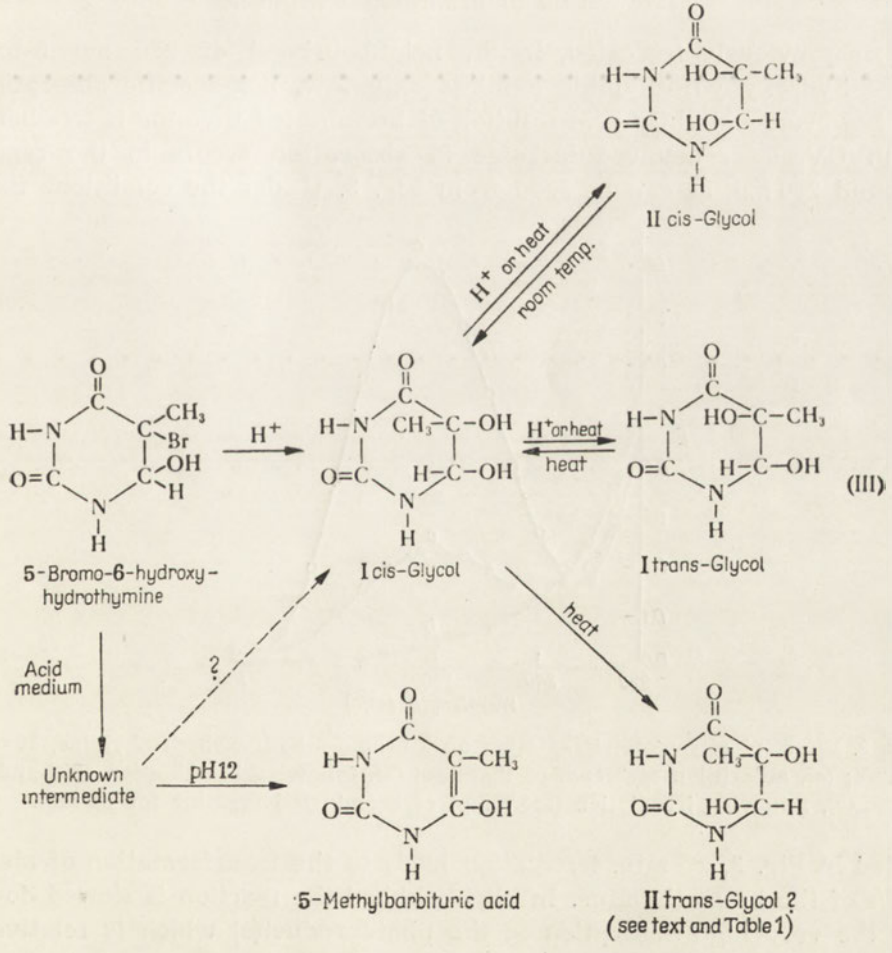
Fig. 2

Fig. 1. Formation of 5-methylbarbituric acid by alkalization of a solution of 5-bromo-6-hydroxyhydrothymine which had been previously incubated for several days at room temperature, in acid medium. Curve *a* is the absorption spectrum of the incubated solution of bromothymine at pH 2.9. When this solution is brought to pH 6.9, we obtain curve *b*. If the solution is brought at once to pH 12.5, we obtain curve *c*, which is that of 5-methylbarbituric acid. This is further confirmed by bringing the alkaline solution in turn to pH 7.4 (curve *d*) and pH 1.7 (curve *e*), the changes with pH corresponding to those for methylbarbituric acid.

Fig. 2. Formation of 5-methylbarbituric acid by alkalization to pH 12 of 5-bromo-6-hydroxyhydrothymine heated at 100° in solution: (*a*), unbuffered, pH 4 - 5; (*b*), buffered at pH 9.2, 0.02 M-borate. Ordinates give the extinction at pH 12 of the methylbarbituric acid formed.

transformed to 5-methylbarbituric acid at pH 12. Identification of the intermediate could not be achieved. But glycol thymine itself does not give rise to formation of 5-methylbarbituric acid.

Fig. 2 shows the rate of formation of 5-methylbarbituric acid as a function of time of heating bromothymine in acid medium. No formation of methylbarbituric acid is observed on incubation of bromothymine in alkaline medium. From Fig. 2 it is also clear that extended heating of bromothymine in acid medium decreases the yield of 5-methylbarbituric acid, suggesting that the intermediate from which the latter is formed is thermolabile. The precipitous drop in yield of methylbarbituric acid following 40 - 50 min. heating (Fig. 2) suggests that under these conditions the presumed intermediate has been irreversibly destroyed. Two possible explanations may account for this behaviour, viz. (a) oxygen is necessary for the transformation of bromothymine to the presumed intermediate, or (b) only a fraction of the bromothymine is transformed to the intermediate.



The first of these was excluded by heating the bromothymine solution under a layer of paraffin. Under these conditions, the residual oxygen in the solution is rapidly expelled during initial heating. If oxygen were necessary, then the curve in Fig. 2 should exhibit a break much earlier than it actually does. From the data of this experiment, it was calculated that the amount of methylbarbituric acid observed was 7% of the initial bromothymine; but the actual yield of methylbarbituric acid must have been considerably higher if we take into account the apparent thermolability of the intermediate at elevated temperatures.

Chromatography in solvent *C* of the foregoing solutions also demonstrated the formation of methylbarbituric acid as well as the decrease with time of incubation of bromothymine and the corresponding increase in glycol thymine. The transformations undergone by bromothymine in aqueous medium at room temperature are represented schematically (see above schema III).

Irradiation of brominated thymine

As previously indicated, in the neighbourhood of 260 mμ 5-bromo-6-hydroxyhydrothymine exhibits only low, non-specific absorption. If, however, a slightly acid solution of brominated thymine is irradiated with UV, a pronounced increase in absorption occurs in the region around 260 mμ, as can be seen from Fig. 3. Under the conditions indi-

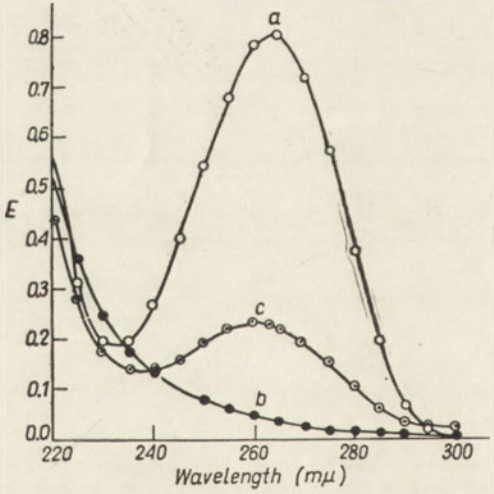


Fig. 3. Effect of ultraviolet irradiation on 5-bromo-6-hydroxyhydrothymine, 10^{-4} M, pH~4; (*a*), absorption spectrum of thymine; (*b*), following bromination of thymine; (*c*), following irradiation (253.7 mμ) of brominated thymine for 90 min.

cated in Fig. 3, 90 min. irradiation leads to the transformation of about 20% of the bromothymine. In all likelihood the reaction is slowed down by the very high absorption of the photoproduct(s) which is relatively

radiation resistant but which protects the starting substance by internal shielding. During the course of irradiation, the pH of the solution drops by about 2 units, while the solution itself turns yellowish to a degree dependent on the initial concentration subjected to irradiation. Paper chromatography of the irradiated solution demonstrated the appearance of not one, but several ultraviolet absorbing photoproducts, the nature of which depended to some extent on the wavelength of the irradiation source employed (see Table 2).

It is important to emphasize at this point that, although a solution of brominated thymine contains, as shown above, a mixture of several compounds, the photochemical reactions to be described below are due to the influence of radiation solely on 5-bromo-6-hydroxyhydrothymine.

Table 2

Nature and relative yields of photoproducts resulting from irradiation of 5-bromo-6-hydroxyhydrothymine with different lamps and cut-off filters

Photoproducts were separated on Whatman no. 1 with solvent *A*. Intensity of spots is indicated by number of plus signs

Type of lamp and filter	Products and their R_F values	Relative intensity on chromatogram	Identification
British Thermal Syndicate resonance lamp with 5 mm. 1:1 aqueous acetic acid filter	0.13	+ +	unidentified
	0.23	+ + + +	5-hydroxymethyluracil
	0.52	+ + + + +	thymine
Above lamp with 1 mm. saturated $NiSO_4$ filter	0.13	+	unidentified
	0.23	+ + + + +	5-hydroxymethyluracil
	0.52	+ + + +	thymine
Above lamp without filter	0.23	+ + + +	5-hydroxymethyluracil
	0.52	+ + +	thymine
40 watt Phillips germicidal lamp without filter	0.23	+ + + +	5-hydroxymethyluracil
	0.35	+ +	uracil
	0.52	+ + +	thymine

The absorption maximum of an irradiated (253.7 mμ) solution of bromothymine was at 263 mμ. Chromatography of a lyophilized solution in solvent *A* exhibited the presence of ultraviolet absorbing spots with R_F values of 0.52, 0.23 and 0.13; and a spot exhibiting fluorescence with R_F 0, which was not studied. The absorbing spots were eluted and their spectra examined as a function of pH; this, together with chromatography with known derivatives, made it possible to identify the spot with R_F 0.52 as thymine, and that with R_F 0.23 as 5-hydroxymethyluracil. The compound with R_F 0.13 exhibited an absorption spectrum al-

most identical to that of uracil. At pH 7.2, λ_{max} 260 mμ, λ_{min} 247 mμ. At pH 13, λ_{max} 276 mμ, λ_{min} 255 mμ. It cannot, however, be uracil since in solvent *A* its R_F was 0.13 while that of authentic uracil is 0.35. From the spectral data for the absorption of the individual spots, it was calculated that the yield of the three products in terms of the amount of bromothymine irradiated was 11% for thymine, 8% for 5-hydroxymethyluracil and 3% for the unidentified compound with R_F 0.13.

Spectral examination of the eluates of the above substances exhibited also the presence of other products, exhibiting only end absorption in the quartz ultraviolet, which deformed the absorption spectra at shorter wavelengths down to 210 mμ. These products, which originate from the

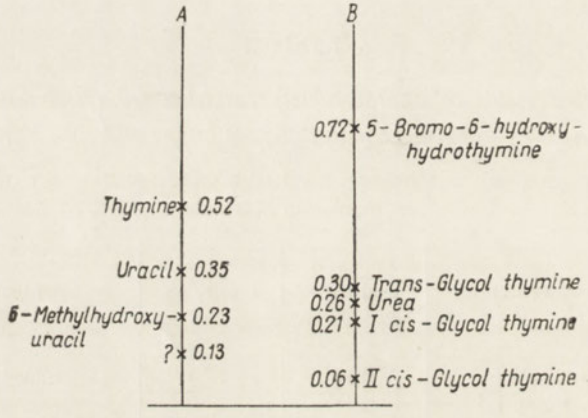


Fig. 4. Chromatographic separation in solvent *A* of the products of spontaneous and ultraviolet transformation of 5-bromo-6-hydroxyhydrothymine at pH~4; (*A*), ultraviolet absorbing products; (*B*), products revealed by spraying with alkali and then *p*-dimethylaminobenzaldehyde

spontaneous decomposition of bromothymine, were difficult to separate from the other photoproducts and, in particular, from 5-hydroxymethyluracil. The location of all the foregoing is shown schematically in Fig. 4. In addition to these substances, which have already been described above, chromatography in solvent *A* of an irradiated solution exhibited the presence of a spot at R_F 0.26 identified as urea. Since urea is not a product of the spontaneous transformation of brominated thymine, it may originate in the photochemical decomposition of glycol thymine as in the alkaline hydrolysis of this latter [3], but its formation from bromothymine itself cannot be excluded.

When a solution of bromothymine was irradiated with the same source as above, but without exclusion of traces of radiation below 253.7 mμ, the only ultraviolet absorbing photoproducts were thymine and 5-hydroxymethyluracil; that with R_F 0.13 in solvent *A* was entirely

absent and must consequently be quite radiation sensitive to shorter wavelengths.

When, however, the bromothymine solution was irradiated at considerably higher intensities, using a germicidal lamp, the photoproducts included thymine, 5-hydroxymethyluracil and one further product with R_F 0.35 in solvent *A*, identified as uracil. The substance with R_F 0.13 again was absent. The yield of uracil was somewhat less than for the other two photoproducts.

While no attempt was made to study in detail the kinetics of photoproduct formation, the photochemical reaction was found to be accompanied by an initial lag phase (see Fig. 5), the extent of which was related to the pH of the medium. In 0.01 M-phosphate buffer, pH 7.2, there was no sign of any reaction even after 45 min. irradiation. In acetate buffer of the same molarity the lag was about 20 min. at pH 5.2 (*c*) and less than 2 min. at pH 4 (*b*). It is consequently not surprising that in unbuffered medium (*a*) the initiation of photoproduct formation commences after 2 - 3 min. irradiation, during which period the pH falls to pH 4.2. This suggests the involvement of some acid-catalysed reaction, which is confirmed by the fact that there is no lag at all at pH 1 (0.1 N-HCl, *d*) plus the fact that in this medium a similar reaction

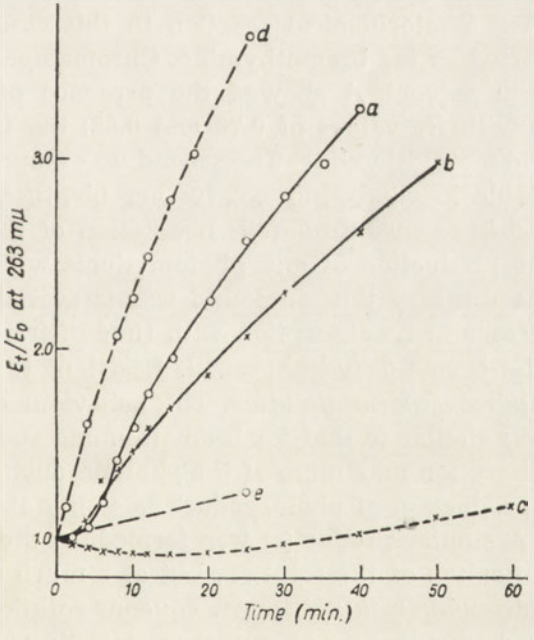


Fig. 5. Influence of pH on the photochemical transformation of 5-bromo-6-hydroxyhydrothymine, irradiated at 253.7 mμ with elimination of shorter wavelengths by filter. Medium: (*a*), unbuffered, pH 4.5 - 3.6; (*b*), 0.02 M-acetate buffer, pH 4.0; (*c*), 0.01 M-acetate buffer, pH 5.2; (*d*), 0.1 N-HCl; (*e*), 0.1 N-HCl without irradiation

appears to take place spontaneously *in the absence of irradiation*, but at a much lower rate (Fig. 5*e*). This reaction in the absence of irradiation has been previously reported [5], the product being characterized as thymine on the basis of melting point and ultraviolet absorption spectrum at one pH value [14]. We have confirmed this result by chromatographic examination of the acidified solution, left for some time at ambient temperature. The rate of formation of thymine under these conditions was, however, very low by comparison with that resulting from irradiation.

Irradiation results not only in debromination with formation of the products outlined above, but also in the appearance of some products of degradation of the pyrimidine ring. This is shown, amongst others, by the appearance of urea, already referred to above.

Behaviour of other saturated pyrimidine analogues

1,3-Dimethyl-5-bromo-6-hydroxyhydrothymine. Irradiation of this compound at 253.7 mμ with exclusion of shorter wavelengths, again led to the appearance of ultraviolet absorbing substances with maximum at 270 mμ, in 20% yield following irradiation periods of 50 and 80 min. at concentrations of 10^{-3} and 10^{-4} M respectively. The yield was decreased by one-half if the solution was heated for 15 min. at 100° prior to irradiation. The photochemical reaction in this case did not exhibit an initial lag period as for bromothymine. Chromatography of the irradiated solution in solvent *A* showed the presence of two ultraviolet absorbing spots with R_F values of 0.79 and 0.63; the first of these was shown to be 1,3-dimethylthymine. The second was a pyrimidine analogue which was unstable in alkali, but not further identified.

5,5-Dibromo-6-hydroxyhydrouracil. Irradiation of this compound did not result in the production of any photoproducts with an unsaturated 5,6 bond. On the contrary the compound was irreversibly destroyed, as shown by a decrease in its absorption with time of irradiation.

5,6-Dimethyl-5-bromo-6-hydroxyuracil. Resistant to irradiation.

5-Bromo-6-hydroxyhydrothymidine. The behaviour of this compound on irradiation was similar to that for bromothymine and dimethylbromothymine. The absorption maximum of the photoproduct(s) was at 265 mμ, but the rate of production of photoproduct was only about 40% that for bromothymine. A similar product(s) was formed, in the absence of irradiation, by acidification of the solution, but at a much slower rate.

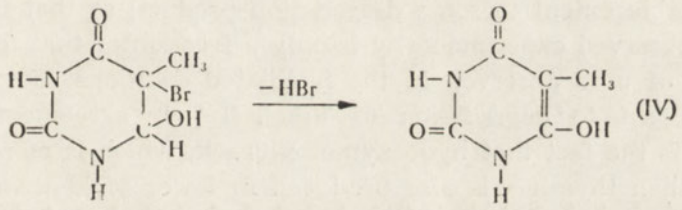
5-Nitro-6-hydroxyhydrothymine. An aqueous solution of this derivative exhibited a maximum at 315 mμ characteristic of the nitro group, and a minimum at about 257.5 mμ. The principal effect of irradiation at 253.7 mμ was to destroy the maximum at 315 mμ. No evidence for formation of new ultraviolet absorbing products could be observed.

DISCUSSION

Although the scheme outlined for the transformation of bromothymine in aqueous medium is by no means fully complete, it does establish a reasonably good basis for a more adequate elucidation of the steps involved. Larger scale experiments, conducted on a quantitative basis, would be necessary to determine whether any additional transformation pathways exist. In particular, the foregoing findings should be taken into account in investigations of the effects of bromination on nucleic acids and, especially, on DNA [21, 14].

Two points undoubtedly merit further examination. First, while the evidence for the existence of two *cis* and two *trans* isomers of glycol thymine is reasonably convincing, our data do not enable us to distinguish either between the two *cis*, or the two *trans*, forms.

Second, the mechanism by means of which bromothymine is transformed to 5-methylbarbituric acid remains to be established and, indeed, seems rather puzzling. Theoretically, the simplest pathway for this reaction would be the acid-catalysed elimination of a molecule of HBr (IV). Apparently, however, this is not the case; since the pK of methyl-



(IV)

barbituric acid is 4.7, its formation in the pH range 4 to 6 should be accompanied by the appearance of its characteristic absorption spectrum, notwithstanding the reported instability of this compound in acid medium [11], which we have verified. The presumed intermediate formed in acid medium, which gives rise to methylbarbituric acid on alkalization, must be reasonably stable since the quantity of methylbarbituric acid formed increases with time of incubation. The use of ^{14}C-labelled thymine is most likely indicated in any further attempts at the isolation, and identification, of this intermediate.

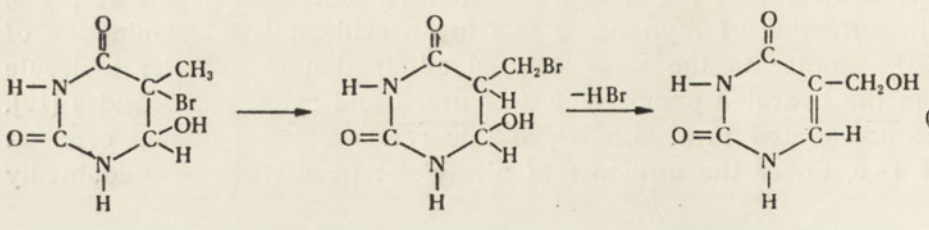
It is clear that the slow transformation of bromothymine at pH 1, with formation of thymine, is due to an acid-catalysed elimination of HOBr, similar to the acid-catalysed elimination of a water molecule from the hydrated photoproducts of uracil and cytosine glycosides [19]. The accelerated rate with which this reaction takes place, even at pH 4 - 5, under the influence of ultraviolet irradiation, is undoubtedly

an example of a specific photocatalytic reaction. The specificity of this reaction is attested to by the fact that it also proceeds with 1,3-dimethylbromothymine and brominated thymidine, but is entirely absent in the case of 5,5-dibromo-6-hydroxyhydrouracil and 6-methylbromothymine.

Rather striking is the photoproduction from bromothymine of such compounds as uracil and 5-hydroxymethyluracil. These are certainly not produced *via* thymine as an intermediate, since thymine itself under these conditions undergoes largely photochemical degradation with low quantum yield [19]. On the other hand the presence of a lag phase, during which the irradiated solution becomes acidified, suggests that the formation of uracil and hydroxymethyluracil may result from some interaction between an excited state of bromothymine and the liberated HOBr. Another possibility is some intramolecular rearrangement as described below for hydroxymethyluracil formation. It should be noted, in this connection, that the relatively low formation of uracil (3%) as compared to the other photoproducts (thymine, 11%; hydroxymethyluracil, 8%) is probably more apparent than real. Both thymine and hydroxymethyluracil are more radiation resistant than uracil and the production of the latter is undoubtedly accompanied by its further photochemical transformation, in part to the hydrated form and to a considerable extent to some degradation product; so that the amount of uracil observed experimentally is only a fraction of that formed. The formation of urea observed in the irradiated solutions could certainly be due in part to degradation of uracil [19]. In agreement with the foregoing is the fact that hydroxymethyluracil, which is more radiation sensitive than thymine, is also produced in lower yield than thymine.

It has previously been shown that demethylation may occur at N_1 and N_3 of saturated *N*-methylated diketopyrimidines under the influence of ionizing radiations in aqueous medium [2]. Even more unusual, however, is the observed demethylation at the 5-position of the ring leading to the formation of uracil from bromothymine.

The formation of hydroxymethyluracil is likewise not readily explicable and must, of necessity, involve at least one transition state. A possible scheme for this is a rearrangement, followed by the elimination of HBr, with a simultaneous second rearrangement (V).



The foregoing reactions are of particular interest in that they involve the photochemical formation of nucleic acid derivatives from saturated pyrimidine rings. Attention has previously been drawn to the potential significance of such reactions in connection with the problem of the primitive synthesis of organic matter. It would be of value, in relation to this, to see whether similar reactions may not occur with 5-bromo-dihydropyrimidines.

SUMMARY

5-Bromo-6-hydroxyhydrothymine is unstable in aqueous medium and undergoes at least two spontaneous transformations leading to the production of: (a) three isomeric forms of glycol thymine, two *cis* and one *trans*; (b) an unidentified compound, formed in slightly acid medium and which, on alkalization, is instantaneously transformed to 5-methyl-barbituric acid. At pH about 1, bromothymine is slowly transformed to thymine by the acid-catalysed elimination of HOBr; bromothymidine undergoes a similar reaction. Synthetic glycol thymine is one of the two possible *cis* isomers; at room temp. in slightly acid medium, or at elevated temperature in neutral medium, the glycol is transformed in part to the second *cis* form, to one of the *trans* configurations and probably also to the second *trans* isomer.

UV irradiation of bromothymine in slightly acid medium leads to the formation of thymine, uracil, 5-hydroxymethyluracil and one unsaturated pyrimidine derivative, not identified. Bromothymidine undergoes a similar photochemical transformation, but at a slower rate. Irradiation of 1,3-dimethylbromothymine gives two photoproducts, one of which was identified as dimethylthymine. These photochemical reactions are quite specific since 5,5-dibromo-6-hydroxyhydrouracil and 5,6-dimethyl-5-bromo-6-hydroxyuracil are not affected. The nature and significance of these photochemical transformations are discussed.

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BROMOWANIE TYMINY ORAZ FOTOCHEMIA 5-BROMO-6-HYDROKSYHYDROPOCHODNYCH PIRYMIDYNOWYCH

Streszczenie

5-Bromo-6-hydroksyhydrotymina jest nietrwała w wodnym środowisku i ulega co najmniej dwu spontanicznym przemianom prowadzącym do powstania: (a) trzech izomerycznych form glikolu tyminy, dwóch form *cis* i jednej *trans*; (b) jednego niezidentyfikowanego związku, powstającego w słabo kwaśnym środowisku, który po alkalizacji ulega natychmiastowemu przekształceniu na kwas 5-metylobarbiturowy. W środowisku silnie kwaśnym, które działa katalitycznie na odszczepienie HOBr, bromotymina przekształca się powoli w tyminę; podobnej reakcji ulega bromotymidyna. Wykazano, że syntetyczny glikol tyminy jest jednym z dwóch możliwych *cis* izomerów; izomer ten w słabo kwaśnym środowisku w temperaturze pokojowej lub przy podwyższonej temperaturze w środowisku obojętnym przekształca się częściowo w drugi izomer *cis*, oraz w formę *trans*, a prawdopodobnie również w drugi izomer formy *trans*.

Działanie na bromotyminę promieniowaniem ultrafioletowym w słabo kwaśnym środowisku prowadzi do powstania tyminy, 5-hydroksymetylouracylu, uracylu oraz niezidentyfikowanej pochodnej z nienasyconym wiązaniem 5,6. Bromotymidyna podlega podobnym przekształceniom fotochemicznym jednak z mniejszą szybkością. Naświetlona 1,3-dwumetylobromotymina daje dwa fotoprodukty, z których jeden zidentyfikowano jako dwumetylotyminę. O specyficzności powyższych reakcji fotochemicznych świadczy brak podobnych przemian dla 5,5-dwubromo-6-hydroksyhydrouracylu i 5,6-dwumetylo-5-bromo-6-hydroksyuracylu. Przedyskutowano charakter i znaczenie powyższych przekształceń fotochemicznych.

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ULTRAVIOLET ABSORPTION SPECTRA AND STRUCTURE OF HALOGENATED URACILS AND THEIR GLYCOSIDES

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During the course of some preliminary observations on the photochemical behaviour of several 5-substituted halogeno uracils, their glycosides and polymers, it was found necessary to obtain some information with regard to the dissociation constants of this important class of compounds as well as the dependence of their absorption spectra on pH.

Although the biological properties of the halogeno uracils and their glycosides have become the object of widespread investigations, it is somewhat surprising that only sporadic, and frequently non-quantitative, data are to be found scattered in the literature with respect to their absorption spectra and dissociation constants. Quantitative information of this nature is of importance in determining the tautomeric species involved in biological activity; this follows from the fact that the introduction of an electronegative substituent at the 5-position of the uracil ring should lead to a marked strengthening of its acid character, so that even at neutral pH one might expect to find a mixture of ionic species. Some steric effects may also be anticipated, particularly with regard to the 4-carbonyl in the pyrimidine ring, which is ortho to the 5-position; as well as in the case of nucleosides and nucleotides due to possible modifications of conformation. Quantitative data should also be of value in investigations on the physico-chemical and enzymic properties of synthetic polynucleotides containing halogenated residues [21, 15], as well as for analytical purposes in investigations on natural nucleic acids which contain biologically incorporated halogenated rings.

Particularly pertinent is the suggestion, advanced by Watson & Crick [23] and Freese [5] amongst others, that incorporation of 5-bromouracil into DNA, which is associated with an increase in mutation rate, might result from the occasional pairing of guanine with tautomeric forms of thymine or bromouracil. Experimental evidence

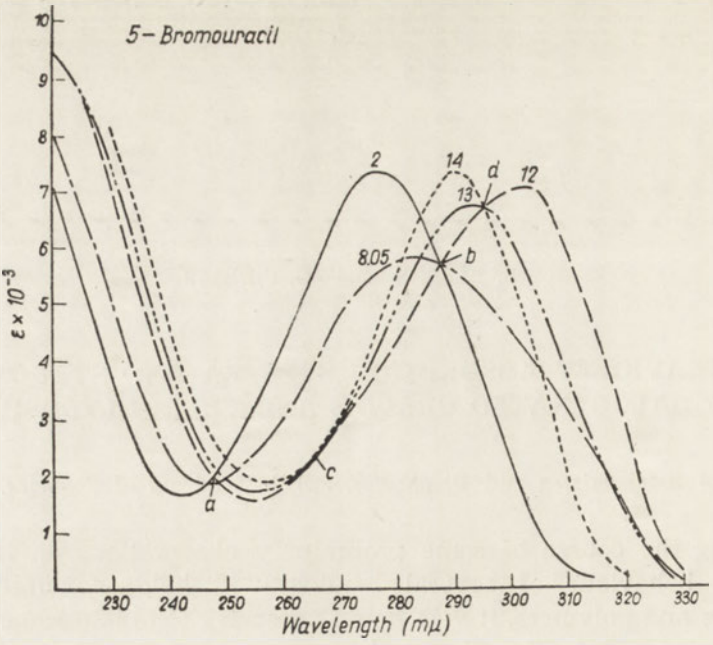

Fig. 1. 5-Bromouracil at various pH values selected to indicate the two ionic equilibria. Isosbestic points for 1st equilibrium, with pK_1 of 8.05, are denoted by dashes *a* and b; isosbestic points for 2nd equilibrium, with $pK_2 \sim 13$, are denoted by c and *d*. The figures on each curve, in this and subsequent diagrams, indicate pH values; see Tables for further data

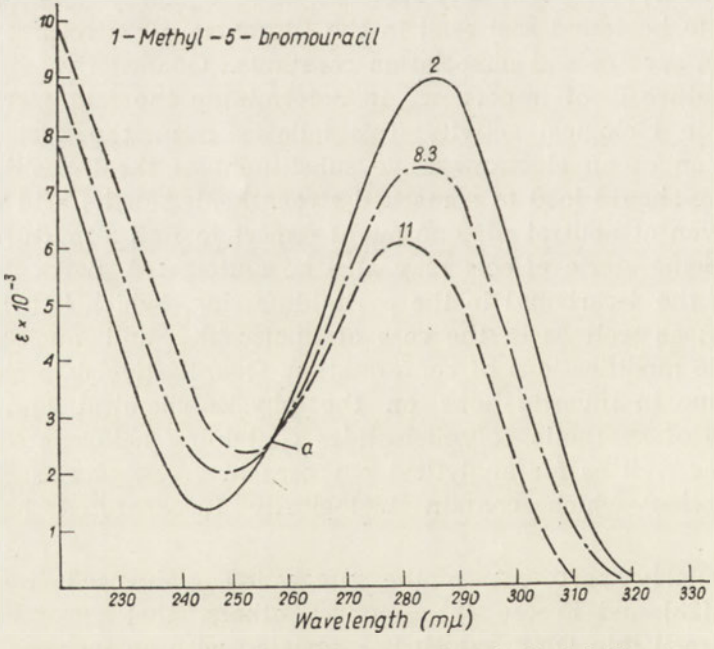

Fig. 2. 1-Methyl-5-bromouracil at various pH values indicated. Spectrum for pH 11 remains unaltered when pH increased to 14. Single ionic equilibrium with isosbestic point at *a* and pK 8.30

in favour of such an hypothesis has been recently adduced by Trautner *et al.* [22], who demonstrated that the *in vitro* enzymic replication of the copolymer poly-A-BrU is accompanied by a small, but measureable, incorporation of guanine.

EXPERIMENTAL

Materials

5-Fluorouracil [3] was a gift of Dr. R. Duschinsky and Hoffman-LaRoche Inc.

1-Methyluracil was prepared according to Hilbert & Johnson [8] and 3-methyluracil by a new general procedure for N_3 methylation of uracil analogues by treatment of the 1-acetyl derivatives [20] in anhydrous medium with diazomethane. Both the foregoing, as well as uracil and 1,3-dimethyluracil, were brominated in aqueous medium according to Hilbert [7]. The brominated products were repeatedly crystallized from water to accepted melting points (with decomp.) as follows: 5-bromouracil, 285°; 1-methyl-5-bromouracil, 266°; 3-methyl-5-bromouracil, 220°; 1,3-dimethyl-5-bromouracil, 179 - 180°. For additional criteria of purity, see below.

5-Chlorouracil was prepared according to the suggested procedure of Johnson & Johns [12], as follows: 1 g. uracil was suspended in 20 ml. chlorinated water and chlorine passed into the suspension until all the uracil had dissolved, following which excess chlorine was removed by aeration. Zinc filings were then added and, after the solution had been brought to 2 N with conc. HCl, it was heated to 100°. Several drops of concentrated HCl were then added and heating on the steam bath continued for 3 hr., during which a white precipitate formed. After cooling, the precipitate of chlorouracil was collected on a G-4 glass filter, washed with 1 N-HCl, water, alcohol and ether. Recrystallization from water yielded 440 mg. of product with m.p. 321° (decomp.).

5-Iodouracil was prepared by two methods, one according to Prusoff *et al.* [17], the other as worked out by Miss Z. Rybakow in this laboratory as follows: 170 mg. (1.5 mM) of uracil in 8 ml. of 80% acetic anhydride was heated to 70°. With constant stirring, 518 mg. (3.2 mM) of iodine chloride in 1 ml. 6 N-HCl was added dropwise, following which the temperature was maintained at 70 - 75° for 1.5 hr. The reaction mixture was then cooled, saturated with SO_2 and concentrated to about 4 - 5 ml. On cooling, the resulting white precipitate of iodouracil (250 mg., 83% theor.) was filtered off and recrystallized from water to obtain white, glistening needles, m.p. 273 - 274° (decomp.). As for other halogenated uracil derivatives, the melting point varied with rate of heating. Both of the foregoing preparations, as well as a sample kindly provided

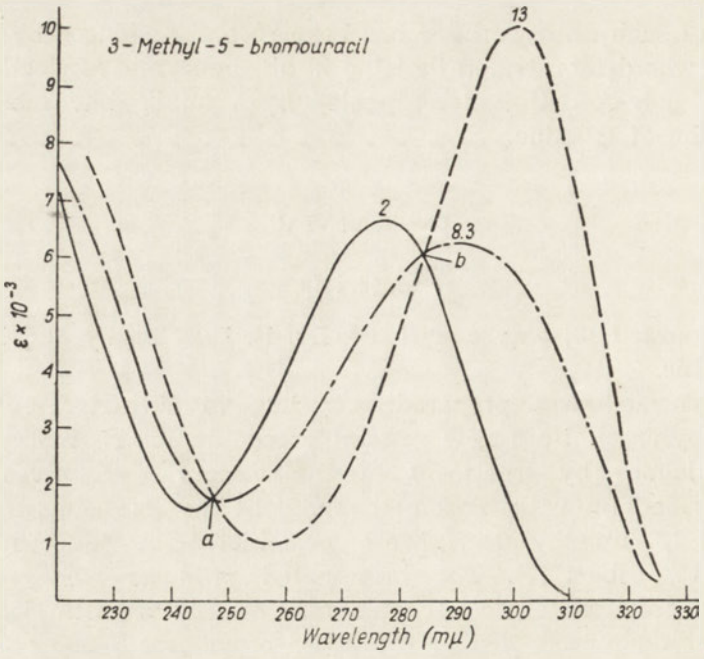

Fig. 3. 3-Methyl-5-bromouracil at several pH values showing existence of single ionic equilibrium with isosbestic points *a* and *b*, pK 8.30

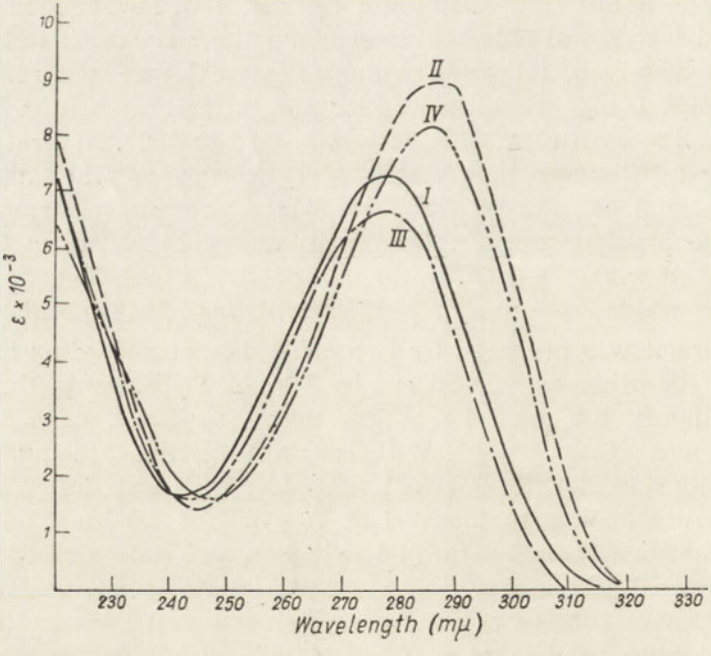

Fig. 4. Absorption spectra at pH 4, where only non-dissociated forms are present, of (*I*), 5-bromouracil; (*II*), 1-methyl-5-bromouracil; (*III*), 3-methyl-5-bromouracil; (*IV*), 1,3-dimethyl-5-bromouracil

by Dr. A. M. Michelson, all exhibited identical properties chromatographically and spectroscopically, with extinction coefficients agreeing to 1.5%.

Bromouridine was prepared according to Hilbert & Johnson [9] and iodouridine as described by Prusoff [16]. Chlorouridine was prepared by treatment of uridine with chlorosuccinimide in anhydrous dimethyl formamide, but difficulty was encountered in freeing the final product from some non-absorbing impurities; a CalBiochem. product was therefore used for determining the extinction value. Fluorouridine [26] was a gift of Dr. R. Duschinsky and Hoffman-LaRoche Inc.

The ribonucleotides were prepared in connection with the synthesis of oligo- and polyribonucleotides (Szer & Shugar, in preparation) and some analogues of the nucleotide coenzyme UDPG (Schmidt, Szer & Shugar, in preparation) and were isolated either as the free acids or the calcium salts.

Fluorodeoxyuridine [10] was kindly provided by Hoffman-LaRoche Inc.; and samples of the other deoxyribosides by California Corporation for Biochemical Research and by Dr. W. Szybalski.

METHODS

For making up stock solutions, all compounds were dried at temperatures between 110 and 135° and, where necessary, under vacuum using an oil pump. We have confirmed the rather unexpected finding of Greer & Zamenhof [6], who demonstrated that 5-bromodeoxyuridine undergoes cleavage at the glycosidic bond on heating at 100° at neutral pH. We have found, however, that under the same conditions 5-bromouridine exhibits no detectable degradation [1]. Nonetheless, in view of the foregoing, care was exercised in preparation of stock solutions of the glycosides to use the lowest possible temperature to induce solution of the more difficultly soluble compounds like the iodo analogues.

The purity of the compounds was also tested by paper electrophoresis, as well as chromatography with different solvent systems, using excessive quantities so as to make possible detection of less than 1% of non-halogenated absorbing impurities, by means of a dark ultraviolet lamp. An additional criterion was the sharpness of the isosbestic points for the individual compounds [18, 19]. The possible presence of 5,5′-dihalogeno derivatives was tested for in some instances by an examination of the spectrum in alkaline medium at about 230 mμ, under which conditions such compounds should exhibit a maximum at this

[1] This marked lability of bromodeoxyuridine in aqueous medium at neutral pH may very well be the source of the well-known and oft-reported lability of DNA containing biologically incorporated halogeno uracils.

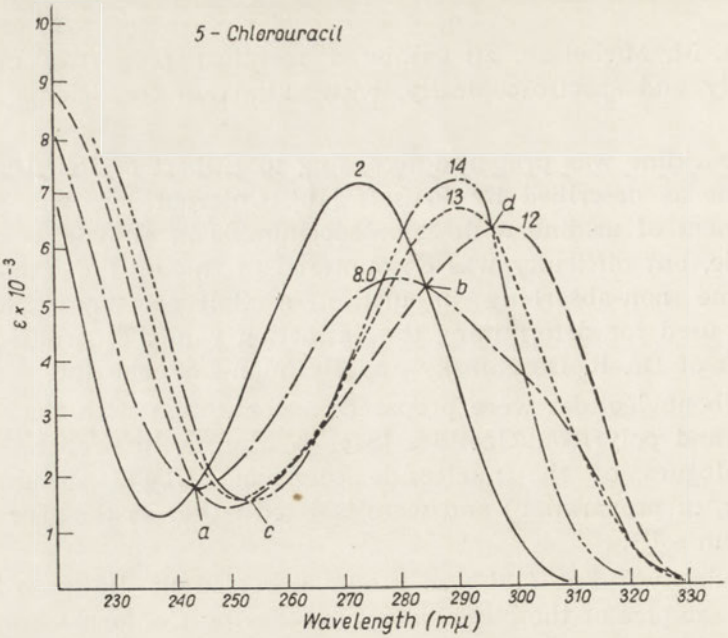

Fig. 5. 5-Chlorouracil at various pH values and exhibiting two ionic equilibria. Isosbestic points at *a* and *b* for first equilibrium with pK_1 8.0; and for second equilibrium at *c* and *d* with $pK_2 < 13$

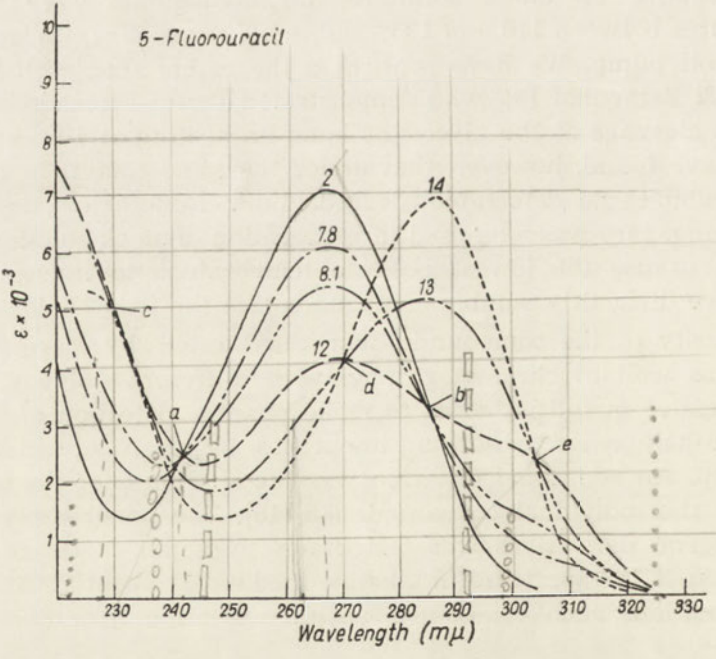

Fig. 6. 5-Fluorouracil at various pH values. Isosbestic points at *a* and *b* correspond to first ionic equilibrium with pK_1 8.0; those at *c*, *d* and *e* are for second equilibrium with $pK_2 \sim 13$

wavelength which decreases with time due to opening of the 3,4 bond of the saturated pyrimidine ring [1, 11].

Buffers used were phosphate, borate and tetraborate - phosphate, usually at molarities of about 0.05 M. No influence of buffer molarity on spectra or dissociation constants was observed over the range 0.05 - 0.1 M; nor did variations in salt concentration, up to 1 M, reveal any important differences. For extreme acid and alkaline pH values, dilutions were made with HCl and NaOH, accepting 0.01 N-HCl as pH 2, 0.1 N-HCl as pH 1 and 0.01 N, 0.1 N and 1 N-NaOH as pH 12, 13, 14, respectively.

A Radiometer Model 22 meter was used for all pH measurements, at a temperature of 20 - 22°, and apparent pK values are considered accurate to 0.05 pH units.

Most of the spectral measurements were made with a Hilger Uvispek, using 10-mm. cuvettes, so that concentrations of the various compounds were of the order of 10^{-4} M. Some measurements were also made with a Unicam SP-500, the two instruments agreeing to better than 1%. All results are expressed as molar extinction coefficients (ε). Taking into account weighing and other manipulations involved, it is estimated that extinction values are correct to better than 1.5%. It must, however, be emphasized that careful cleaning of the cuvettes and application of the usual blank corrections are of considerable importance [cf. 18].

RESULTS AND DISCUSSION

The absorption spectra of the halogenated bases, as well as those of the various *N*-methylated bromouracils, are exhibited in Figs. 1 through 8. Some of the pertinent spectral data have been compiled in Table 1. The dissociation constants, calculated from the variations of optical density with pH at several wavelengths, are shown in Table 2.

The acid strengthening effect of halogenation on all compounds is quite clear (see also Table 2) and it will be seen that it is, in general, only below pH 6 that all the bases exist in the non-dissociated form. That this is the diketo form, as for uracil and thymine, is shown unequivocally by a comparison of bromouracil with its 1-methyl, 3-methyl and 1,3-dimethyl analogues (Fig. 4). As in the case of the corresponding uracil and 5-methyluracil (thymine), the 5-halogenated derivatives also possess two dissociation constants involving the hydrogens on the two ring nitrogens [19]. In fact, as may be seen from Figs. 1, 5, 6 and 7, the acid strengthening effect of halogenation manifests itself likewise for the second dissociation constants, which are lowered sufficiently in the case of fluoro- and iodouracils so that the pK_2 values may be measured quantitatively (Table 2).

Table 1

Spectrophotometric data for various halogenated uracil derivatives

The molar extinction coefficients have been determined with an accuracy of ±1.5%

Compound	Absorption maxima			ε ratios		Isosbestic points		
	pH range	λ (mμ)	ε × 10⁻³	250/260	280/260	pH range	λ (mμ)	ε × 10⁻³
5-Fluorouracil	1.0 - 5.0	268	7.00[a]	0.63	0.78	1.0 - 10.0	242.5	2.50
	7.8	269	6.10				285	3.40
	8.1	269.5	5.40					
	10.0 - 12.0	270	4.10			12.0 - 14.0	230	5.00
	13.0	285	5.20				270	4.10
	14.0	286	6.90				304	2.30
5-Chlorouracil	1.0 - 5.0	275	7.06	0.53	1.25	1.0 - 10.0	244	1.70
	8.0	281	5.40				285	5.30
	8.2	281	5.10					
	10.0 - 12.0	300	6.30			12.0 - 14.0	253.5	1.60
	13.0	292.5	6.60				296	6.20
	14.0	289	7.10					
5-Bromouracil	1.0 - 5.0	277.5	7.14	0.52	1.73	1.0 - 10.0	247.5	3.90
	8.05	282.5	5.40				288.5	5.60
	8.3	287	5.45					
	10.0 - 12.0	302.5	6.90			12.0 - 14.0	266.5	2.20
	13.0	294	6.70				297	6.60
	14.0	291	7.20					

5-Iodouracil	1.0 - 5.0	285	6.15	0.61	1.85	1.0 - 10.0	248.5	1.90
	8.2	292.5	5.80				293	5.80
	8.4	296	5.85					
	10.0 - 12.0	305	7.10			12.0 - 14.0	296.5	6.40
	13.0	298	6.40					
	14.0	292.5	6.40					
1-Methyl-5-bromouracil	1.0 - 5.0	286	8.90	0.50	2.64	1.0 - 14.0	256.5	2.50
	8.0	285	8.03					
	8.3	283	7.41					
	10.0 - 14.0	281	6.03					
3-Methyl-5-bromouracil	1.0 - 5.0	276	6.60	0.52	1.66	1.0 - 14.0	247.5	1.70
	8.0	284	6.00				284	5.90
	8.1	285	6.11					
	8.4	295	6.60					
	10.0 - 14.0	301	10.02					
1,3-Dimethyl-5-bromouracil	1.0 - 14.0	285	8.10	0.53	2.59	—	—	—

[a]Duschinsky *et al.* [3] give a value of 7.07×10^3 at 265-266 mμ in 0.1 N-HCl.

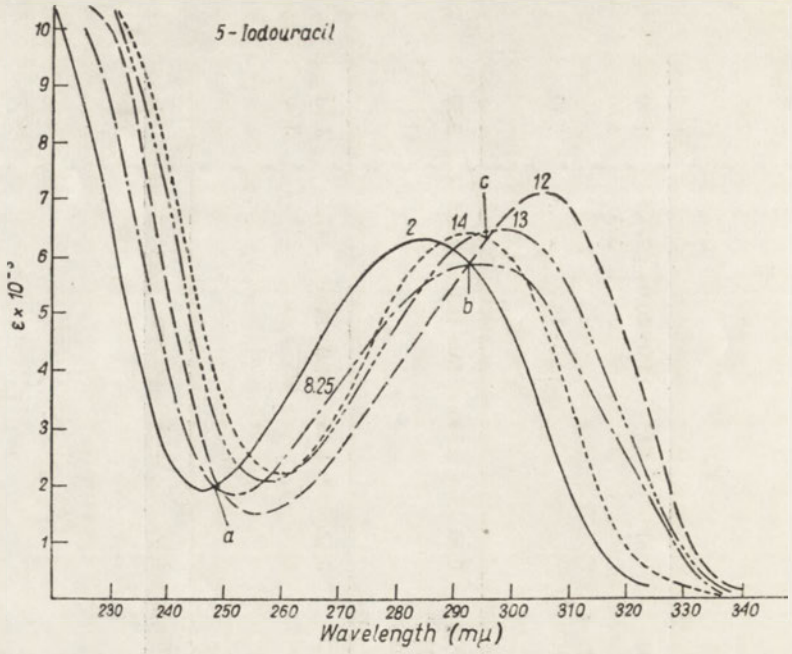

Fig. 7. 5-Iodouracil at various pH values. Isosbestic points at *a* and *b* correspond to first ionic equilibrium with pK_1 8.25; that at *c* is for second equilibrium with $pK_2 < 13$

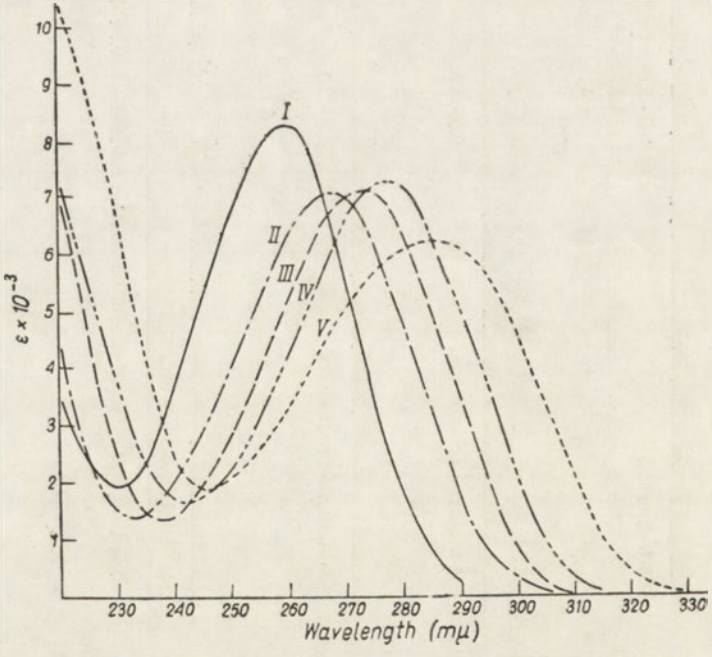

Fig. 8. Absorption spectra at pH 4 of non-dissociated forms of (*I*), uracil; (*II*), 5-fluorouracil; (*III*), 5-chlorouracil; (*IV*), 5-bromouracil; (*V*), 5-iodouracil

From the figures for 1-methyl-5-bromouracil and 3-methyl-5-bromouracil, and by analogy with the corresponding spectra for 1-methyluracil and 3-methyluracil [19], it may be inferred that in bromouracil it is likewise the 2-carbonyl which dissociates first and that the second dissociation involves the number 4-carbonyl. The same conclusion may be drawn from a comparison of the spectra for chloro- and iodouracils with that for uracil itself [19]. For fluorouracil, however, the situation is not unambiguous (see Fig. 6) and it will obviously be necessary to prepare the 1- and 3-methyl derivatives in order to settle this question unequivocally. On the other hand, the spectrum of 5-fluorouridine (Fig. 9), which may be regarded as an analogue of 1-methyl-5-fluorouridine, suggests that the order of dissociation is probably the same as for the other derivatives. Nonetheless the unusual biological properties of 5-fluorouracil and its glycosides indicate the desirability of a more detailed investigation of the singly dissociated form, particularly when it is recalled that at physiological pH values (pH 7.2) at least 13% is in the dissociated form.

Table 2

Spectrophotometrically determined apparent pK values for halogenated uracil derivatives

Values significant to 0.05 pH units

Compound	pK_1	pK_2	Dissociated at pH 7.2 (%)
Uracil	9.50[a]	>13[a]	<1
5-Fluorouracil	8.00[b]	~13	13
5-Chlorouracil	7.95	<13	15
5-Bromouracil	8.05[c]	~13	12
1-Methyl-5-bromouracil	8.30[d]	—	7
3-Methyl-5-bromouracil	8.30	—	7
5-Iodouracil	8.25	<13	8

[a] Taken from Shugar & Fox [19].
[b] Wempen *et al.* [24] give 7.98.
[c] Katritzky & Waring [13] give 7.83 by titration.
[d] Katritzky & Waring [13] give 7.84 by titration.

Contrary to the observation of Katritzky & Waring [13] for 1-methyl-5-bromouracil, it will be seen from Figs. 2 and 3 (cf. Table 2) that the acid weakening effect of *N*-methylation [19] is also present in the case of bromouracil.

The bathochromic shift in the principal absorption maxima due to halogenation of uracil is seen from Fig. 8 to increase in the order F, Cl, Br, I, i.e. in order of decreasing electronegativity *or* in order of

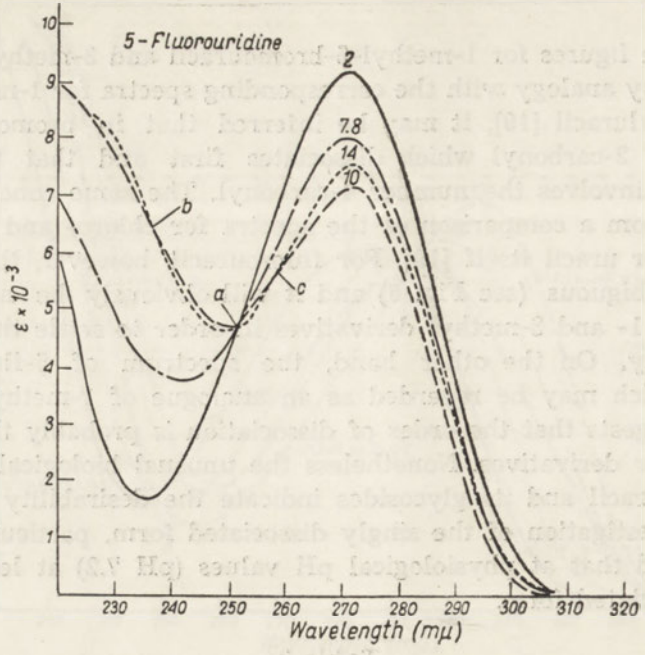

Fig. 9a. Absorption spectrum of 5-fluorouridine at pH values indicated. Isosbestic point *a* is for equilibrium corresponding to dissociation of 4-carbonyl of pyrimidine ring with pK 7.75; isosbestic points *b* and *c* are for dissociation of carbohydrate hydroxyl(s)

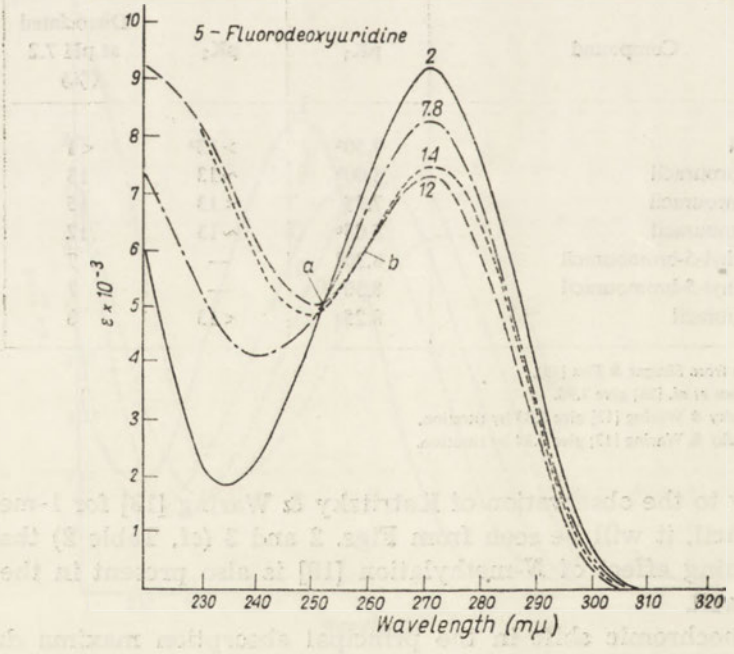

Fig. 9b. 5-Fluorodeoxyuridine at pH values indicated, with isosbestic point *a* for 4-carbonyl dissociation (pK 7.8) and *b* for sugar dissociation

decreasing "bulkiness" or van der Waals' radii of the halogeno substituents. While it is possible that some steric effect results from the physical dimensions of the halogeno substituents, it is most unlikely that this is a major factor in the bathochromic shift since a 5-methyl substituent, the van der Waals' radius of which is only slightly less than that for I (2.0 A as compared to 2.15 A), produces only a small bathochromic shift [19]. (The bathochromic shift in the absorption spectra resulting from halogenation is such that the extinction coefficients at 253.7 mμ are considerably reduced as compared to uracil. This is the wavelength of the "dark" ultraviolet lamps used for detecting nucleic acid derivatives on paper chromatograms; and it is for this reason that 2 to 3-fold larger quantities of halogeno uracils, or their glycosides, must be spotted on the chromatograms to provide a similar level of detection with such lamps).

It should also be noted that the magnitude of the bathochromic shift for the principal absorption maximum is decreased in the dissociated form of 1-methyl-5-bromouracil (Fig. 2), as observed previously, but to a considerably smaller extent, for 1-methyluracil [19]. The magnitude of this decrease is again related to the degree of electronegativity of the halogeno substituent, as will be seen below for the nucleoside analogues.

Attention is directed to Fig. 4, which exhibits the spectra in acid medium, hence of the non-dissociated forms, of bromouracil, 1-methyl--5-bromouracil, 3-methyl-5-bromouracil and 1,3-dimethyl-5-bromouracil. If we compare these four spectra with the corresponding ones for uracil and its *N*-methylated analogues [19], neglecting the bathochromic effect of bromination on the absorption spectra, the correspondence between the two is seen to be quantitatively excellent, i.e. the effect of *N*-methylation of bromouracil on the absorption spectrum is similar to that for uracil itself. In particular, the effects of N_1 and N_3 methylation are additive as in the case of uracil [19]. The self--consistency exhibited between the four curves in Fig. 4, as compared to the corresponding ones for uracil (Fig. 12 in ref. [19]), may therefore be regarded as confirmation of the correctness of the extinction coefficients for the four compounds. Brown *et al.* [2] have reported a molar extinction coefficient for 3-methyl-5-bromouracil of 6.96×10^3 at 275 mμ in acid medium, to be compared with our value of 6.6×10^3 at a maximum wavelength of 276 mμ. The results shown in Fig. 4 substantiate, in our view, the latter value. Brown *et al.* [2] also report an extinction for 1-methyl-5-bromouracil in acid medium as 9.0×10^3 at 265 mμ. Although the discrepancy in location of the maximum is, in this case, probably due in large part to the fact that the maximum is extremely broad (see Fig. 7), and is therefore more apparent than real,

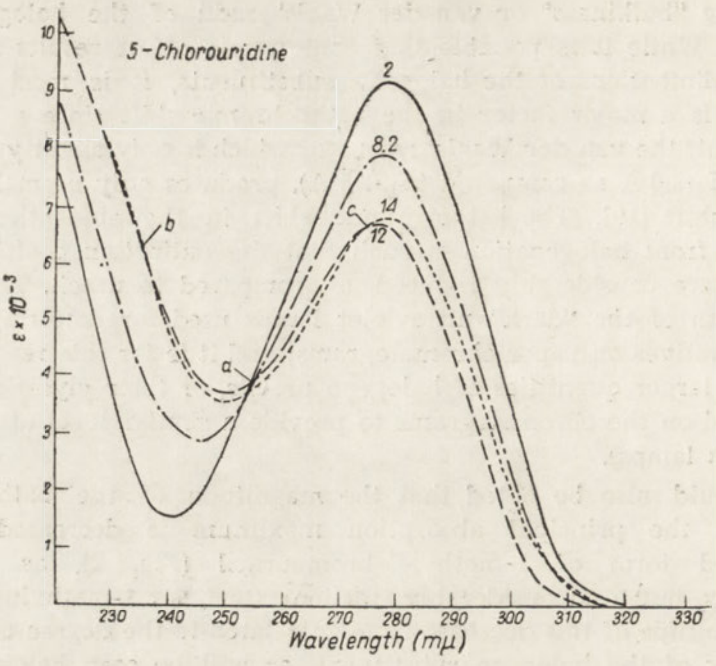

Fig. 10a. 5-Chlorouridine at pH values indicated, with isosbestic point *a* for 4-carbonyl dissociation (pK 8.2) and *b* and *c* for sugar dissociation

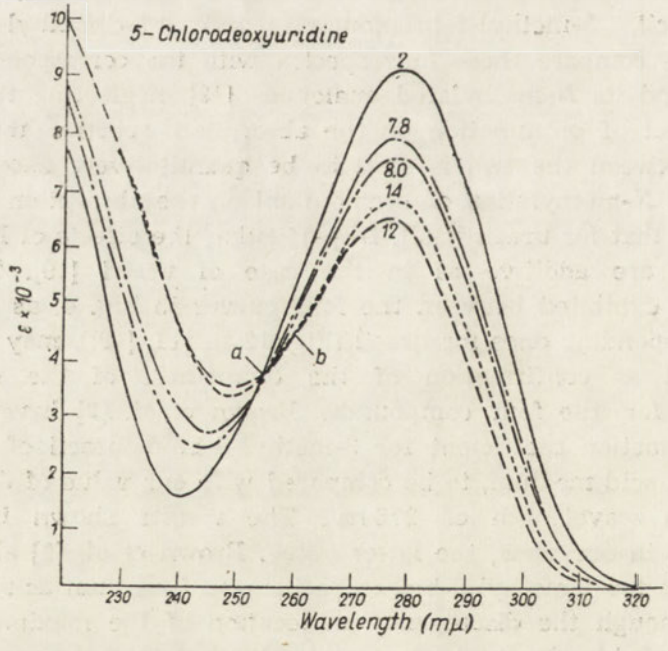

Fig. 10b. 5-Chlorodeoxyuridine at pH values indicated, with isosbestic point *a* for 4-carbonyl dissociation (pK 7.9) and *b* for carbohydrate dissociation

the agreement between extinction coefficients is as good as might be expected.

1,3-Dimethyl-5-bromouracil was found to be unstable in alkaline medium, as previously reported for a number of *N*, *N'*-dimethyl diketopyrimidines (see ref. [11] for literature), and due, at least in part, to opening of the pyrimidine ring at the 3,4 position [11]. The bromo analogue is, however, considerably more unstable in alkali than the non-halogeno diketopyrimidines, so that it is relatively rapidly degraded even at pH 12, like *N*-methyl-5-bromouridine [21]; e.g., for dimethyluracil a similar rate of degradation at room temperature requires a pH between 13 and 14.

The absorption spectra for the halogenated ribonucleosides and deoxyribonucleosides are represented in Figs. 9a through 12b; and the corresponding spectral data and pK values in Tables 3 and 4.

From a general point of view the spectra are qualitatively in accord with what one might expect on the basis of the spectra of the halogenated bases and, in particular, that for 1-methyl-5-bromouracil. There are, however, a few features of some interest which merit comment.

Halogenation produces a bathochromic shift of the principal absorption maxima, relative to the uracil glycosides [4] which, as in the case of the bases, increases with decreasing electronegativity (or increasing bulkiness) of the halogen substituent. However, the hypochromic effect (i.e. reduction in extinction coefficient as compared to uracil glycosides) is only 7 - 8% for the fluoro, chloro and bromo analogues, but very marked (about 23%) for the iodo derivatives. In this respect the results are comparable to those for the free bases (cf. above).

Furthermore, while λ_{max} for the fluoro glycosides is unchanged in alkaline medium, i.e. for the dissociated form, it undergoes a net hypsochromic shift for the chloro and bromo derivatives and a very pronounced one for the iodo glycosides. The order is again, as before, F, Cl, Br, I.

Of some interest in connection with the foregoing is a comparison of 1-methyl-5-bromouracil (Fig. 2) with its analogue 5-bromouridine (Fig. 11a). For the non-dissociated forms, λ_{max} for the former is at 286.5 mμ and for the latter 278.5 mμ. For the dissociated forms, there is a hypsochromic shift of λ_{max} for the former of 6.5 mμ, while for the latter this is only 1.5 mμ. Since the pK values are, if we take the extreme limits of experimental error, similar (Tables 2 and 4), it may be inferred that it is rather the "bulk effect" of the Br substituent which is of importance here. In the corresponding 1-methyluracil and uridine, a similar, but much less pronounced, effect is encountered. It would obviously be of interest to compare the other halogenated ribosides with their corresponding 1-methyl analogues.

Table 3

Spectrophotometric data for various halogenated uracil ribosides and deoxyribosides

Absorption maxima have been determined with an accuracy of ±1.5%

Compound	Absorption maxima			ε ratios		Isosbestic points		
	pH range	λ (mμ)	ε×10⁻³	250/260	280/260	pH range	λ (mμ)	ε×10⁻³
5-Fluorouridine	1.0 - 5.0	271	9.22[a]	0.59	1.03	1.0 - 12.0	251.5	4.80
	7.8	271	8.05					
	7.5	271	8.46					
	10.0 - 12.0	272	7.15			12.0 - 14.0	236	6.40
	14.0	271.5	7.60				258.5	5.70
5-Fluorodeoxyuridine	1.0 - 5.0	271	9.17	0.61	1.01	1.0 - 12.0	252	5.05
	7.8	271	8.20					
	10.0 - 12.0	271	7.30			12.0 - 14.0	262	6.50
	14.0	271	7.45					
5-Chlorouridine	1.0 - 5.0	278	9.11[b]	0.51	1.77	1.0 - 12.0	256	3.85
	8.2	278	7.80					
	10.0 - 12.0	278	6.73			12.0 - 14.0	235	6.30
	14.0	278	6.80				275	6.60
5-Chlorodeoxyuridine	1.0 - 5.0	279	9.16	0·51	1.75	1.0 - 12.0	254	3.70
	7.8	278	7.85					
	8.0	278	7.55					
	10.0 - 12.0	276.5	6.50			12.0 - 14.0	260	4.30
	14.0	276.5	6.75					

5-Bromouridine	1.0 - 5.0	279	9.30[c]	0.54	1.79	1.0 - 12.0	255	3.80
	8.0	279	8.15					
	8.2	279	7.85					
	10.0 - 12.0	278	6.40			12.0 - 14.0	280	6.30
	14.0	278	6.35					
5-Bromodeoxyuridine	1.0 - 5.0	280	9.23	0.51	2.03	1.0 - 12.0	256	3.50
	7.8	280	8.18					
	8.0	280	7.70					
	10.0 - 12.0	280	6.50			12.0 - 14.0	273.5	6.20
	14.0	280	6.80					
5-Iodouridine	1.0 - 5.0	291	7.66[d]	0.65	2.02	1.0 - 12.0	264	3.95
	8.2	287.5	6.95					
	8.4	287	6.63					
	10.0 - 12.0	282.5	5.45			12.0 - 14.0	265	4.00
	14.0	283	5.95					
5-Iododeoxyuridine	1.0 - 5.0	287.5	7.50	0.60	2.05	1.0 - 12.0	260	3.55
	8.2	283.5	6.45					
	10.0 - 12.0	278.5	5.55			12.0 - 14.0	261	3.60
	14.0	282.5	5.90					

[a] Yung *et al.* [26] give a value of 8.95 at 269 mμ in 0.1 N-HCl.
[b] Letters & Michelson [15] give 9.2×10^3 at 274 mμ in 0.01 N-HCl.
[c] Letters & Michelson [15] give 9.4×10^3 at 277 mμ in 0.01 N-HCl.
[d] Letters & Michelson [15] give 8.7×10^3 at 286 mμ in 0.01 N-HCl.

Note: Following completion of the above, samples of all the glycosides, with the exception of the fluoro, were obtained from CalBiochem. The extinctions of all of these in acid medium, as given on the packages, agreed with the above values to within 0.5—1%.

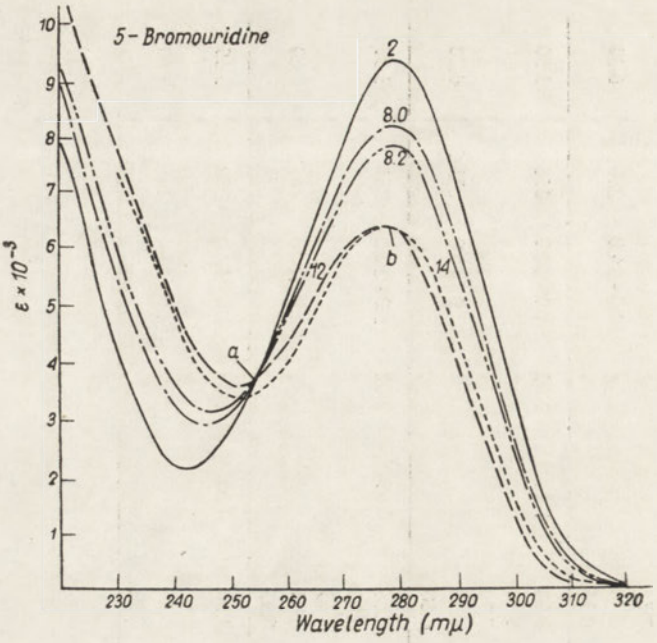

Fig. 11a. 5-Bromouridine at pH values indicated, with isosbestic point *a* for 4-carbonyl dissociation (pK 8.2) and b for carbohydrate dissociation

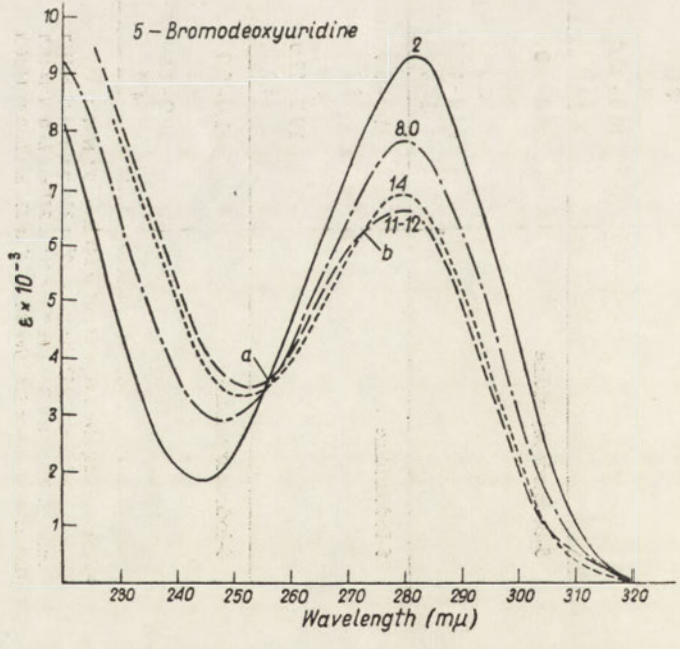

Fig. 11b. 5-Bromodeoxyuridine at pH values indicated, with isosbestic point *a* for 4-carbonyl dissociation (pK 7.9) and b for sugar dissociation

For all the halogenated glycosides, the influence of sugar hydroxyl dissociation is reflected, as in the case of uracil glycosides [4], by a further modification in the absorption spectrum above pH 12 - 13. No attempts were made to estimate the magnitude of the pK value for carbohydrate hydroxyl dissociation, but an examination of Figs. 12a and 12b shows that the spectral shift is appreciable enough for the iodo derivatives to make such measurements feasible. The influence of sugar dissociation on the absorption spectra exhibits in all instances some difference between the riboside and deoxyriboside, testifying to the role of the 2′ hydroxyl in the interaction between the carbohydrate and aglycon rings; but not, as previously pointed out from other considerations [25], to the exclusion of the remaining hydroxyls.

Table 4

Spectrophotometrically determined apparent pK values for halogenated uracil ribosides and deoxyribosides

The pK values are for 4-carbonyl dissociation to within 0.05 pH units. Sugar pK values are all above pH 13 (see Figs. 9 to 12)

Compound	pK_1	Dissociated at pH 7.2 (%)
Uridine	9.25[a]	< 1
Deoxyuridine	9.30[a]	< 1
5-Fluorouridine	7.75[b]	22
5-Fluoroisopropylideneuridine	7.50	33
5-Fluorodeoxyuridine	7.80[c]	17
5-Chlorouridine	8.20[d]	9
5-Chlorodeoxyuridine	7.90	16
5-Bromouridine	8.20[e]	9
5-Bromodeoxyuridine	7.90[f]	16
5-Iodouridine	8.50[g]	5
5-Iododeoxyuridine	8.20	9

[a]Taken from Fox & Shugar [4].
[b]Wempen *et al.* [24] give 7.57.
[c]Wempen *et al.* [24] give 7.66.
[d]Letters & Michelson [15] give 8.5.
[e]Letters & Michelson [15] give 8.5.
[f]Lawley & Brooks [14] give 8.1.
[g]Letters & Michelson [15] give 8.8.

When we examine the effect of halogenation on the dissociation of the pyrimidine 4-carbonyl, it will be noted (Table 4) that the acid strengthening effect is quite similar to that for the bases. But, with the exception of the fluoro glycosides (and it should be borne in mind that fluorine is not a typical halogen), there is a net difference of 0.3 pH

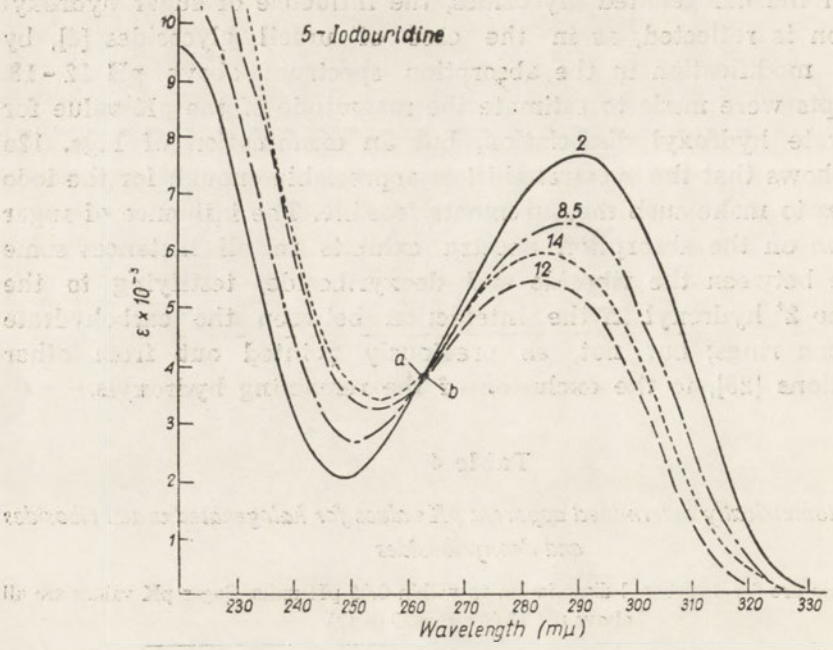

Fig. 12a. 5-Iodouridine at pH values indicated, with isosbestic point *a* for 4-carbonyl dissociation (pK 8.5) and *b* for sugar dissociation

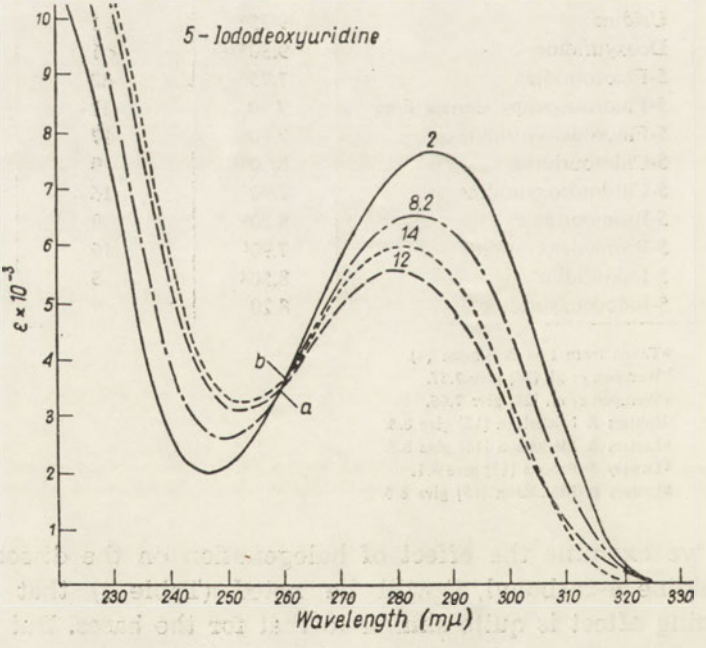

Fig. 12b. 5-Iododeoxyuridine at pH values indicated, with isosbestic point *a* for 4-carbonyl dissociation (pK 8.2) and *b* for carbohydrate dissociation

units between the pK values for the ribosides and deoxyribosides, the latter being more acidic. This again reflects the influence of the 2′ hydroxyl. We have, on the other hand, examined the spectrum of 5-fluoro--2′ : 3′-*O*-isopropylideneuridine [2] as a function of pH and find that the pK for carbonyl dissociation is 7.5 as compared to 7.8 for 5-fluorouridine (Table 4). Furthermore the absorption spectrum at pH 14 indicated that the degree of carbohydrate hydroxyl dissociation, while altered, is still marked, showing that it is not only the 2′ hydroxyl which is responsible for these effects (see ref. [25] for discussion of this point).

No attempts were made to estimate directly the extinction coefficients of the nucleotides. That for 5-bromouridine-2′(3′)-phosphate was measured indirectly by hydrolysing it with acid phosphatase at pH 5 to bromouridine; no significant change in the absorption spectrum could be discerned. For the other halogenated nucleotides, only the 5′-phosphates were available. Measurements of the pK values for carbonyl dissociation of all four nucleotides gave values of 7.75, 8.2, 8.2 and 8.5 for the fluoro, chloro, bromo and iodo derivatives, respectively, i.e. the same values as for the nucleosides (Table 4). These determinations are in disagreement with those of Letters & Michelson [15] who report 8.5, 8.5 and 8.8 for the 2′(3′)-phosphates of chloro, bromo and iodo uridine, respectively.

A titrimetric procedure was used to estimate the pK for dissociation of the secondary phosphate hydroxyl in 5-bromouridine-2′(3′)-phosphate. The titration curve is complex, as might be expected, since the phosphate hydroxyl and 4-carbonyl dissociations overlap, but the results indicated that the pK for secondary phosphate hydroxyl was unaltered relative to that for uridine-2′(3′)-phosphate.

From Tables 2 and 4, as well as from the data cited in the preceding two paragraphs, it is clear that the halogeno uracils and all their glycosides exist in neutral, aqueous medium as a mixture which includes an appreciable percentage of the anionic form. It is consequently of importance, when using these derivatives in biological experiments, to determine whether it is the non-dissociated form which is the biologically active species, or the anion. In most instances a small change in pH will likely suffice for this purpose; where conditions permit, one may employ a pH of 6 for the completely non-dissociated form. It should be noted that, in the experiments of Trautner *et al.* [22], referred to in the introduction, the pH employed was such that the free bromouracil residues would be expected to be largely in the dissociated form. This may not necessarily be the case once the residues are incorporated into a twin-stranded helix.

[2] This compound will be reported on elsewhere (Szer & Shugar, Acta Biochim. Polon., 1963, in press) in connection with the preparation of poly-5-fluorouridylic acid.

Attention should be drawn to the fact that the Br, Cl and I derivatives of uracil and its glycosides show appreciable absorption at wavelengths to the red of 300 mμ. This is certainly not unrelated to the frequent reports on the sensitivity of halogenated deoxyribonucleic acids to the radiation from ordinary "daylight" lamps. Such lamps are known to emit traces of radiation down to as low as 300 mμ, where the halogenated residues absorb and would consequently be expected to react photochemically.

We are indebted to the following for making available compounds useful in the foregoing study: Dr. R. Duschinsky and Hoffman-LaRoche Inc., California Corporation for Biochemical Research, Dr. A. M. Michelson and Dr. W. Szybalski. Our thanks are also due to Mgr. Z. Rybakow and Dr. W. Szer for their assistance in the synthetic work.

SUMMARY

A systematic quantitative tabulation has been made of the ultraviolet absorption spectra of the halogenated uracils, their ribosides and deoxyribosides, as a function of pH; and of the dissociation constants of these, as well as their nucleotides.

The results demonstrate the normal, acid strengthening effect of halogenation on all compounds, so that an appreciable percentage is dissociated at physiological pH. The spectra exhibit the usual bathochromic and hypochromic shifts relative to the non-halogenated compounds, in the order F, Cl, Br, I.

The bases all exhibit two pK values, as for uracil and thymine, corresponding to the 2- and 4-carbonyls. The former is the first to dissociate; but the data for fluorouracil require additional confirmation. The acid strengthening effect of halogenation is pronounced for the pK_2 values, involving the 4-carbonyl; in fact the pK_2 values are sufficiently reduced, by comparison with uracil and thymine, that they are susceptible of accurate measurement.

The similarities and differences between the ribosides and deoxyribosides are discussed. The spectra of all of these are also slightly modified by dissociation of the carbohydrate hydroxyls at high pH. The observation of Greer & Zamenhof as to the thermolability of the glycosidic linkage of 5-bromodeoxyuridine has been confirmed; but, under the same conditions, bromouridine is stable.

The nucleotides exhibit pK values for 4-carbonyl dissociation very similar to those for the nucleosides. In 5-bromouridylic acid the pK of the secondary phosphate hydroxyls is only slightly modified.

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WIDMA ABSORPCYJNE W ULTRAFIOLECIE CHLOROWCOWYCH POCHODNYCH URACYLU I JEGO GLUKOZYDÓW

Streszczenie

Wykonano ilościowe pomiary widm absorpcyjnych w ultrafiolecie chlorowcowych pochodnych uracylu, oraz ich rybozydów i deoksyrybozydów, w zależności od pH. Oznaczono również stałe dysocjacji wyżej wymienionych związków.

Uzyskane dane ilustrują efekt zwiększenia kwasowości tych wszystkich związków przy podstawieniu chlorowcem, co powoduje znaczną dysocjację w fizjologicznym zakresie pH. Widma wykazują zwykłe batochromowe i hypochromowe przesunięcia w stosunku do związków nie podstawionych chlorowcem, wzrastające w szeregu: F, Cl, Br, J.

Wszystkie badane zasady, analogicznie do uracylu i tyminy, wykazują dwie wartości pK odpowiadające dysocjacji grupy 2- i 4-karbonylowej. Grupa 2-karbonylowa dysocjuje pierwsza, lecz dane uzyskane dla fluorouracylu wymagają dodatkowego potwierdzenia. Zwiększenie kwasowości przy podstawieniu chlorowcem jest wyraźne dla wartości pK_2 dotyczącej grupy 4-karbonylowej; w porównaniu z uracylem i tyminą, wartości pK_2 są w dostatecznym stopniu obniżone, co umożliwia dokładny pomiar.

Przedyskutowano analogie i różnice pomiędzy rybozydami a deoksyrybozydami. Dysocjacja grup hydroksylowych reszty cukrowej przy wysokich pH zmienia nieco widma tych związków. Potwierdzono zaobserwowaną przez Greer'a i Zamenhof'a termolabilność glikozydowego wiązania w 5-bromodeoksyurydynie. W tych samych warunkach 5-bromourydyna jest trwała.

Wartości pK dla dysocjacji grupy 4-karbonylowej w nukleotydach są bardzo zbliżone do wartości dla nukleozydów. W kwasie 5-bromourydylowym pK drugorzędowej grupy hydroksylowej fosforanu jest nieco zmienione.

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ISOLATION AND AMINO ACID COMPOSITION OF THE CATHODIC PROTEIN FROM HOG KIDNEY MITOCHONDRIA

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Proteins, which had been extracted from hog kidney homogenate with sulphosalicylic acid, in paper electrophoresis separated into several anodic and cathodic fractions [7]. It has been also demonstrated that the cathodic protein fractions originate from mitochondria. The fastest moving fraction *A* was the largest one, the smaller fraction *B* migrated slower.

In the present paper the isolation and amino acid composition of the cathodic protein fraction *A* from hog kidney mitochondria is reported.

METHODS

From hog kidney homogenate in 0.25 M-saccharose solution the proteins soluble in 0.2 M-sulphosalicylic acid were extracted and concentrated as described previously [7]. The concentrated protein solution was dialysed for 24 hr. against distilled water, then lyophilized and stored at -10°.

The sulphosalicylic acid-soluble proteins were fractionated on carboxymethylcellulose (Serva) columns at 2°. Carboxymethylcellulose was suspended in 0.05 M-acetate buffer, pH 4.2, and packed into the column (1 × 15 cm.) at a pressure of 120 cm. of water. Then 50 mg. of protein dissolved in 5 ml. of acetate buffer was applied to the column and the fractions eluted with a gradient of increasing concentration of potassium chloride in acetate buffer. The mixing vessel containing 150 ml. of acetate buffer was connected by rubber tubings with the column, and with a vessel containing 0.6 M-KCl solution. An electromagnetic mixer was used and during the elution the volume of solvent in the mixing vessel was constant. Fractions of 3 ml. were collected every ten minutes. The concentration of KCl in each fraction was calculated from the equation given by Cherkin *et al.* [2].

Protein was determined in the fractions by the tannin method [5] and by measuring the extinction at 280 mμ in 1 cm. cuvette. The fractions comprising a peak were combined, dialysed, and after lyophilization submitted to paper electrophoresis as previously described [7].

The electrophoretical fraction *A* was hydrolysed for 20 hr. with 6 N-HCl or 14% $Ba(OH)_2$ (Block *et al.* [1], p. 80 and 83), and then its amino acid composition examined by paper chromatography. Six mg. of protein was used for acid, and 3 mg. for alkaline hydrolysis, and the evaporated hydrolysates were dissolved in 1 and 0.5 ml. of water, respectively. Samples of 20 μl. were applied on 3×30 cm. strips of Whatman no. 1 paper. The chromatograms were developed with the ascending technique three times in the solvent system of *n*-butanol - acetic acid - water (4:1:1, by vol.) (solvent 1). For two-dimensional chromatography (developed twice) the same solvent was used in the first run; in the second run 2,6-lutidine - collidine - water (1:1:1, by vol.) with 2% addition of diethylamine was used (solvent 2). The identification of amino acids was made by comparing their R_F values with those of standards chromatographed simultaneously. In dubious cases the areas corresponding to the non-identified amino acids were cut out, eluted with water and rechromatographed in suitable solvents.

Methionine was also checked by oxidation with H_2O_2; SO_2-methionine formed during this procedure has in solvent 1 a different R_F value than methionine. Cystine or cysteine was also checked with sodium nitroprusside [1].

For the quantitative determination of amino acids, except proline, the method of Meyer as modified by Krebs & Bellamy [3] was used, the chromatograms being developed three times in solvent 1. From the same chromatograms, the spots of proline localized with ninhydrin were eluted to 1 ml. of water, and for the quantitative determination of proline the procedure of Messer [6] was adopted.

The amino acids migrating together were determined together: leucine and/or isoleucine; threonine and glutamic acid; glycine with serine and aspartic acid; lysine and histidine. The ratio of threonine to glutamic acid was determined in the following way. From the developed chromatogram the corresponding area, partly including the neighbouring amino acids (alanine, serine and glycine) was cut out and eluted, then the amino acids were rechromatographed using twice the solvent 2. The chromatograms were sprayed with Krebs ninhydrin reagent with 4% (v/v) acetic acid added, and left for 2 hr. at room temperature. A good separation of threonine and glutamic acid from each other, as well as from the neighbouring amino acids was obtained. Threonine and glutamic acid were determined according to Krebs & Bellamy [3] and their molar ratio calculated. The separation and determination of serine, glycine and aspartic acid were made in a similar way. The rechromato-

graphy was carried out twice in phenol - water (10:20, v/v) with 0.3% NH_3 added, then sprayed with ninhydrin solution (without acetic acid) and left for 2 hr. at room temperature. Histidine was separated from lysine after rechromatography in *tert*-butanol - ethyl methyl ketone - water - diethylamine (40:40:20:4, by vol.) [1], but it was not possible to determine their ratio, as the amount of lysine was great, and that of histidine very small.

The test for protein-bound hexoses was made after Winzler [9], and for sialic acid after Svennerholm [8].

RESULTS AND DISCUSSION

The fractionation by column chromatography of sulphosalicylic acid-soluble proteins from hog kidney gave three protein peaks as determined with the tannin method (Fig. 1). Peak *I* was characterized by a considerable absorption at 280 mμ. On paper electrophoresis at pH 8.6 this fraction was homogeneous and moved towards the anode (Fig. 2). The protein peak *II* showed smaller absorption at 280 mμ, and on electrophoresis separated into two anodic fractions, and one cathodic frac-

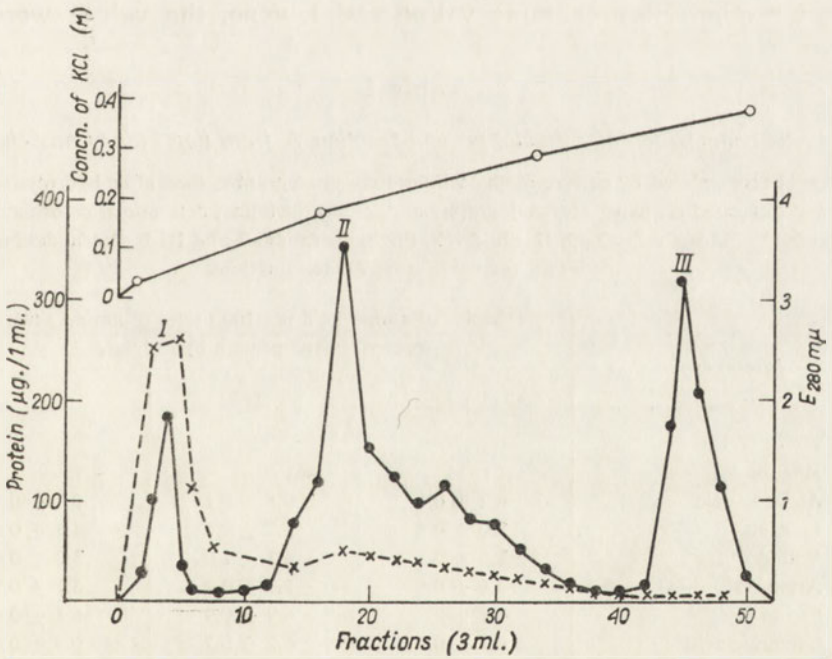

Fig. 1. Gradient elution of sulphosalicylic acid-soluble proteins from hog kidney homogenate, from carboxymethylcellulose (0.05 M-acetate buffer, pH 4.2, with increasing KCl concentration). Experimental details are given in the text. (○), Protein determined by tannin method [5]; (×), extinction at 280 mμ in 1 cm. cuvette

tion corresponding to the fraction *B* in mitochondria [7]. The peak *III* was electrophoretically homogeneous and corresponded to the mitochondrial cathodic fraction *A*. Its absorption at 280 mμ was almost negligible.

The amino acid analysis of fraction *A* carried out by two-dimensional chromatography (Fig. 3) revealed the presence of leucine and/or isoleucine, valine, proline, alanine, threonine, glutamic acid, glycine, serine, aspartic acid, lysine with arginine and histidine.

During the acid hydrolysis, glutamine and asparagine are converted to their amino acids, therefore it is not known whether the demonstrated aspartic and glutamic acids were not derived from asparagine and glutamine. No aromatic amino acids were found which agrees with the lack of extinction at 280 mμ. The presence of tryptophan was excluded by chromatography of alkaline hydrolysates. There were also no sulphur-containing amino acids.

The tests for protein-bound hexoses and for sialic acid in fraction *A* were negative. These results seem to indicate that this protein contains no carbohydrate component.

The quantitative amino acid composition of fraction *A* was made on three different preparations. The results presented in Table 1 show that a notable reproducibility has been obtained for several amino acids, while for others, for example valine and leucine, the values were di-

Table 1

Amino acid composition of cathodic protein fraction A *from hog kidney mitochondria*

The protein was isolated by carboxymethylcellulose chromatography, then after hydrolysis amino acids were separated on paper chromatography and, except histidine, determined colorimetrically. Details see text. Mean values, ±S.D. are given. For preparations I and III 6 determinations were made; for preparation II, 7 determinations

Amino acid	Moles of amino acid per 100 moles of amino acids present in the protein hydrolysate		
	I	II	III
Histidine	<1	<1	<1
Aspartic acid	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
Leucine	2.0 ± 0.5	5.2 ± 0.5	4.1 ± 0.6
Valine	2.6 ± 0.5	4.1 ± 1.0	3.3 ± 0.9
Arginine	3.6 ± 0.3	2.7 ± 0.1	3.1 ± 0.5
Serine	4.7 ± 0.7	6.9 ± 0.9	6.1 ± 0.6
Glutamic acid	6.1 ± 0.5	6.2 ± 0.7	6.9 ± 0.6
Threonine	6.1 ± 0.5	6.2 ± 0.6	6.9 ± 0.6
Glycine	8.1 ± 0.6	11.4 ± 0.8	10.1 ± 0.9
Proline	16.0 ± 2.8	15.4 ± 1.4	14.2 ± 1.5
Alanine	17.2 ± 1.0	17.8 ± 0.7	16.7 ± 1.3
Lysine	32.3 ± 2.1	23.1 ± 2.5	27.5 ± 3.4

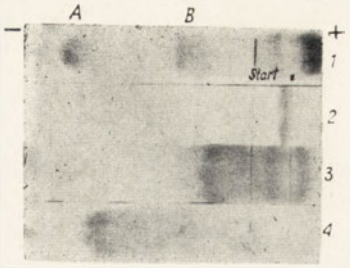

Fig. 2. Paper electrophoresis in veronal buffer, pH 8.6, of sulphosalicylic acid-soluble proteins from hog kidney homogenate, before and after carboxymethylcellulose chromatography . (*1*), Sulphosalicylic acid-soluble proteins from hog kidney homogenate; (*2*), peak *I* from carboxymethylcellulose chromatography; (*3*), peak *II*; (4), peak *III*. *A*, Fast migrating, and *B*, slowly migrating cathodic fractions

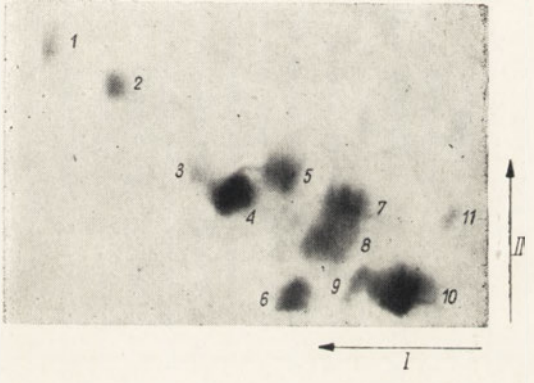

Fig. 3. Paper chromatography of acid hydrolysate of fraction *A* protein. *I* run, *n*-butanol - acetic acid - water (1 : 1 : 1, by vol.); *II* run, 2,6-lutidine - collidine - water (1 : 1 : 1, by vol.) with 2% diethylamine. *1*, Leucine or isoleucine; *2*, valine; *3*, proline; *4*, alanine; *5*, threonine; *6*, glutamic acid; *7*, glycine; *8*, serine; *9*, aspartic acid; *10*, lysine with arginine; *11*, histidine

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vergent. These discrepancies could result either from non-homogeneity of the preparations or from errors in the determinations, as the values of standard deviation were rather high.

The fraction *A* protein is composed in one quarter of basic amino acids. Lysine was found in greatest quantities, about 25%, and arginine formed only 3%. Histidine was found in less than 1%, and was not determined. Alanine constituted about 17%, proline about 15%, and glycine about 10%. Other amino acids were within the range of 2 - 7%, except aspartic acid which amounted to less than 1%.

Recently Machinist *et al.* [4] reported that basic proteins and basic peptides (i.e. synthetic polylysine) can bind phospholipids and acidic glycoproteins, and in this way significantly influence certain enzymic reactions. It may be possible that the demonstrated basic protein in kidney mitochondria has similar properties.

SUMMARY

Hog kidney proteins soluble in 0.2 M-sulphosalicylic acid were separated on carboxymethylcellulose column into three peaks.

The peak *III* was homogeneous in paper electrophoresis and was identified with the cathodic fraction *A* from hog kidney mitochondria. The amino acid composition of this protein was determined.

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IZOLOWANIE ORAZ SKŁAD AMINOKWASÓW BIAŁKA KATODOWEGO Z MITOCHONDRIÓW NEREK WIEPRZA

Streszczenie

Przy pomocy chromatografii na karboksymetylocelulozie rozdzielono rozpuszczalne w kwasie sulfosalicylowym białka nerki wieprza na trzy składowe.

Jedną z nich (*III*) zidentyfikowano jako elektroforetyczną frakcję katodową *A* z mitochondriów nerki. Zbadano jakościowy i ilościowy skład aminokwasów w tym białku.

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NITROGEN COMPOUNDS IN SNAIL *HELIX POMATIA* EXCRETION

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It is generally accepted that in snails there is a seasonal change in the excreted end-product of protein metabolism following the ecological conditions. During dormancy (hibernation or estivation) when the water supply to the body is restricted, uric acid is the end-product. On the other hand, during the period of activity when the snail is feeding on plants the organism is amply provided with water and then urea is a major constituent of nitrogenous wastes. This view originated from the data collected by J. Needham and is discussed by E. Baldwin in his well known book [2]. In Baldwin's interpretation the shift from urea to uric acid excretion permits to avoid the rise of osmotic pressure due to retention of excreta [1]. According to these data, the snail *Helix pomatia* excretes during the feeding period 13.7% of total nitrogen as ammonia, 20% as urea, 10.7% in the form of uric acid, and 6% as amino acids, creatinine and other compounds. It follows that 50% of nitrogen in excreta remains unaccounted for.

The aim of the present study was to fill this gap and to examine the wastes accumulating in nephridia during hibernation as well as during the active period of snail's life. Parallelly the nephridial excreta were collected and analysed.

EXPERIMENTAL

Preparation of material

During hibernation the snails with opercula closed by calcified epiphragm were stored in a refrigerator at 4°. In spring the animals were transferred to glass beakers and kept at room temperature. The bottom of the vessels was covered with a 2 - 4 millimeters high layer of water. After awakening the snails were fed with garden lettuce and cauliflower leaves. They spent most of the time on the walls of the beakers where faeces and nephridial excreta were deposited.

Table 1

Nitrogen compounds in nephridium and excreta from nephridium in snail Helix pomatia

The average values of two determinations are given. The difference between the determinations did not exceed 0.5 mg. per nephridium or per 100 mg. of excreta. The results are presented in mg. N per one nephridium or per 100 mg. of excreta, and in per cent of total nitrogen. The values for uric acid are recalculated from the data of Table 2

Material	Expt. no.	N total (mg.)	Nitrogen of										N accounted for	
			uric acid		xanthine		guanine		total purines		unknown compound			
			(mg.)	(%)	(mg.)	(%)	(mg.)	(%)	(mg.)	(%)	(mg.)	(%)	(mg.)	(%)
Nephridium														
during hibernation	1	43.9	25.0	56.9	8.6	19.5	5.8	13.2	39.5	90.0	0.0	0.0	39.4	90.0
	2	38.1	24.3	63.7	4.8	12.5	5.0	13.1	34.1	89.5	0.0	0.0	34.1	89.5
	3	59.4	44.6	75.0	4.8	8.0	5.9	9.9	55.3	93.0	0.0	0.0	55.3	93.0
	average	47.1	31.3	66.4	6.0	12.7	5.6	11.8	42.9	91.0	0.0	0.0	42.9	91.0
during the feeding period	4	21.9	6.0	27.3	8.1	36.9	4.2	19.1	18.3	83.5	3.3	15.0	21.6	98.6
	5	22.1	5.0	22.6	8.7	39.3	5.3	24.0	19.0	85.9	3.2	14.0	22.2	100.4
	6	26.3	11.1	42.2	8.4	31.9	3.6	13.6	23.2	88.1	1.4	5.3	24.6	93.5
	7	15.1	7.8	51.6	3.6	23.8	3.6	23.8	15.0	99.3	0.0	0.0	15.0	99.3
	average	21.3	7.5	35.2	7.2	33.9	4.2	19.7	18.9	88.7	1.9	8.6	20.8	97.6
Excreta														
first after hibernation	8	33.6	21.7	64.6	12.6	37.5	0.0	0.0	34.3	102.1	0.0	0.0	34.3	102.1
during the feeding period	9	33.1	12.8	38.7	12.0	36.3	3.8	11.5	28.6	86.4	2.1	6.3	30.7	92.7
	10	34.7	15.8	45.5	8.8	25.3	7.4	21.3	32.0	92.2	2.1	6.1	34.1	98.3
	11	31.1	14.1	45.3	9.0	28.9	6.9	22.2	30.0	96.5	0.5	1.6	30.5	98.0
	average	33.0	14.2	43.0	9.9	30.0	6.0	18.2	30.2	91.5	1.5	4.5	31.7	96.1

For analysis during both hibernation and active period the snails were dissected and the nephridia were isolated. In snail the nephridium functions as storage-tissue collecting the end-products of N metabolism transported by haemolymph from pericardial space. During the feeding period these products are partially excreted. The average weight of nephridium is 240 mg. of which only a small part consists of tissue proper. The yellowish, semiliquid content consists mainly of concrements of what was generally regarded to be only uric acid.

The content of each nephridium was rinsed out with distilled water into a 100 ml. flask. A saturated solution of lithium carbonate was instilled under shaking and moderate heating until all concrements were dissolved. Distilled water and a few crystals of thymol were added to make up the volume to 100 ml., and the solution was stored in a refrigerator.

The excreta deposited by the feeding snails on the walls of glass beakers consisted of two separable parts, the one being nephridial excreta and the other faeces. The yellowish nephridial excreta were collected and analysed during April, May and June. A 100 mg. sample of air dry excreta was dissolved in 50 ml. of distilled water with thymol, and lithium carbonate added as mentioned above.

In solutions of nephridial content and of excreta the total nitrogen as well as the nitrogen of some compounds was estimated. The results are presented in Table 1.

Total nitrogen

This was estimated after Kjeldahl in 5 ml. of the solution using sulphuric acid supplemented with potassium sulphate and small amounts of cupric sulphate. Results are calculated as mg. N in the whole nephridium or in 100 mg. of excreta.

During hibernation the average content of N in one nephridium was 47 mg. During the feeding period only 21 mg. was found. In excreta the total N amounted to 33 mg. per 100 mg. of air dry weight.

Ammonia

For ammonia estimation the solutions were prepared from fresh material omitting the application of lithium carbonate. Ammonia was assayed by steam distillation and titration [13, p. 890]. No ammonia was found, and the direct testing with Nessler reagent was also negative both in nephridia and in excreta.

Urea

The presence of urea was checked by Engel & Engel [10] modification of the xanthydrol method and by chromatography using p-dimethylaminobenzaldehyde [6] for locating the spots. With neither method

the urea was detected. This, however, does not exclude the presence of traces of urea.

Creatine and creatinine

These compounds were assayed with Jaffe's reaction [13, p. 899 and 903]. The results for nephridia indicated the presence of creatine and creatinine amounting to 0.5% of total N, but this figure can include also some other chromogens, as specificity of the method is not very high. The same content was also found in extracts from excreta.

Amino acids

Chromatograms were prepared after Leone & Guerritore [17] and Markham & Smith [21]. The composition of the solvents was the same as for guanine and xanthine estimation (see below). Nephridia from hibernating and feeding snails as well as collected excreta were tested but in no case any ninhydrin-positive spots were detected.

Proteins

In solutions prepared from nephridia of hibernating snails small amounts of protein (from traces to 12 mg. per nephridium) were detected. The protein was estimated after Lowry *et al.* [19] in natrium tungstate precipitate dissolved in NaOH. The results were calculated as N using the standard value of 16% for the N content in proteins. The protein N averaged 2% of total N, ranging between traces and 4%. During the active period of snail's life no protein was found in extracts from nephridia. We interpret this difference as being due to an artifact, attributing the protein content during the winter experiments to the excessive mechanical treatment of the nephridia, which was necessary to liberate the thickly packed concrements of uric acid.

Allantoin

Neither in nephridia nor in excreta the presence of allantoin was detected by paper chromatography separation and by testing with p-dimethylaminobenzaldehyde [11].

Uric acid

The assays were performed by the colorimetric method after Caraway [5]. The low specificity of this method was no objection since it has been stated that in extracts from nephridia as well as those from excreta no phosphotungstic chromogens were left after decomposition of uric acid by uricase (prepared after Leone [16]). In Table 2 are presented the results of uric acid estimation expressed in mg. per one nephridium

Table 2

Purines in nephridium and excreta from nephridium in snail Helix pomatia

The values for uric acid were determined colorimetrically [5]; for guanine and xanthine they were recalculated from the Table 1. The values represent the quantity of purines in mg. per one nephridium or per 100 mg. of excreta

Material	Expt. no.	Uric acid	Xanthine	Guanine	Sum of purines
Nephridium					
during hibernation	1	75.0	23.4	12.5	110.9
	2	73.0	12.9	10.8	96.7
	3	134.0	12.9	12.7	159.6
	average	94.0	16.4	12.0	122.4
during the feeding period	4	18.0	22.0	9.1	49.1
	5	15.0	23.6	11.4	50.0
	6	33.5	22.8	7.8	64.1
	7	23.5	9.7	7.8	41.0
	average	22.5	19.5	9.1	51.1
Excreta					
first after hibernation	8	65.4	34.2	0.0	99.6
during the feeding period	9	38.5	32.6	8.2	79.3
	10	47.5	23.9	15.9	87.3
	11	42.4	24.4	15.0	81.8
	average	42.8	26.9	12.9	82.7

or per 100 mg. of excreta, and in Table 1 calculated as mg. N or as percents of total nitrogen.

During hibernation the nephridia contained on the average 94 mg. of uric acid representing 66% of total nitrogen. In feeding snails (April, May, and June) the average content of uric acid was only 22 mg., amounting to 35% of total N.

During the same period the content of uric acid in excreta was estimated. It was found that the uric acid N contributed to 43% of the total N content in excreta. In one experiment concerning the excreta of a single snail immediately after awakening, the content of uric acid was higher reaching the level found in nephridia of hibernating snails, i.e. 64% of total N.

An assay was also made for the presence of uric acid riboside which is known as a component of ox erythrocytes and has been found in this laboratory in insects [14]. The solutions obtained from nephridia and excreta of snails were submitted to paper chromatography after Leone & Guerritore [17] but uric acid riboside was not detected. Also the amount of uric acid was not augmented when these solutions were hydrolysed at 100° [7].

Guanine and xanthine

Needham [22] reported that in nephridia and excreta of snails some other purines besides uric acid are present. An attempt was made to identify them by means of chromatography in the following solvent systems: (1), butan-1-ol - formic acid - water (77:13:10, by vol.) [21]; (2), 60% solution of propan-1-ol in water [17]; (3), propan-1-ol - water (3:1, v/v) [24]; (4), butan-1-ol - acetic acid - water 4:1:1, by vol.) for the first run and acetone - butan-1-ol - water (80:10:10, by vol.) [9] for the second run in the same direction. The ascending one-dimensional chromatography on Whatman no. 3 paper was used. The chromatograms were examined in ultraviolet light with a 260 mμ filter and four spots were found. One of them tested with *N*,2,6-trichlorobenzoquinoneimine after Berry *et al.* in the modification of Forrest *et al.* [12] proved to be uric acid. Two other spots stained red with the reagent of Reguera & Asimow [25]. This reaction is characteristic for purine bases and the R_F of the spots, as compared with that of uric acid, pointed to their identity

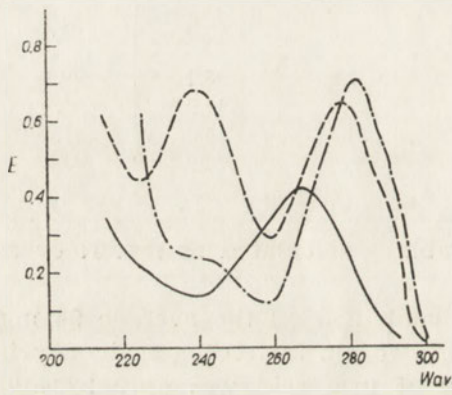

Fig. 1

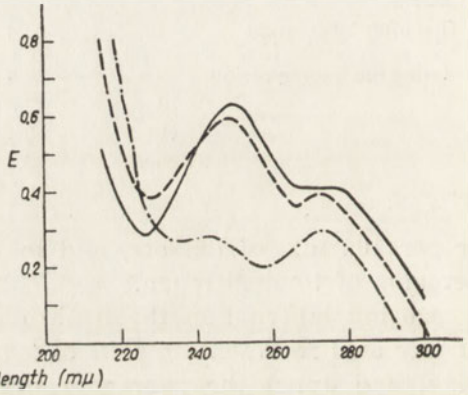

Fig. 2

Fig. 1. The absorption spectra of the eluate from the spot next to that of uric acid. Details see Methods. (———), pH 2.5; (— — —), pH 9; (— - —), pH 12

Fig. 2. The absorption spectra of the eluate from the spot next but one to that of uric acid. Details see Methods. (———), pH 2; (— — —), pH 7; (— - —), pH 9

with xanthine and guanine. To identify them, the absorption spectra of the eluates were examined. For this purpose chromatography in the solvent system no. 3 was carried out. In this system uric acid moved as the last one, and so the tails which always follow this spot did not interfere with isolation of other compounds. The corresponding areas were cut out and eluted in successive experiments with 0.01 N-HCl, 0.01 N-NaOH, and distilled water. The pH during elution was kept adjusted under control of a pH-meter. The absorption of the eluates

was examined in the Unicam spectrophotometer in 1 cm. layer. The curves obtained in this examination were compared with those published by Beaven *et al.* [3]. The absorption spectrum of the spot next to that of uric acid (Fig. 1) corresponded to xanthine whereas that of the next but one (Fig. 2), to guanine. Both these bases were detected on each chromatogram derived from nephridia or from excreta. The only exception was the experiment no. 8 where guanine has not been detected, but we don't feel justified to attribute significance to this single result.

The fourth spot, detected in chromatograms by UV examination, had the highest R_F value. It did not stain with Reguera & Asimow reagent, and the pentose test after Mejbaum [20] in the eluate was also negative. The absorption spectrum of the eluate is presented in Fig. 3. So far

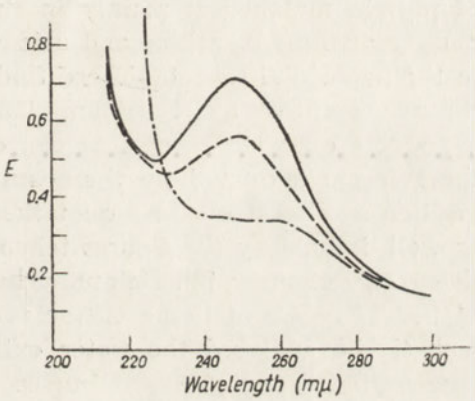

Fig. 3. The absorption spectra of the spot with the highest R_F. Details see Methods. (———), pH 3; (— — —), pH 7; (— - —), pH 14

the spot remains unidentified except that the presence of nitrogen in this compound has been stated. This spot was always found in chromatograms from nephridia and excreta of snails during the period of activity, it was, however, absent during hibernation.

The amounts of guanine, xanthine and the unidentified compound were determined by the estimation of nitrogen. For this purpose 5 ml. of the solution of nephridial content or of excreta was instilled along one side of a sheet of Whatman no. 3 paper and chromatographed in solvent system no. 3. The obtained bands were eluted with 0.1 N-KOH. The whole eluates after being tested spectrophotometrically between 210 and 300 mμ were digested, and N was estimated after Kjeldahl.

In nephridia of hibernating snails guanine-N and xanthine-N contributed each over 10% of total nitrogen, forming together about 25% of total N. This means that all purine derivatives, including uric acid (66%), form 90% of total N content in nephridia.

In nephridia of feeding snails the content of uric acid was greatly decreased due to its preferential elimination in excreta. Since the

decrease was not so marked for other purines, their percentage contribution to total N was almost doubled reaching 53%. Including the uric acid N, we obtained the figure of 89% of total N for the three purine derivatives. The above mentioned unidentified UV absorbing compound represented about 8% of total N.

The ratio of guanine-N to xanthine-N to uric acid-N in nephridia was 1:1.1:5.5 during hibernation, and 1:1.7:1.75 in spring, as against 1:1.7:2.4 in excreta. The amount of purine derivatives in excreta reflected the composition of the nephridial content except that uric acid was eliminated in preference to both bases.

DISCUSSION

The presented data indicate that the snail deposes the waste product of protein metabolism mainly in the form of three purine derivatives namely guanine, xanthine and uric acid, which make up 90% of total N in the nephridial excreta. These findings are strikingly at variance with those presented by Needham [22] and Baldwin [2]. Looking for a possible explanation of this discrepancy we have checked carefully the references quoted by these authors. We have found that the key position in almost all their quotations, and those of many other authors as well, is held by the figures taken from a table first published in the thesis of Delaunay [8]. Delaunay had investigated nitrogen compounds of body fluids and tissue extracts of various invertebrata. In *Helix pomatia* he analysed the water extract of nephridia prepared in such a way that about 80% of purine compounds must have remained unextracted. This is clearly shown by the fact that his extract represented only 522 mg. N per 100 g. of nephridium tissue of feeding snails, whereas our figures recalculated in the same way as his, attain about 10 000 mg. When Delaunay's figures are referred to total N values as established in our investigation, then his evaluation of urea, ammonia and amino acids would cover only 1% of total N. Such an amount could very likely be overlooked in our analysis, concerned with the major constituents and therefore carried out on small samples of material. Delaunay himself was aware of the inadequacy of his extraction procedure and he referred to his material as "water soluble nitrogen substances".

Notwithstanding this restriction he overestimated the role of urea and ammonia in nephridial content, and this seems to have influenced his views about the excretion in snails. He assumed that there are two different kinds of excreta in this species i.e. solid ones consisting mainly of purine compounds, and liquid ones containing mainly ammonia and urea. To collect the latter he kept the snails partially immersed in distilled water and estimated the nitrogen compounds

accumulating in this water. His figures for various substances in this series are expressed only in percentages of total nitrogen found. These results were regarded by Delaunay as representing the chemical composition of the "liquid excretion", but later they were accepted by other authors as representing snail excretion in general. Actually, they probably represent the product of diffusion into hypotonical medium under quite unphysiological conditions. In our culture the snails had ample opportunity to dip in water but we have never observed them to do so, or to void a liquid form of excreta.

Our results leave little doubt as to the uricotelic character of the metabolism in the snail. Delaunay interpreted his findings as a proof of ureotely operating in the snail in the period of feeding. He was also the author of the suggestion that uric acid in hibernating snail could be derived from urea and tricarbonic acids according to Wiener's hypothesis. Delaunay's conclusion, accepted and popularized by Needham and Baldwin, impressed heavily further research in this field for almost four decades. Many investigations have been undertaken to elucidate the way of urea synthesis in snail and to check the occurrence of uric acid synthesis after Wiener's scheme. Only in 1962 we have met with three papers stimulated by these erroneous views. One of them originated in our own laboratory and aimed at the demonstration of urea cycle enzymes in *Helix pomatia* [23]. We have found ornithine transcarbamylase and very active arginase, but it proved impossible to demonstrate the transition from citrulline to arginine, and we feel justified to exclude the presence of the full ornithine cycle in this species. The second paper (Linton & Campbell [18]) reports on similar investigation in *Otala lactea*. The authors have found an enzyme splitting argininesuccinic acid but were also unable to demonstrate the transition from citrulline to arginine. Nevertheless, their conclusion was that the ornithine cycle is operating in Gastropoda, and they hoped to prove it in further research. The third paper, published by Bricteux-Grégoire & Florkin [4] deals with an attempt to examine the hypothetical transition of urea to uric acid in *Helix pomatia*, applying ^{14}C-labelled urea. The result was quite negative but at the same time it was demonstrated that the label was situated in C_6 of uric acid. So far as we know this is the first experimental result indicating that the synthesis of uric acid in *Helix pomatia* is following the same pattern as in birds, insects and microorganisms.

It seems obvious that all three quoted investigations were based on the erroneous interpretation of Delaunay's experiments. It is to be hoped that in future the misconception of ureotely in Gastropoda as well as that of Wiener's synthesis will vanish from the Comparative Biochemistry.

Interpreting the high content of guanine and xanthine in nephridium and excreta we would like to call the attention to their very different solubility. At 20° the solubility per 100 ml. of water is 0.5 mg. for guanine, 50 mg. for xanthine and 2.5 mg. for uric acid [15]. During dormancy the snail is compelled to store considerable amounts of uric acid which leads as well to accumulation of its next precursor, the relatively water-soluble xanthine. It seems that this could affect the osmotic equilibrium, and that the reversible amination of a part of xanthine into poorly soluble guanine would act as a sort of buffer of the osmotic balance. Besides, guanine allows to store 20% more of nitrogen per one purine ring. This hypothesis is to some extent supported by our finding that the ratio of guanine to xanthine rises during hibernation and falls during the period of snail's activity.

SUMMARY

In the snail *Helix pomatia* 90% of total nitrogen in the content of nephridia and in excreta consists of uric acid, xanthine and guanine. During the feeding period the total N content in the nephridia is only 50% of that in hibernating snails. This decrease is due to the elimination of uric acid. Urea, ammonia and allantoin were not detected. The proportion of uric acid to both purine bases is discussed regarding their different solubility and its bearing on osmotic pressure.

The causes of the discrepancy between these findings and those of other authors are discussed and explained.

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ZWIĄZKI AZOTOWE W WYDALINACH ŚLIMAKA WINNICZKA

Streszczenie

W nefridium ślimaka winniczka *(Helix pomatia)* podczas hibernacji i żerowania 90% azotu ogólnego stanowią guanina, ksantyna i kwas moczowy. Ilość azotu ogólnego w nefridium zmniejsza się podczas żerowania do połowy w porównaniu do okresu hibernacji, przy czym głównie spada ilość kwasu moczowego. Skład wydalin jest zbliżony do składu zawartości nefridium. Zarówno w nefridium jak i w wydalinach nie stwierdzono mocznika, amoniaku ani allantoiny. Porównując rozpuszczalność stwierdzonych u ślimaków puryn rozważono możliwość regulacji ciśnienia osmotycznego przy pomocy ich wzajemnego stosunku ilościowego.

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THE ANABOLIC PATHWAY OF URACIL IN HIGHER PLANTS

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In earlier studies we have reported that plants fed with carbamylaspartic acid [5, 6] and orotic acid [3, 4] respectively synthesize rapidly considerable quantities of uracil. Therefore we have tentatively suggested that in higher plants uracil may be an intermediate on the path of pyrimidine nucleotides biosynthesis from carbamylaspartic acid and from orotic acid. This suggestion, however, has thus far not been experimentally confirmed and therefore an attempt has been made to obtain some information concerning the possible role of uracil in nucleotide synthesis. This was particularly interesting in view of recent experiments by Barnes & Naylor [1], who have proved the existence of a very active pathway of pyrimidine catabolism in higher plants.

MATERIALS AND METHODS

All reagents employed were obtained from commercial sources. [2-^{14}C]Uracil was a sample from The Radiochemical Centre, Amersham, England.

The plant material used, consisted of excised 5 days old wheat blades (variety Dańkowska 40). Uracil was introduced into the plant tissue as previously reported [8], a solution of [2-^{14}C]uracil with specific activity of 30,000 counts/sec./μmole having been used. In all cases samples of 2 g. of fresh weight of excised wheat blades were employed.

In the first experiment (Table 1) three samples of plant material were incubated with 2 ml. portions of 2 mM solution of [2-^{14}C]uracil and one was incubated with distilled water, serving as a control sample. Feeding with the uracil solution was interrupted after 30 min., 2 hr. and 4 hr., respectively. The ingestion of water into the control sample was stopped after 4 hr.

In the second experiment (Table 2) the quantities of uracil used were greatly reduced. The plant material was incubated with 2 ml. portions of a 0.05 mM solution of [2-^{14}C]uracil to which 10 μmoles each of ATP, ADP and KH_2PO_4, respectively, were added in order to

Table 1

Amounts and specific activities of pyrimidine derivatives isolated from wheat blades fed with [2-^{14}C]uracil

Incubation at 25° with 4 μmoles uracil per 2 g. of wheat blades. Controls were incubated for 240 min. without uracil added. Amounts are expressed in μmoles per 2 g. of fresh tissue weight, specific activities (Sp. act.) in counts/sec./μmole

Expt. no.	Time of feeding (min.)	Uracil				In acid-soluble fraction						In acid-insoluble fraction			
		absorbed (μmoles)	metabol. (μmoles)	recovered		Uridine		5′-UMP		5′-CMP		2′(3′)-UMP		2′(3′)-CMP	
				(μmoles)	(Sp. act.)	(μmoles)	(Sp. act.)	(μmoles)	(Sp. act.)	(μmoles)	(Sp. act.)	(μmoles)	(Sp. act.)	(μmoles)	(Sp. act.)
1	Control	—	—	0.39	—	0.63	—	0.08	—	0.03	—	1.08	—	1.20	—
2	30	0.63	0.58	0.44	3,900	0.86	51	0.14	7.6	0.07	6.1	0.97	1.5	1.13	1.0
3	120	1.12	0.94	0.57	7,440	0.69	150	0.09	31.6	0.06	17.6	1.02	9.3	1.15	6.5
4	240	2.66	2.23	0.82	16,100	0.67	304	0.08	102.7	0.05	48.4	0.95	17.0	1.16	14.0

ascertain a possible influence of the various sources of phosphates upon the anabolic reactions of uracil. All the solutions were brought to pH 6.0 and the incubation of the samples was stopped after 6 hr.

The extraction, separation, identification and the quantitative determination of the particular pyrimidine derivatives, as well as the measurements of radioactivity were performed as already previously described [8, 6].

RESULTS

Results presented in Table 1 show that under the experimental conditions described, only a small proportion of the uracil intake accumulated in the plant tissues. Well over 80% of uracil underwent rapid catabolic and anabolic changes. Already after 30 min. of incubation of the wheat blades with uracil, some measurable radioactivity was detected in various pyrimidine derivatives, although the sum total recovered in all these compounds amounted to less than 0.5% of the uracil catabolised by the reductive pathway and by degradation to CO_2. The highest radioactivity was observed in uridine, less in the acid-soluble fraction (5′-UMP and 5′-CMP) and still less in pyrimidines of the polynucleotides, 2′(3′)-UMP and 2′(3′)-CMP. No radioactivity was detected in purines and their derivatives after hydrolysis of the acid-insoluble fraction.

Prolongation of incubation of the tissue with uracil lead to very considerable increases in specific activity of the pyrimidine derivatives, with the activity of the nucleotides growing faster than that of uridine.

The incorporation of labelled uracil into uridine and the pyrimidine nucleotides was not accompanied by any increases of these compounds in the plant tissue, as the quantities recovered were approximately the same as in the control sample.

In the second experiment (Table 2) despite a 40-fold dilution of the uracil used, it was also possible to detect marked incorporation of the uracil label into uridine and pyrimidines of the polynucleotides. However, this time no radioactivity was found in the mononucleotides of the acid-soluble fraction (No. 1, Table 2). The addition of ATP, ADP and inorganic phosphate, respectively, caused considerable stimulation of the incorporation of [2-^{14}C]uracil into uridine as well as into pyrimidines of the polynucleotides (No. 2, 3, 4, Table 2). Similarly as in the experiments described in Table 1, despite increases in radioactivity, the molar concentration of the investigated pyrimidine derivatives remained essentially unchanged.

DISCUSSION

The presented data suggest that [2-^{14}C]uracil was incorporated into uridine and the pyrimidine nucleotides already after 30 min. of incubation, whereas the purines and their derivatives remained inactive. When

Table 2

Amounts and specific activities of pyrimidine derivatives isolated from wheat blades fed with [2-^{14}C]uracil with the addition of ATP, ADP and inorganic phosphate

Incubation 6 hr. at 25°. Amounts expressed in μmoles /2 g. of fresh tissue weight, specific activity (Sp. act.) in counts/sec./μmole, ± S.E. Standard error was calculated using equation according to Verchovskaja [12]

Expt. no.	Substrate	μmoles/ 2 g. blades	In acid-soluble fraction								In acid-insoluble fraction			
			Uracil		Uridine		5′-UMP		5′-CMP		2′(3′)-UMP		2′(3′)-CMP	
			(μmoles)	(Sp. act.)	(μmoles)	(Sp. act.)	(μmoles)	(Sp. act.)	(μmoles)	(Sp. act.)	(μmoles)	(Sp. act.)	(μmoles)	(Sp. act.)
1	[2-^{14}C]Uracil	0.1	0.24	433±28	0.30	207±10	0.08	<0.25	0.03	<0.25	1.08	1.37 ± 0.25	1.25	0.56 ± 0.25
2	[2-^{14}C]Uracil ATP	0.1 10	0.22	468±28	0.35	294±11	0.09	<0.25	0.04	<0.25	1.14	2.44 ± 0.29	1.44	1.33 ± 0.27
3	[2-^{14}C]Uracil ADP	0.1 10	0.19	511±34	0.32	238±11	0.09	<0.25	0.03	<0.25	1.10	2.83 ± 0.30	1.28	1.35 ± 0.27
4	[2-^{14}C]Uracil KH_2PO_4	0.1 10	0.16	440±31	0.33	435±14	0.09	<0.25	0.03	<0.25	1.05	3.16 ± 0.30	1.24	1.36 ± 0.27

the time of incubation was extended to 6 hr. (No. 1, Table 2) the specific activity of uridine was extremely high and amounted to about half of the specific activity of the uracil isolated from the plant tissue. This would confirm our previous suggestion that uracil may be an intermediate in the biosynthesis of nucleotides in higher plants [3, 6, 4].

Somewhat similar findings were reported by King & Wang [7], Šebesta *et al.* [10], Bonner & Zeevaart [2] using various pyrimidine analogues, as well as by Tunis & Chargaff [11] who described the synthetic ability of the nucleoside phosphotransferase in higher plants. The anabolic metabolism of uracil in animal tissues and microorganisms has been repeatedly reported. However, with the exception of fast regenerating tissue and cancerous growth, the incorporation of uracil into the nucleotides was negligible and the introduced uracil underwent rapid degradation [see: Schulman, 9].

The specific activity of the pyrimidine nucleotides was very low in comparison with the specific activity of the uracil isolated from the plant tissue and therefore it is not probable that the anabolism of uracil constitutes the only source of pyrimidine nucleotide synthesis in higher plants.

On the other hand the specific activity of uridine isolated from the tissue was of the same order as that of the isolated uracil (No. 1, Table 2). This fact in conjunction with the previously reported rapid synthesis of uridine in plants fed with carbamylaspartic acid [8, 5, 6] and orotic acid [3, 4] leads to the assumption that uridine may play a much bigger part in the biosynthesis of nucleotides than thus far reported. Further studies using labelled uridine are indicated.

Contrary to our anticipations, we have observed a measurable incorporation of the label into pyrimidines of the polynucleotide chain, whereas the pyrimidine mononucleotides remained inactive (Table 2). These results may sugest that 5′-UMP need not be an intermediate in the synthesis of the polynucleotides from uracil, particularly in view of the fact that the dilution of [2-^{14}C]uracil in the medium caused an incomparably bigger drop in the specific activity of 5′-UMP and 5′-CMP than of 2′(3′)-UMP and 2′(3′)-CMP (compare Tables 1 and 2). It is not unlikely that in the anabolism of uracil in higher plants some enzyme system may participate which catalyses the synthesis of polynucleotides to the exclusion of the mononucleotide level.

The data presented in Table 2 indicate that ATP, ADP and inorganic phosphates have a marked stimulating effect upon the anabolic pathway of uracil metabolism in higher plants. As all the three sources of phosphate have shown similar influence upon the utilisation of uracil, it is concluded that the adenine nucleotides act merely as phosphate donors for the synthesis of the polynucleotide chain. The stimulating

effect of phosphate upon the synthesis of uridine must be of some indirect nature and further results will be reported later.

SUMMARY

1. Excised wheat blades were fed with [2-^{14}C]uracil which was rapidly converted to uridine.

2. When the wheat blades were incubated with 2 mM of uracil, then radioactivity was found both in the pyrimidine mononucleotides and pyrimidines of the polynucleotides.

3. When the concentration of uracil was 0.05 mM, radioactivity was observed only in the polynucleotides.

4. Inorganic phosphate stimulated the incorporation of labelled uracil into uridine and polynucleotides.

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ANABOLIZM URACYLU W ROŚLINACH WYŻSZYCH

Streszczenie

1. Dokarmiano odcięte źdźbła pszenicy [2-^{14}C]uracylem i stwierdzono szybką przemianę uracylu do urydyny.

2. Przy użyciu 2 mM roztworu uracylu odnajdywano radioaktywność w mononukleotydach pirymidynowych i w pirymidynach polinukleotydów.

3. Przy użyciu 0.05 mM roztworu uracylu radioaktywność stwierdzono tylko w polinukleotydach.

4. Ortofosforan stymulował włączanie znakowanego uracylu do urydyny i polinukleotydów.

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MICROBIOLOGICAL DETERMINATION OF DEOXYRIBONUCLEIC ACID

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The microbiological DNA assay, introduced by Hoff-Jørgensen in 1951 [6, 7] is based on the fact that *Thermobacterium acidophilum (Lactobacillus acidophilus)* Orla Jensen strain R 26 is unable to synthesize compounds containing deoxyriboside bonds. Such substances must therefore be added to the growth medium. As the organism cannot utilize DNA this must be hydrolysed before the assay. The method has been used to study the DNA synthesis in embryos, and in the bacterium itself (Hoff-Jørgensen & Zeuthen [8], Hoff-Jørgensen [7], Løvtrup [11, 12], Gregg & Løvtrup [3, 4, 5], Grant [2], Okazaki & Okazaki [20], Burns [1]). It has likewise been used for determination of DNA precursors (Schneider *et al.* [26, 27], Okazaki *et al.* [21]).

Hoff-Jørgensen found that for preparations of thymus DNA as well as for DNA in various rat tissues digestion with pancreatic DNase gave microbiologically satisfactory results when compared with estimations of tissue DNA according to Schmidt-Thannhauser. Other authors, like ourselves, have found that by this treatment only a part of the nucleic acid in a biological sample becomes active in the microbiological test (Grant [2], Siedler & Schweigert [29]). If thymidine or any other deoxynucleoside is used as standard, too low DNA values are obtained.

It is now known that the growth-promoting effect of various low-molecular deoxyriboside compounds varies considerably for *Th. acidophilum* (Siedler, Nayder & Schweigert [28], Schneider & Potter [26], Schneider & Rotherham [27], Løvtrup & Roos [15], Løvtrup & Shugar [19]). Thus the polyphosphates of various nucleosides, as well as many di- and trinucleotides, are rather ineffective, whereas certain dinucleotides are well utilized. The microbiological activity per mole varies even for such simple compounds as nucleosides and nucleotides. This phenomenon affords a reasonable explanation of the irregular and often low values obtained when biological samples are digested solely with pancreatic DNase, since Kunitz [10], Privat de Garilhe & Laskowski [22],

and Vanecko & Laskowski [30] have shown that this treatment mainly results in the formation of various oligo-, tri-, and dinucleotides. Further degradation is therefore required. As the highest microbiological activity is obtained with deoxyribosides (Siedler *et al.* [28]) the ideal degradation must reach this level. Further hydrolysis will lead to losses in deoxyriboside bonds, and thus to decreased microbiological activity.

In a recently published paper it was shown by Williams, Sung & Laskowski [31] that a purified fraction of snake venom may degrade high-polymeric calf thymus DNA to free nucleotides. When crude snake venom is used, the presence of 5-nucleotidase activity, and thus the formation of nucleosides, may be expected (Schmidt [23]).

The efficiency of the enzymes may vary with the type of DNA, but it nevertheless seems probable that the combined enzyme treatment or in certain cases treatment with snake venom alone, is the safest way to achieve a more or less complete degradation of DNA to deoxyribosides. As these possess varying microbiological activity it is important to use an equimolar mixture of deoxyribosides for the standard curve (Løvtrup & Roos [15]).

The main part of this paper presents the results of a search for optimal conditions for digestion of DNA samples to be analysed microbiologically. Besides, some other modifications which have been introduced in the original Hoff-Jørgensen technique are also described.

Apart from the rare occurrence of capricious behaviour, which is unavoidable in microbiological assays, the method now works in our laboratory as a reliable analytical tool. We therefore feel that it may be warranted in brief outline to describe our present technique.

EXPERIMENTAL

Analytical procedure

Cleaning of glassware. Careful cleaning of glassware is a prerequisite for successful work. Detergents are potent inhibitors of the DNase activity and therefore should be avoided. We place tubes and pipettes in chromic acid overnight, other glassware is placed in a bath of equal volumes of 96% alcohol and concentrated HCl. Subsequently we rinse many times with water, ending with redistilled water.

Preparation of double strength basal medium (DBM). Chemicals of highest purity are employed. The following stock solutions are prepared. *Amino acids:* 2.0 g. L-glutamic acid, 1.5 g. DL-alanine, 1.0 g. L-aspartic acid, 0.4 g. L-arginine and L-leucine, and 0.2 g. each of L-cysteine HCl, glycine, L-histidine HCl H_2O, L-isoleucine, L-methionine, L-phenylalanine, L proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, and L-valine, dissolved in water to 1 liter. The solution is stored frozen in 250 ml.

portions in polyethylene bottles. *Vitamins:* 2 mg. riboflavin, 2 mg. folic acid, 10 mg. pyridoxin HCl, 10 mg. nicotinic acid, 10 mg. calcium pantothenate to 50 ml. The folic acid is first dissolved in 2 ml. 1 N-NaOH, 30 - 40 ml. water is added, the solution neutralized, and the other vitamins added before the volume is made up to 50 ml. (Løvtrup & Roos [13]). *Salts:* 4 g. $MgSO_4 \cdot 7H_2O$, 0.8 g. $MnSO_4 \cdot 4H_2O$, 0.3 g. $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$ are dissolved in water, adjusted to about pH 1 with HCl and water added to 100 ml. *Tween 80:* 10% solution. *Thioglycollic* acid: 10% solution. New solution is made every third month. The three latter stock solutions are kept in the refrigerator.

For one liter of DBM, 2.5 g. K_2HPO_4, 2.5 g. KH_2PO_4, 25 g. potassium acetate and 30 g. glucose are dissolved in about 500 ml. of water. To this is added 250 ml. of the amino acid solution, 12.5 ml. salt solution, 10 ml. Tween solution, and a freshly prepared solution containing 20 mg. adenine, 20 mg. guanine, 40 mg. uracil and 50 mg. cytidylic acid in 10 ml. 1 N-HCl. Adenine and cytidylic acid are not necessary for growth, but may have a slightly enhancing effect. The pH of the mixture is adjusted to about pH 6.9 with 2 N-NaOH, 10 ml. thioglycollic acid and 4 ml. freshly prepared vitamin solution are added, pH readjusted to 6.9 and the volume made up to 1 liter. The DBM is kept frozen in polyethylene bottles in appropriate quantities (e.g. 50 and 100 ml.) and may be stored for at least one month. For further details about the DBM, see Løvtrup & Roos [14].

Deoxynucleoside standard. 25 μmoles thymidine, deoxycytidine, deoxyadenosine, and deoxyguanosine are dissolved in 100 ml. 25% ethanol (total concentration 1 mM). The stock solution is kept in the refrigerator (Løvtrup & Roos [15]).

Milk tubes for the stock culture. Skim milk is enriched with 0.5% Difco yeast extract and 0.1% L-cysteine HCl, and after adjusting pH to 6.7, distributed in 3 ml. portions in Pyrex tubes (10×100 mm.) containing about 0.2 g. $CaCO_3$. The tubes are stoppered with cotton plugs and autoclaved for 10 min. at 120° in a pressure cooker and stored at 4° (Hoff-Jørgensen [6]).

Inoculum tubes. To 50 ml. DBM is added 1 ml. deoxynucleoside stock solution and the volume made up to 100 ml. The solution is distributed in 5 ml. portions in tubes (14×120 mm.) with cotton plugs, autoclaved for 6 min. in a pressure cooker and stored at -20°.

Buffer. Imidazole buffer (0.1 M), pH 7.0, made up with $MgSO_4$ (0.01 M) is used.

Preparation of standard curves. The deoxynucleoside stock solution is diluted 250 times with 10 times diluted imidazole buffer. Of this solution 1.00, 0.75, 0.50, 0.25, 0.10, and 0 ml. is pipetted into Pyrex tubes (8×100 mm.), and the volume made up to 1 ml. with diluted buffer. All pipettings are done with Carlsberg constriction pipettes. For each

nucleoside concentration two tubes are used. To each tube 1 ml. DBM is added with a syringe pipette.

Preparation and digestion of biological samples. The sample is homogenized, and may be used directly or stored for later analysis. In the latter case it must be dried, with acetone or *in vacuo*, since losses occur otherwise. We do not know for how long time the dried samples may be stored, but it seems that a decrease may occur after some months of storage. When the analysis is to be made, NaOH is added to give a final concentration of 0.5 N, and the sample placed in boiling water for 15 min. After neutralization with HCl, buffer is added to a known volume. The DNA content before enzyme addition should be in the range of 1 - 20 μg. per ml. and therefore the amount of buffer must be suitably adjusted. At least two volumes of buffer must be added. As will be described in the sequel, it is necessary to investigate optimum conditions for digestion when a new type of tissue DNA is to be determined.

As a general rule we have found optimum digestion of DNA by the following procedure. To four parts of the sample treated as described above is added 1 part of DNase solution (Worthington Biochemical Corporation, Freehold, N.J., U.S.A., 1 mg. per ml. buffer) and the mixture incubated at 37° for 3 hr. Subsequently the enzyme is inactivated by heating for some minutes at 90° (Kunitz [9]) and 0.25 volume of crude snake venom solution (*Crotalus atrox*, Ross Allen's Reptile Institute, Silver Springs, Fla., U.S.A., 0.5 mg. per ml. buffer) is added, and the sample incubated for another 3 hr. The digestion is interrupted by heating as above, and the tubes are centrifuged. By dilution with water two different concentrations of the digested sample are prepared from the supernatant. One ml. samples are added to tubes similar to those used for the standard curve. Three tubes are used for each dilution. After addition of DBM the tubes (samples and standards) are covered with loosely fitting glass capsules, and autoclaved in a pressure cooker for 6 min. It is important for the reliability and reproducibility of the method that the two dilutions are adjusted to lie within the range 0.1 - 1 μg. DNA.

Microbiological technique. The incubation time is 22 hr. The stock culture is preserved by weekly transfers to new milk tubes. The inoculum is always prepared from fresh milk tubes, which are made during the week according to demand. After incubation the bacteria in the inoculum are centrifuged down, and resuspended in 10 ml. sterile 0.65% NaCl solution. The tubes containing the standard and the unknown samples are inoculated from the same freshy prepared inoculum, by adding one drop of suspension to each tube (sterile pipette).

Estimation of the growth response. The bacterial growth is determined turbidimetrically in semimicrocuvettes at 650 mμ. The tubes are vigorously shaken before transfer to the cuvettes (Hoff-Jørgensen [6]).

The growth may also be estimated by titration of the lactic acid formation, but the deoxynucleoside concentration may under these conditions be decreased 10 times, and the sensitivity of the DNA determination is thus increased correspondingly. Estimating deoxynucleosides the incubation volume may be decreased 10 - 20 times, which further increases the sensitivity (Løvtrup & Roos [16]). So far we have not been able to achieve a successful digestion of DNA in this decreased volume.

Calculation of DNA content in a biological sample. The DNA values of the diluted samples are read from the standard curve. Assuming that 1 μmole deoxyriboside is equivalent to 309 μg. DNA, the highest point on the curve corresponds to 1.24 μg. DNA per ml. of the diluted digested sample (turbidimetric determination). Occasionally the mean values of the two dilutions deviate by more than 10%. When this happens, we routinely repeat the analysis. If the highest dilution systematically gives too low values, there is reason to suspect that the digestion is incomplete (cf. below).

Observations on some of the steps in the analytical procedure

Choice of buffer. The pH optimum for pancreatic DNase is 7.0. The maleic acid originally used has a pK_a value of 6.07, and therefore at pH 7.0 it hardly has any buffer capacity. (It may be mentioned that an erroneous pK_a value of 6.58 is listed in some tables, e.g. Handbook of Chemistry and Physics, 29th ed.). An increase in the maleic acid concentration might increase the buffer capacity, but causes at the same time inhibition of the enzyme activity. A phosphate buffer of pH 7.0 must for the same reason be diluted so much that the buffer capacity is unsatisfactory. Imidazole (pK_a 6.95) has maximum buffer capacity at the right pH, and may furthermore be used in rather high concentration (0.1 M) without influencing the enzyme activity. After dilution of the neutralized DNA sample with the buffer- and enzyme-solutions, the Mg^{2+} concentration is optimal for DNase activity, and at the same time the content of NaCl reaches a non-inhibitory concentration (Kunitz [9]). The digestion procedure also secures acceptable conditions for the snake venom activity (Williams *et al.* [31]). The latter seems not to be inhibited by imidazole.

Effect of the treatment with NaOH. The nucleic acid is extracted with hot NaOH. We have made some experiments to study the effect of this treatment on DNA preparations. The subsequent digestion with DNase seems in some cases to proceed somewhat slower, whereas the snake venom effect is slightly enhanced. Since this step cannot be omitted, it is important to note that the yield in no case was influenced.

Enzymic digestion of high-polymeric DNA. Various commercial preparations of salmon sperm and calf thymus DNA have been employed.

Table 1

The effect of pancreatic DNase, crude snake venom and alkaline phosphatase on the digestion of salmon sperm DNA, as measured by the microbiological method

The results are expressed as percentage yield, calculated on the basis of the P content

Treatment	Incubation time (hr.)			
	1	3	6	23
DNase	41	70	72	94
Crude snake venom	95	95	92	95
Alkaline phosphatase	80	100	95	93
DNase and snake venom simultaneously	103	103	108	108
DNase and phosphatase simultaneously	93	88	90	90
DNase digestion followed by snake venom or phosphatase:				
DNase 1 hr., snake venom	90	100	93	105
DNase 1 hr., phosphatase	113	103	82	74
DNase 3 hr., snake venom	106	106	95	98
DNase 3 hr., phosphatase	108	104	100	80
DNase 6 hr., snake venom	103	103	98	90
DNase 6 hr., phosphatase	110	100	87	82
DNase 23 hr., snake venom	103	95	96	100
DNase 23 hr., phosphatase	108	105	93	90

The DNA content was calculated from the P content, corrected for RNA, when present. From Table 1 it appears that digestion with pancreatic DNase gives a rather satisfactory decomposition of a salmon sperm DNA preparation, when measured by the microbiological method. In spite of notable differences in the experimental conditions we have thus partly been able to confirm the results of Hoff-Jørgensen. However, calf thymus DNA gave at most 75% yield when digested in the same way.

The good yield with salmon sperm DNA is theoretically rather astonishing, since, as mentioned above, the treatment with DNase does not lead to complete digestion, but to compounds, many of which have a rather low microbiological activity. In this context may be mentioned the experimental observations of Okazaki & Okazaki [20], who found that after addition of a deoxynucleoside to a bacterial perchloric acid extract, its microbiological activity might be increased up to 10 times. The resulting activity corresponded to about 60% of the activity found after digestion of the extract with snake venom. This finding demonstrates that the presence of a low molecular deoxyribose compound enables the bacteria to utilize more complex deoxyribose compounds, and may thus explain the finding reported above. The utilization of deoxyribose compounds by the bacteria is apparently a complicated question, depending probably on many different factors (Siedler & Schweigert

Table 2

The effect of DNase and crude snake venom on the digestion of DNA in rat brain powder, as measured by the microbiological method

The yields are expressed as percentages of the results obtained by the chemical method

Treatment	Incubation time (hr.)			
	1	3	6	23
No enzyme	15	12	18	23
DNase	40	47	54	73
Crude snake venom	78	100	99	98
DNase and crude snake venom simultaneously	87	98	107	124
DNase digestion followed by snake venom:				
DNase 1 hr.	95	105	103	107
DNase 3 hr.	98	103	103	103
DNase 6 hr.	96	104	100	112
DNase 23 hr.	99	96	96	104

[29]). In order to minimize the sources of error it is important that the digestion should be as complete as possible.

Contrary to DNase, snake venom and alkaline phosphatase alone, or in combination with DNase, give a microbiologically acceptable depolymerization after a rather short incubation (Table 1). If DNase-treated DNA is subjected to treatment with snake venom or alkaline phosphatase, the incubation time may be varied within rather wide limits, but prolonged incubation with alkaline phosphatase results in a gradually decreasing microbiological activity. This is probably due to splitting of deoxyriboside bonds, and we have therefore refrained from further use of this enzyme preparation.

Enzymic digestion of tissue DNA. It is a prerequisite for estimating the efficiency of various procedures for transforming DNA to a microbiologically active form that the DNA content may be determined by a reliable, independent method. Our work has been hampered for several years by the fact that the available chemical methods under certain circumstances are rather unsatisfactory. We have therefore been forced to study even this question and as a result of this we have developed a chemical method for DNA determination (Løvtrup & Roos [17, 18] *). Using this as a reference we have estimated the yield obtained with the microbiological method.

In Table 2 are shown the results of DNase and snake venom treatment of rat brain powder. Prolonged treatment with DNase alone gives an unsatisfactory yield. Snake venom alone, or in combination with

* For review see *Acta Biochim. Polon.* 9, 411, 1962.

DNase gives after a relatively short time a 100% microbiological response. It appears from the Table that the digestion time under these conditions may be varied within wide limits. By prolonged treatment a microbiological activity exceeding 100% is sometimes observed. This excess, which has been found in several cases, may often be too large to be accounted for by the analytical error.

We do not know the correct explanation of this phenomenon. However, as we do not work with sterile digestion mixtures, the possibility of an infection during the digestion cannot be excluded. Another, less likely, explanation is that certain digestion products may be more active, on a molar basis, than the free deoxynucleosides. If the former explanation is correct, sterile work should cancel this source of error. This may be rather tedious and difficult to achieve, but since a lag phase of at least 5 - 6 hr. is to be expected, the simplest way to avoid such excess results appears to be the use of short digestion times.

In Table 3 are listed the results of DNase and snake venom treatment of DNA in some other organs. Only calf thymus DNA is satisfactorily digested by DNase alone. Rabbit kidney DNA also seems to be rather easy to digest, whereas DNA in rat liver and heart, and in rabbit brain appears to be quite resistant to DNase. A certain parallelism seems to exist between the ease with which DNA is extracted with hot perchloric acid and digested with DNase (cf. Løvtrup & Roos [17]).

A comparison between Tables 1 and 3 indicates that nothing can be inferred about the efficiency of DNase towards tissue DNA from results

Table 3

Digestion with DNase and crude snake venom of DNA in various kinds of tissue powder

The yields are expressed as percentages of the results obtained by the chemical method

Source of DNA	DNase		Crude snake venom		DNase and crude snake venom simultaneously		DNase followed by crude snake venom
	Incubation time (hr.)						
	3	23	3	6	3	6	3 + 3
Rat liver	19	60	108	93	93	96	105
Rat kidney							95
Rat heart	25	55	68	87	81	81	97
Rabbit liver	25	75	67	86	79	79	108
Rabbit kidney	36	82	86	105	99	121	102
Rabbit heart	25	69	64	99	75	103	106
Rabbit brain	30	55	95	85	93	99	97
Calf thymus		95					102

obtained by treatment of purified DNA preparations. Neither does recovery of DNA added to tissue samples furnish any proof of the validity of the digestion procedure as regards the DNA contained in the tissue sample.

Digestion with snake venom is considerably more efficient, but certain types of tissue DNA, e.g., from rat heart and rabbit liver, are not satisfactorily digested by this enzyme preparation alone. Simultaneous treatment with both enzymes gives in principle the same result as treatment with snake venom alone.

As appears from Table 3, the most satisfactory results are obtained when a 3-hr. DNase digestion is followed by a 3-hr. treatment with snake venom. The results thus obtained agree with those of the chemical method within the limits of error of the methods.

Although this enzyme treatment may be considered a standard procedure, it is nevertheless advisable to check the efficiency of the enzymic digestion for every type of biological material. With respect to this point, it may be mentioned that on some occasions we have obtained too low results for rat liver DNA, when the standard digestion procedure was followed. This DNA seems to be the most difficult to digest, and we believe that the occasionally observed failure may be due to variations in the activity of the DNase preparations. In these cases prolongation of the digestion with this enzyme has led to satisfactory results.

Enzyme concentration. In the procedure recommended by us the final enzyme concentration is 0.2 mg. DNase and 0.1 mg. snake venom per ml. of the digestion mixture. By using such high enzyme concentrations we follow the original suggestion of Hoff-Jørgensen. In some instances optimal digestion of biological samples may be achieved with lower concentrations of enzyme. However, as mentioned above, certain types of DNA are very difficult to digest, and therefore the quantities added may warrant a necessary margin of safety. Due to the almost complete digestion by snake venom in many cases, it is difficult to ascertain the effect of decreased addition of DNase. Another reason for using rather high concentrations of this enzyme is that it is considerably more stable in concentrated solution (Kunitz [9]).

The quantities of enzyme added are so small compared with the amounts of nutritive material in the DBM that they hardly can be of influence on the analytical results, unless they are contaminated with DNA. This point has been investigated by incubating enzyme solutions in the usual way, and adding them in a final dilution of 1:5 - 1:25 to standard samples. No effect could then be observed.

The effect of dilution after digestion on the yield. In our early work, in which maleic acid buffer was used, we observed that the yield was dependent upon the degree of dilution after digestion. When only DNase was used for digestion, and dilutions of 5, 10, and 20 times were em-

ployed, the agreement between the results were often found to be poor, the yield tending to decrease as the dilution was increased. Further dilution resulted in still lower yields. We also found that the concentration of maleic acid was of decisive influence on the yield when the samples were greatly diluted after digestion. Under these circumstances better results were obtained when the buffer concentration was low, indicating a certain inhibitory effect of maleic acid on the DNase activity. The previously mentioned influence of simple deoxyribose compounds on the utilization of more complex digestion products may explain this phenomenon. It is likely that the amount of the former is decreased below a certain treshold value when either the enzyme is inhibited by maleic acid, or the digestion mixture is diluted excessively.

When both DNase and snake venom are used for digestion, and maleic acid is replaced by imidazole, it is possible in many cases to dilute the digestion mixture considerably without observing any deleterious influence on the yield (Fig. 1). It is seen from the figure that one may dilute the digestion mixture of high-polymeric DNA and calf thymus DNA 200 times without influencing the yield. In contrast to this the microbiological yield is considerably reduced at high dilutions of rabbit brain and rat liver DNA. This observation may presumably be correlated with the fact that these types of DNA are difficult to digest. As

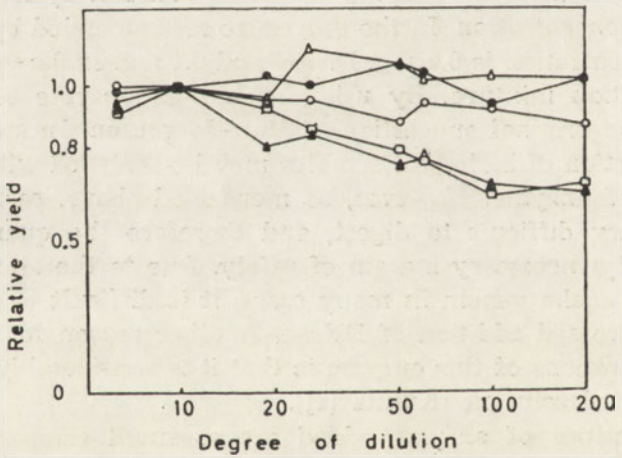

Fig. 1. The influence of dilution after digestion with DNase and snake venom, on the microbiological yield. The yields have been expressed relative to that obtained at 10-fold dilution. (●), Salmon sperm DNA; (△), calf thymus powder; (○), rabbit kidney powder; (□), rabbit brain powder; (▲), rat liver powder

a general rule it may therefore be recommended never to dilute the digestion mixture more than 20 times, unless it has been ascertained experimentally that this can be done without influencing the microbiological result.

The influence of the digestion buffer on the yield. As previously shown (Løvtrup & Roos [14]), the bacterial growth is to a large degree dependent on the buffer capacity of the medium. When digested samples are analysed, the buffer capacity is increased by the addition of the digestion mixture. It is therefore important that the standard curves are prepared in such a way that the buffer capacity is the same as in the tubes containing the digested samples. If the biological sample is diluted between 5 and 20 times it suffices to dissolve the deoxyribosides in 10 times diluted buffer, since a change in buffer concentration by a factor of 2 has been found to be of very slight influence on the standard curve.

The error of the method. To evaluate the error of the method the following experiment was carried out. Salmon sperm DNA, rabbit liver and rat liver powder were treated with NaOH, and from each preparation 10 samples were separately digested, and assayed after 5, 10, and 20 times dilution. The standard deviation between and within the 10 digestion samples was calculated. The results are reported in Table 4. Contrary to expectation, maybe, the standard deviation within samples tends to be larger than the one between samples. This phenomenon is to some extent caused by the dilution effect described above, as shown by the fact that the standard deviation for each series of the same dilution (not shown in the Table) is smaller than the standard deviation

Table 4

The error of the method

The standard deviation is expressed as percentage of the mean value

	Standard deviation	
	within samples	between samples
Salmon sperm DNA	2.6	1.9
Rabbit liver powder	2.9	3.1
Rat liver powder	4.8	3.1

within samples. In agreement with this interpretation is also the fact that the largest value is found for rat liver powder. It is seen that as a general rule the error increases as the DNA becomes more difficult to digest.

CONCLUSION

Several of the existing methods for DNA determination give quite diverging results. This is obviously a serious drawback in the study of biological processes involving DNA, and none the least in the work on development of new methods for DNA analysis.

Under such conditions the introduction, by Hoff-Jørgensen, of a microbiological method, must obviously be considered an important achievement. The principle of this DNA assay is determination of the amount of deoxyribose nucleoside bonds present. It may *a priori* be expected that it is more specific than most other methods. It should therefore be of particular advantage in work with biological material in which the concentration of DNA is so low that the interference from other substances may invalidate the results. On the whole this expectation has been met, although the interference by ribonucleotides probably, under certain circumstances, may be significant. Another advantage is that the method is very sensitive. In our titrimetric modification 0.02 μg. DNA may be determined.

These are the reasons justifying our attempts to combat some of the caprices of the original method. The greatest difficulty consisted in getting more or less complete digestion of DNA.

We have been able to show that the relative error of the method as modified by us is of the same order as that of other microbiological assays. The question about the absolute accuracy may not be definitely settled, but the very good agreement observed with our chemical method is encouraging.

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SUMMARY

The resistance of various types of DNA towards enzymic degradation is highly varying. This fact may explain why the yield with the original method for DNA determination as developed by Hoff-Jørgensen varies with the source of DNA. A number of modifications have been necessary to achieve a yield close to 100%. The present paper contains a report on experiments designed to establish optimal conditions for degradation of DNA. A complete description of the final procedure adopted is also included. If the growth of the bacteria is determined turbidimetrically, 0.2 μg. DNA may be estimated. If lactic acid formation is measured, the sensitivity is increased 10 times (0.02 μg. DNA).

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MIKROBIOLOGICZNE OZNACZANIE KWASU DEOKSYRYBONUKLEINOWEGO

Streszczenie

Odporność różnych rodzajów DNA na działanie enzymów wykazuje duże różnice. Tym tłumaczy się fakt, że wydajność oznaczonego DNA przy zastosowaniu pierwotnej mikrobiologicznej metody Hoff-Jørgensena jest zmienna w zależności od źródła DNA. Aby otrzymać oznaczenie z prawie 100-procentową wydajnością, konieczne było wprowadzenie szeregu modyfikacji. W pracy przedstawiono eksperymenty zmierzające do określenia optymalnych warunków degradacji DNA i podano pełny opis przyjętej metody. Przy turbidometrycznym określaniu wzrostu bakterii można oznaczać 0.2 μg. DNA; przy oznaczaniu powstałego kwasu mlekowego dokładność wzrasta 10-krotnie (0.02 μg. DNA).

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SECONDARY STRUCTURE OF POLY-URIDYLIC AND POLY-RIBOTHYMIDYLIC ACIDS, THEIR *N*-METHYLATED ANALOGUES, AND THEIR 1:1 COMPLEXES WITH POLY-A

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Of all the synthetic polyribonucleotides hitherto investigated, the lowest hyperchromicity at room temperature, about 9%, is exhibited by poly-U[1] which, under these conditions, is believed to exist as a random coil. (The value of 5 - 6% hyperchromicity originally reported for poly-U by Warner [42], although widely quoted, is somewhat on the low side [36, 37]). The significant observation was made by Lipsett [20], and subsequently confirmed [37] that, at lower temperatures, about 6°, the polymer possesses enhanced hypochromicity amounting to about 16% and testifying to the formation under these conditions of a certain degree of secondary structure.

In view of the foregoing, it is rather surprising that poly-rT, first prepared by Griffin *et al.* [12], was reported to exhibit a hyperchromicity of up to 33% on hydrolysis to mononucleotides. (Note that both the terms hypochromicity and hyperchromicity are used in this text [see ref. 33]). This is all the more unusual in that the behaviour of the base pair A-U in the twin-stranded complex poly-A+poly-U has hitherto been tacitly accepted as reflecting the behaviour of the base pair A-T in DNA [5, 27]. Attention was drawn to the importance of checking the unusually high reported hyperchromicity of poly-rT [33] and it was proposed that, if the observations of Griffin *et al.* [12] were to be con-

[1] The following abbreviations are used in this text: U, uridylic acid; MeU, *N*-methyluridylic acid; rT, ribothymidylic acid; Me-rT, *N*-methyl-ribothymidylic acid; dT, decxyribothymidylic acid; A, adenylic acid; C, cytidylic acid; G, guanylic acid. The prefix "poly-" indicates a polyribonucleotide obtained by the action of polynucleotide phosphorylase on the appropriate nucleoside-5′-pyrophosphate [13], e.g. poly-U is poly-uridylic acid. The prefix "oligo-" refers to a polymer of relatively short chain length, which does not normally exhibit secondary structure. Numbering of atoms in the pyrimidine ring according to Chemical Abstracts.

firmed, it would indicate that poly-rT, in contrast to poly-U, exists at room temperature in a highly ordered configuration and with a mid-point transition temperature T_m [21] probably above room temperature [37]. The resultant implications with respect to interpretation of polynucleotide structure are immediately obvious, and led us to prepare both poly-rT and poly-Me-rT with a view to comparing their properties with those of the corresponding poly-U and poly-MeU.

The preparation of the *N*-methylated polymer lent additional interest to such a study in that it was previously shown that poly-MeU was entirely devoid of secondary structure even at temperatures as low as 0°, nor did it exhibit any ability to complex with poly-A even under the most favourable conditions [37]. This result was to be anticipated on the basis of the Watson-Crick hypothesis [3] for DNA structure in which hydrogen bonding between adenine and thymine (uracil) should involve the N_3 position of the thymine (or uracil) rings. It might, of course, be argued that it is not so much the blocking of the N_3 ring nitrogen, as the steric hindrance due to the van der Waals' radius of the methyl group, which prevents complexing with poly-A. But in either case the inference may be drawn that the N_3 position is involved in the formation of secondary structure. It is worth noting that *N*-methylation of poly-U leads to a loss in ability of the latter to code for phenylalanine (S. Ochoa, private information).

RESULTS AND DISCUSSION

Synthetic. The starting substance for the syntheses described below was 1-(β-D-ribofuranosyl)thymine (*I*), which was prepared according to the method of Fox *et al.* [8]. Crystalline 2′:3′-*O*-isopropylideneribosethymine (*II*) was then obtained from *I* according to the method of Levene & Tipson [19]. Phosphorylation of *II* with β-cyanoethylphosphate, as described by Tener [40], gave 1-(β-D-ribofuranosyl)thymine-5′-phosphate (*III*) which was once more phosphorylated with dibenzylphosphochloridate [22] to give the 5′-pyrophosphate (*IV*).

Treatment of *II* with an excess of diazomethane in anhydrous medium [36] gave, quantitatively, 1-(β-D-ribofuranosyl-2′:3′-*O*-isopropylidene)-*N*-methylthymine (*VI*) in crystalline form. The 5′-monophosphate of 1-(β-D-ribofuranosyl)-*N*-methylthymine (*VII*) was then obtained by phosphorylation of *VI* with P_2O_5 in H_3PO_4 [14, 22]. The application in this instance of the milder and more efficient phosphorylating agent, β-cyanoethylphosphate [40], was not feasible since it requires subsequent removal of the cyancethyl group in strong alkali at elevated temperature. Under these conditions the *N*-methyl-ribosethymine was found to undergo degradation, a finding which might have been anticipated from the known instability in alkaline medium, even at room temperature, of *N*,*N*′-alkylated uracils [7, 35, 15, 36].

Phosphorylation of *VII* was then carried out with dibenzylphosphochloridate, as above, to yield 1-(β-D-ribofuranosyl)-*N*-methylthymine-5′-pyrophosphate (*VIII*).

Both the 5′-pyrophosphates, *IV* and *VIII*, were found to be active as substrates of polynucleotide phosphorylase [13], although to markedly different degrees. Polymerization of *IV* did not give uniform results, the yield of polymer varying in different experiments from 20 to 40%. More disturbing was the fact that the degree of polymerization of the various preparations, as measured quantitatively by the degree of temperature hyperchromicity (see below), likewise varied for the different preparations. Griffin *et al.* [12] also obtained preparations of poly-rT with varying degrees of hyperchromicity on hydrolysis to mononucleotides. It will be seen, below, that our best preparations exhibited a higher degree of hyperchromicity, and hence were more highly polymerized, than the preparations reported by the preceding authors.

Under identical conditions as for poly-rT, poly-U could be obtained consistently with a polymer yield of 60%.

Out of 7 experiments, 4 gave preparations of poly-rT (*V*) with very high temperature hyperchromicity and the data presented below refer to these 4 samples, unless otherwise indicated. The source of this variation in degree of polymerization is discussed in a subsequent section, below.

Initial attempts at polymerization of *VIII* with polynucleotide phosphorylase gave only traces of poly-Me-rT (*IX*), and this only after an extended "lag phase" of up to 30 - 40 hr. These traces of polymer were eluted from a paper chromatogram and utilized as "primer" in subsequent polymerizations. But even with the use of this "primer", the yield of polymer was only 11 - 14% after 22 - 24 hr. incubation with the enzyme.

Secondary structure of polymers and complexes. In Fig. 1 are presented the temperature profiles for poly-rT in 0.01 M-$MgCl_2$ and in 0.15 M-NaCl + 0.15 M-sodium citrate; as well as that for poly-U in 0.01 M-$MgCl_2$. (The lowest temperature attainable in the spectrophotometer cuvette compartment, in these experiments, was 4.8°). As will be seen from the Figure, the temperature hyperchromicity for poly-rT in 0.01 M-$MgCl_2$ over the temperature range 4.8 - 45° was 85%, the highest yet recorded for a synthetic poly-ribonucleotide. For poly-U the corresponding value is 19%. For poly-rT in 0.01 M-$MgCl_2$ the melting point T_m, i.e. the temperature at which half the secondary structure is dissociated, is 36°; while for poly-U under the same conditions it is only 8.5°. The T_m for poly-rT in 0.15 M-NaCl + 0.015 M-sodium citrate is about 7° lower than in 0.01 M-$MgCl_2$, in agreement with the known effect of ionic strength and, in particular, of the divalent cation Mg^{2+} on the magnitude of T_m [26, 20].

If, in Fig. 1, we ignore the left-hand portion of the "melting" curve for poly-rT, the low slope of which may be due either to some deformations in secondary structure or to the dissociation of the secondary structure of some fractions of low molecular weight, the remainder of the temperature profile, and particularly that embraced by the region $T_m \pm 7°$, is characteristic of an "all or none" process. It is typical of that observed for other polynucleotides, both synthetic and natural, and represents the transition from a helix to a random coil [31], the T_m under

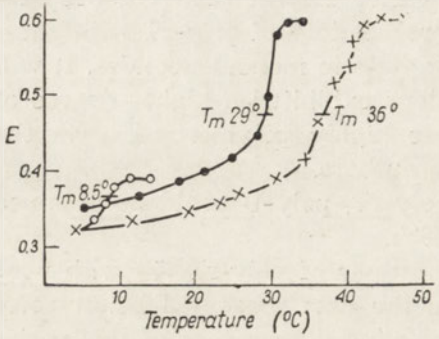

Fig. 1. Temperature profiles of: (×), poly-rT in 0.01 M-$MgCl_2$, pH 7, at 267 mμ; (●), poly-rT in 0.15 M-NaCl + + 0.015 M-sodium citrate, pH 6.9, at 267 mμ; (○), poly-U in 0.01 M-$MgCl_2$, pH 7, at 262 mμ. (Extinction measured at maxima of principal absorption bands)

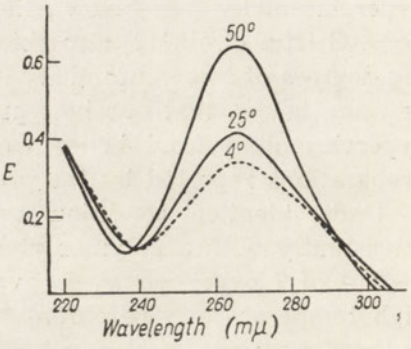

Fig. 2. Absorption spectrum of poly-rT in 0.01 M-$MgCl_2$, pH 7, at temperatures indicated in Figure

defined conditions being a measure of the relative stability of the helix. It follows from Fig. 1 that poly-rT is energetically considerably more stable than poly-U. The nature of the transition and its dependence on temperature suggest that we are dealing, at least in part, with the formation or rupture of hydrogen bonds (see, however, below).

It is consequently of interest that neither poly-Me-U [37], nor poly--Me-rT (see Experimental, below) exhibit secondary structure even under the most favourable conditions of ionic strength or temperature, thus pinpointing the importance of the ring N_3 nitrogen, most likely as a hydrogen donor, in the formation of an intramolecular helix. It would clearly be desirable to conduct a rentgenographic analysis of poly-rT, as well as measurements of the sedimentation coefficients of the "random" and "coil" forms at the appropriate temperatures. Poly--rT is, in fact, an admirable model for such investigations because of the closeness of its T_m to room temperature, and its extremely high temperature hypochromicity indicating that it possesses a highly ordered configuration below room temperature.

When ribonuclease was added to a solution of poly-rT in a spectrophotometer cuvette, buffered at neutral pH in 0.01 M-$MgCl_2$, there resulted an instantaneous increase in extinction to the value obtained by heating to 45° (Fig. 1), and indicative of the dissociation of the secondary structure of the polymer. Following this, there was a further, time-dependent, increase of about 8% accompanying hydrolysis of the shorter chains to mononucleotides. This "residual hyperchromicity" of 8% was also obtained by hydrolysis of the polymer by acid or alkali. That this residual hyperchromicity has nothing to do with secondary structure was shown by the fact that poly-Me-rT exhibited a similar increase in extinction, about 7%, on hydrolysis to mononucleotides by acid or snake venom. Both poly-U at room temperature, where it exists as a random coil, and poly-MeU, which is in the form of a random coil at all temperatures, exhibit residual hyperchromicity of about 9% [36, 37]. It should be recalled that various oligonucleotides exhibit such residual hyperchromicity [for review see ref. 33] which appears most likely due to overlapping of the π-orbitals of the neighbouring bases in the chains [18, 23, 37, 38].

Fig. 2 presents the absorption spectrum of poly-rT in 0.01 M-$MgCl_2$ over the wavelength range 220 - 310 mμ at temperatures of 4°, 25° and 50°. From this Figure the hypochromicity at any wavelength may be read off directly. Attention is drawn, in particular, to the fact that the

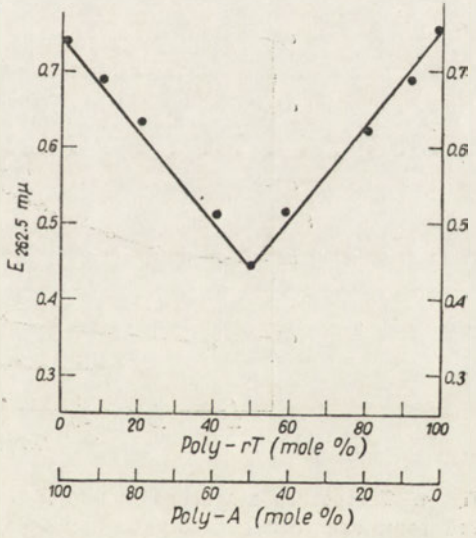

Fig. 3. Formation of the 1 : 1 complex poly-rT + poly-A in 0.15 M-NaCl + + 0.015 M-sodium citrate, pH 6.9 at 25°, as followed by changes in extinction at 262.5 mμ on addition of one polymer to the other. The extinction values for the homopolymers are presented without taking into account the influence of their own secondary structure

helix form of poly-rT exhibits *hyperchromicity*, i.e. an increase in extinction, to the violet of 235 mμ and to the red of 290 mμ, with respect to the random coil. We shall revert to this in the General Discussion, below.

Fig. 3 demonstrates complex formation between poly-rT and poly-A at room temperature under standard conditions. The formation of

a complex was followed by the decrease in extinction of the mixture of the two polymers, with respect to their arithmetical summation. From Fig. 3 it will be seen that maximum hypochromicity of the complex poly-rT + poly-A occurs at a molar ratio of the two components of 1 : 1, as already observed for a number of complexes of synthetic polyribonucleotides [28, 25, 26, 37], its value being 41% as compared to 30% for the 1 : 1 complex of poly-U + poly-A. The hyperchromicities of the complexes, i.e. the increases in extinction accompanying their dissociation, are 64% for poly-rT + poly-A and 39% for poly-U + poly-A [42]. As previously observed for poly-MeU [37], poly-Me-rT did not exhibit any evidence of complex formation with poly-A under the most favourable conditions of temperature and ionic strength.

In Fig. 4 the extinctions of the twin-stranded complexes are shown as a function of temperature. As the temperature is raised the "melting out" of the complex, i.e. the dissociation of the twin strands, is followed by the increase in optical density. It will be seen from the Figure that the T_m for the complex of poly-A with poly-rT is 79° and is 20° higher than that with poly-U. Also shown in Fig. 4 is the temperature profile for the melting out of the complex of poly-A with a sample of poly-rT which had been partially degraded spontaneously by standing in aqueous medium at room temperature (see below) so that it consisted

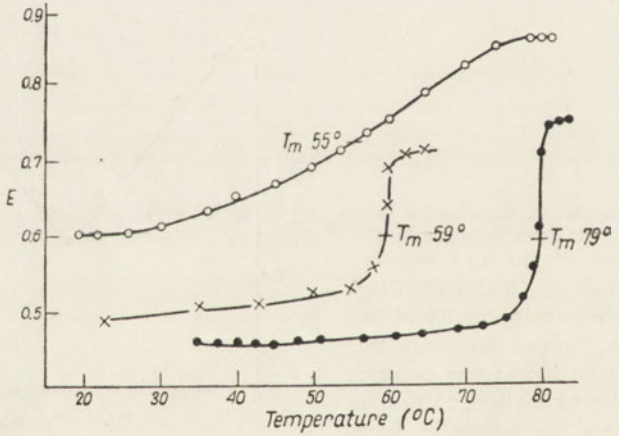

Fig. 4. Temperature profiles in 0.15 M-NaCl + 0.015 M-sodium citrate, pH 6.9, of 1 : 1 complexes of: (×), poly-U + poly-A, at 259.5 mμ; (●), poly-rT + poly-A at 262.5 mμ; (○), oligo-rT + poly-A at 262.5 mμ. (Extinction measured at maxima of principal absorption bands)

of chains of shorter length. This latter curve demonstrates strikingly the importance of chain length as a factor in determining the stability of the twin-stranded helix; for not only is the T_m appreciably lowered,

but the flattening out of the profile points to the gradual melting out of complexes containing varying chain lengths of oligo-rT. A somewhat similar temperature profile was obtained by Rich [27] for a complex of poly-A with a sample of oligo-dT of an average chain length of 12 residues.

Fig. 5 exhibits the absorption spectrum of the 1 : 1 complex of poly-A with poly-rT at room temperature and at 85° where the complex is completely dissociated. It should be noted that to the violet

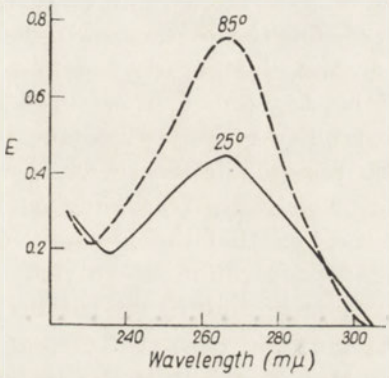

Fig. 5. Absorption spectrum in 0.15 M-NaCl + 0.015 M-sodium citrate, pH 6.9, of 1 : 1 complex of poly-rT + poly-A at 25° and 85°

of 230 mμ and to the red of 295 mμ the complex exhibits an *increase* in absorption, or *hyperchromicity*, with respect to the dissociated form.

Apparent instability of poly-rT. Frequent references are to be found in the literature with respect to the "instability" of some synthetic polyribonucleotides, one of the best-known examples being poly-C [44]. This has been variously ascribed to the presence in the preparations of traces of nucleases; but the principal argument against this has been the apparent stability of poly-U.

Griffin *et al.* [12] noted that their various preparations of poly-rT exhibited different degrees of hyperchromicity, ascribing this to some instability of the polymers. From what has been described above, in particular the fact that the T_m of poly-rT is in the vicinity of room temperature, we now know that the variations in hyperchromicity of their preparations were due at least in part to the fact that their measurements were probably made at varying ambient temperatures.

Nonetheless, despite careful control of temperature, three of our preparations also exhibited low hyperchromicity. More significant was the fact that *all* preparations, when left in aqueous medium at room temperature, underwent slow degradation to the point where they eventually exhibited chromatographically the presence even of mononucleotides. It is difficult, under these circumstances, to conceive of any explanation other than the presence of traces of some nuclease or

phosphodiesterase. The presence of traces of an enzyme such as pancreatic ribonuclease was excluded since poly-Me-rT, which is resistant to pancreatic ribonuclease, exhibited the same behaviour.

An aliquot of poly-rT, when added to a solution of yeast RNA and left for some time at room temperature, was without any effect on the latter. This would appear to exclude the presence of some ribonuclease or phosphodiesterase.

However, when a sample of poly-rT was rapidly heated to 100°, maintained at this temperature for 60 min., and then cooled and left for 18 hr. at room temperature, no further degradation could be observed. On the other hand when the sample was heated at 100° for only 5 min., further degradation did result on subsequent exposure for 18 hr. to room temperature. This is reasonably good evidence that the instability of poly-rT is due to the presence of traces of a highly specific phosphodiesterase which is inactive against poly-U or RNA. That a phosphodiesterase, and not a ribonuclease, is involved is testified to by the fact that the mononucleotide isolated from a spontaneously degraded preparation of poly-rT proved to be the 5′-monophosphate.

Enzymic hydrolysis of polymers. An examination was made of the rate of enzymic (pancreatic ribonuclease) hydrolysis of poly-rT and poly-U at 22° in 0.01 M-$MgCl_2$, i.e. under conditions such that poly-rT possesses a secondary structure corresponding to 90% of maximum, while poly-U is in the form of a random coil. Both polymers were found to undergo hydrolysis (chromatographic determination of rate of appearance of oligonucleotides) at essentially the same rate. It follows that the presence of secondary structure offers no steric or other hindrance to the action of the enzyme. An alternative interpretation may, of course, be offered, viz. the cleavage of only two or three linkages in a chain containing secondary structure may suffice to abolish the latter, the resulting shorter chains then behaving as random coils.

On the other hand, when poly-rT is combined with poly-A in a 1 : 1 helical complex, its rate of hydrolysis by ribonuclease is inhibited to the extent that a five-fold increase in enzyme concentration is required to give a rate of hydrolysis of 40% that for free poly-rT under the same conditions. There consequently appears to be appreciable steric hindrance to formation of an enzyme-substrate complex. The possibility, in the foregoing experiment, that some of the ribonuclease may be bound by the poly-A component, is at once eliminated by the fact that the latter is present at a molar concentration equal to that for poly-rT; the difference in rates of enzymic hydrolysis is too great to be accounted for in this way.

Poly-Me-rT was found to be completely resistant to the action of pancreatic ribonuclease, in agreement with previous observations on the

resistance of poly-MeU to the same enzyme [36 - 39] and with the suggestion regarding the significance of the N_3 position of the pyrimidine ring in the formation of an enzyme-substrate complex [4, 45, 39].

Both poly-rT and poly-Me-rT were readily hydrolysed by snake venom (*Crotalus adamanteus*) to the 5′-monophosphates of ribosethymine and *N*-methyl-ribosethymine, and then to the nucleosides.

Mention should also be made of the observation that the acid-catalysed hydrolysis of poly-rT (measured at room temperature at pH 2.5) proceeded at a rate significantly higher than that for poly-U.

Metachromatic behaviour. Both poly-rT and poly-Me-rT were found to exhibit pronounced metachromasia against a basic dye, toluidine blue. For poly-rT, the degree of metachromasia, estimated visually by comparison with a control dye solution at the same concentration, increased with decreasing temperature; and decreased, but did not disappear, at a temperature above that at which the secondary structure was fully dissociated. These observations may be readily interpreted qualitatively in terms of the linear structure of the polymers as well as the secondary structure in the case of poly-rT. The fact that poly-Me-rT, which is devoid of secondary structure, was found to be metachromatic, is in agreement with the observations of Michelson [23] on the metachromasia of oligonucleotides of short chain length (4 - 13 residues); and with the observations on the metachromasia of the RNA "core" resulting from the exhaustive enzymic (pancreatic ribonuclease) hydrolysis of RNA and which has an average chain length of only 6 - 7 residues [34]. Since the metachromasia of poly-Me-rT was found to be relatively independent of temperature, the dependence of poly-rT metachromasia on temperature implies that the degree to which a basic dye is bound by a polynucleotide chain is, to an appreciable extent, dependent on the manner in which the aromatic bases are oriented, or "stacked", with respect to each other in the chain. Poly-rT and its *N*-methylated analogue provide, in fact, two model polymer chains which should prove extremely useful for examining quantitatively the relative influence of chain length and secondary structure on degree of metachromasia against various basic dyes.

GENERAL DISCUSSION

It is clearly of considerable importance to determine how methylation of the 5-position of the uracil rings in poly-U, with formation of poly-rT, results in such a marked increase in secondary structure of the latter, as well as in its ability to form an appreciably stronger hydrogen bonded complex with poly-A. This is certainly not due to any difference in molecular weight, i.e. chain length, of the polymers since Griffin *et al.* [12] measured the sedimentation constants of their samples of

poly-rT, obtaining values which lie within the range of those for poly-U. It must be also emphasized in this connection that the influence of methylation is negligible in short chains, i.e. where the appearance of secondary structure is excluded. The hypochromicity of the dinucleotide TpT is only 3 - 4% [11]; while that for oligo-dT with a chain length of about 10 residues is of the order of 9 - 10%, i.e. similar to that for oligo-U [36].

The principal effect of the methyl group at the 5-position of the uracil rings is therefore to strengthen the secondary structure. It seems rather unlikely that the inductive effect of the methyl group would be sufficiently pronounced to increase the hydrogen bonding properties of the N_3 or exo-O_4 of the uracil ring. The hydrogen bonding properties may conceivably be modified by hyperconjugation of the methyl groups with the aromatic rings, but the magnitude of such hyperconjugation effects has as yet not been evaluated [1]. Another possibility which must be considered is some type of hydrogen bonding involving the methyl hydrogens themselves; it was proposed some time ago by Evans *et al.* [6] and Watson [43] that methyl hydrogens may participate in hydrogen bonding, but independent confirmation of this is apparently lacking [2]. An examination of molecular models of di- and tri-nucleotides has thrown no additional light on the matter. X-ray diffraction, and rotatory dispersion, studies might be expected to provide some useful clues.

In the light of recent evidence on the involvement of hydrophobic forces in the stabilization of the secondary structure of polypeptides and polynucleotides (see for review [21a]), it seems likely that hydrophobic interactions between adjacent methyl groups of the 5 position of the uracil ring may result in additional stabilization of the helix. In any event it must be concluded, from the difference in behaviour between poly-U and poly-rT on the one hand, and their twin-stranded complexes with poly-A on the other, that the stability of poly-nucleotide helices is not due to the strength of the twin-strand hydrogen bonds alone, and that other or additional factors must be involved. This is discussed in greater detail in a subsequent paper (Szer & Shugar, Acta Biochim. Polon. 10, no. 2, in press).

It would also be of considerable value to compare the properties of poly-C with those of poly-(5-methylcytidylic) acid, the more so in that 5-methylcytosine is an important constituent of some nucleic acids. Additional models which merit investigation are the polynucleotides of halogenated uridylic acids, particularly in view of the current widespread studies on the biological properties of deoxyribonucleic acids containing biologically incorporated halogeno-uracils in place of thymine; Kit & Hsu [17] have, indeed, reported that substitution of

bromouracil for some of the thymine residues in DNA results in a definite increase in the T_m of the melting curve or temperature profile.

It will most likely be necessary in such investigations, as well as in others (see next paragraph) to distinguish between ribo- and deoxy-ribo-polymers. Schildkraut *et al.* [32] have provided evidence indicating that the T_m for the twin-stranded complex of poly-C with poly-dG is 18° higher than that for the corresponding complex of poly-dC with poly-dG; but this finding is perhaps not fully convincing since some doubts existed as to the chain length of the poly-dC component.

From Fig. 4 it follows that the tacit assumption of the equivalence of the base pairs A-U and A-T [see, e.g. ref. 5, 27] cannot be accepted. Rich & Tinoco [30], for example, calculated the influence of chain length on hypochromism in nucleic acids and synthetic polynucleotides, employing as models poly-A and oligonucleotides of dT of varying chain lengths; but their calculations were based on the use of the twin--stranded complex of poly-A with poly-U as a chain of infinite length. The present results show that this is not permissible.

As already noted above, poly-rT at low temperature (where a high degree of secondary structure prevails) and the twin-stranded complex of poly-rT with poly-A both exhibit *hyperchromicity* to the red of 290 - 295 mμ (see Figs. 2 and 5). This effect was predicted from theoretical considerations of the origin of hypochromicity by Tinoco [41], according to whom the hyperchromicity is due to the interaction between the $n \rightarrow \pi^*$ transitions of the individual bases, these transitions being perpendicular to the planes of the bases and hence aligned head to tail along the polymer chain. Such hyperchromicity was observed experimentally by Rich & Kasha [29] for the twin-stranded complex of poly-A and poly-U to the red of 280 mμ and was regarded as confirmation of Tinoco's theoretical treatment. Measurements of the ultraviolet dichroism for mechanically oriented films of poly-C apparently provided additional support for this interpretation [29]. This has, however, been placed in doubt by Gellert [9], who found that poly-dAT exhibited dichroism at 280 mμ similar to that for the main peak, so that the absorption at 280 mμ would be due to a transition *in the plane* of the bases, and concluded from this that the presence of a hyperchromic band is an insufficient criterion for the assignment of an $n \rightarrow \pi^*$ transition. In view of this, attention should be drawn to the fact that both poly-rT and its 1 : 1 complex with poly-A also exhibit hyperchromicity to the violet of 240 mμ and 230 mμ, respectively (Figs. 2 and 5). But the ultraviolet dichroism of oriented poly-C [29] suggests that the observed maximum absorption at 225 mμ is due to $\pi \rightarrow \pi^*$ bands, so that there should be hypochromicity in this region. In fact Rich & Kasha's [29] absorption spectrum for the 1 : 1 complex of poly-A with poly-U shows hyperchromicity to the violet of 230 mμ, in apparent contradiction with

their conclusions and with their observations on oriented poly-C. The foregoing comparisons are, of course, not entirely satisfactory; it will be necessary to carry out measurements of absorption and dichroism on the same polymer.

EXPERIMENTAL

General. Ascending paper chromatography, with Whatman no. 1 paper was used throughout. The composition of two of the solvent systems, *A* and *B*, and the relevant R_F values, are given in Table 1. Two other solvents used were: *C*, propan-2-ol - NH_4OH (d = 0.88) - H_2O (70:10:20 by vol.); *D*, water-saturated butanol.

A "dark" ultraviolet lamp was employed for locating the spots on the chromatograms.

Hilger Uvispek and Unicam SP500 spectrophotometers were used for spectral measurements. The temperature profiles were run on the Hilger instrument, using a specially constructed compartment for three cuvettes; water, or a mixture of water and glycerol, was circulated through the compartment by means of a Hoeppler ultrathermostat. The temperature of the compartment was calibrated against that of the fluid in the thermostat by means of copper-constantan thermocouples.

Table 1

Paper chromatography

The ascending technique on Whatman no. 1 paper and the following solvent systems were used: (*A*), ethanol - 1 M - ammonium acetate, 5:2 (v/v). (*B*), Propan-2-ol - 1% ammonium sulphate, 6:4 (v/v)

Compound	R_F in solvent	
	A	*B*
Ribosethymine (*I*)	0.78	—
2′:3′-*O*-Isopropylidene-ribosethymine (*II*)	0.89	—
Ribosethymine-5′-phosphate (*III*)	0.31	0.35
Ribosethymine-5′-pyrophosphate (*IV*)	0.10	0.16
2′:3′-*O*-Isopropylidene-*N*-methyl-ribosethymine (*VI*)	0.95	—
N-Methylribosethymine-5′-phosphate (*VII*)	0.34	0.40
N-Methylribosethymine-5′-pyrophosphate (*VIII*)	0.14	0.20
Uridine	0.75	—
Uridine-5′-phosphate	0.28	0.37
Uridine-5′-pyrophosphate	0.06	0.18

All pH measurements were made with the glass electrode and a Radiometer 222 pH meter.

Ribosethymine. This was obtained by condensation of the mercury salt of thymine with 1-chloro-2,3,5-tribenzoylribofuranose [16, 24] according to Fox *et al.* [8].

2′:3′-O-Isopropylideneribosethymine. This was prepared as described by Levene & Tipson [19] for isopropylideneuridine. The yield was quantitative and the product exhibited properties identical to those described by Griffin *et al.* [12].

Ribosethymine-5′-phosphate. Isopropylideneribosethymine was phosphorylated with β-cyanoethylphosphate as described by Tener [40]. The reaction was practically quantitative. Following removal of the cyanoethyl group by hydrolysis in 0.5 N-NaOH for 40 min. at 100°, acid hydrolysis (30 min. at 100° in 1 N-HCl) was employed for removal of the protecting isopropylidene group. The ribosethymine phosphate was isolated as the calcium salt and purified by repeated solution in water and precipitation with ethanol. The free nucleotide was then obtained by removal of the calcium with Dowex-50 (H^+), evaporation of the aqueous solution to small volume, removal of water by azeotropic distillation with ethanol and benzene, and addition of an excess of cold ether to a filtered (fritted G-4 glass filter), concentrated (about 50 mg./ml.) ethanolic solution of the acid. Analysis gave, for $C_{10}H_{15}O_9N_2P$: calc. N, 8.28%; P, 9.14%; exp. N, 8.0%; P, 9.3%.

N-*Methyl-2′:3′-O-isopropylideneribosethymine.* 400 mg. (1.35 mM) isopropylideneribosethymine was dissolved in 10 ml. anhydrous methanol, to which was added an ethereal solution of diazomethane until a permanent, pale yellow colour prevailed. Following several minutes, paper chromatography in solvent *D* exhibited the formation in quantitative yield of only one product. Solvent was then removed under vacuum and the residue crystallized from water, followed by recrystallization from ethyl acetate with addition of petroleum ether to slight turbidity, to give 400 mg. of product containing 2 molecules of water of crystallization, softening at 75° and melting completely at 125°. Removal of the water of crystallization at elevated temperature under reduced pressure gave a colourless glass which could not be crystallized. For $C_{14}H_{20}O_6N_2$: calc. N 8.97%; exp. N 8.7%.

N-*Methyl-ribosethymine-5′-phosphate.* Phosphorylation of the foregoing compound was carried out by the "polyphosphate" method as described previously for *N*-methyluridine-5′-phosphate [37] with a yield of 57%. The resultant product was chromatographically homogeneous in solvents *A*, *B* and *C*; gave a positive periodate reaction; and was quantitatively dephosphorylated by snake venom (*Crotalus adamanteus*) to a single product, *N*-methyl-ribosethymine.

Ribosethymine-5′-pyrophosphate. 100 mg. (0.3 mM) of ribosethymine-5′-phosphate was transformed to the mono-(tri-*n*-octyl)amine salt and phosphorylated with an excess of dibenzylphosphochloridate as described by Michelson [22, cf. 37] to give 73 mg. of the pyrophosphate (58.5% theor.) with λ_{max} 267 mμ and ε_{267} 9.4×10^4 (pH 6.9). The product was chromatographically homogeneous in solvents *A* and *B*; gave a positive

periodate reaction; was quantitatively hydrolysed by snake venom (*Crotalus adamanteus*) to ribosethymine; and was active as a substrate for polynucleotide phosphorylase.

N-Methyl-ribosethymine-5′-pyrophosphate. 105 mg. (0.3 mM) of *N*-methyl-ribosethymine-5′-phosphate was phosphorylated as described in the preceding section [cf. 22], to give 82 mg. (63% theor), λ_{max} 267 mμ, ε_{267} 9.0×10^4 (pH 6.9). The product was chromatographically homogeneous and active as a substrate for polynucleotide phosphorylase.

Enzymic synthesis of poly-rT. This was carried out under standard conditions [13, 37] with yields of polymer ranging from 20 to 40% in different experiments. The course of polymerization for the experiment with 40% yield is shown in Table 2. The polymer was isolated by precipitation with ethanol and exhaustive dialysis against 0.005 M-NaCl at 3°. In a couple of experiments isolation was achieved by means of extraction with phenol [10]. The polymer, following dialysis, was either lyophilized or stored in concentrated, aqueous, solution at -60°. Out of 7 experiments, 4 gave polymers with high temperature hypochromicity (see below). Even the best preparations, following storage at -60° for two weeks, and thawed from time to time for the sole purpose of withdrawing samples for experiments, had by the end of this period lost most of their temperature hypochromicity. With longer storage and repeated thawing for short intervals, paper chromatography demonstrated the appearance of oligonucleotides. It is therefore recommended that the polymer solution be divided into aliquots for storage in the deep-freeze. Each aliquot is then thawed as required for a given experiment.

Enzymic synthesis of poly-Me-rT. This was carried out as described above. In the first attempted polymerization, only traces of polymer were detected after 42 hr. (fresh enzyme, and thymol as a bactericidal agent, were added after 24 hr.). This was used as a "primer" in subsequent polymerizations, the resulting incubation periods being then considerably reduced, so that after 24 hr. incubation the yield of polymer ranged from 11 to 14%. The polymer was isolated and stored as for poly-rT. It exhibited the same degree of instability as poly-rT during extended storage.

Secondary structure and residual hyperchromicity of poly-rT and poly-Me-rT. The extinctions of the polymers, dissolved in 0.01 M-$MgCl_2$ or in 0.15 M-NaCl + 0.015 M-sodium citrate at pH 7, were measured over the temperature range 4 - 75° in 10 mm. cuvettes (polymer conc. about 10^{-4} M). The temperature profile for poly-rT, shown in Fig. 1, was completely reversible on reducing the temperature. In the absence of salt and buffer, no temperature hyperchromicity was exhibited by poly-rT. The ultraviolet absorption spectrum of poly-rT in 0.01 M-$MgCl_2$ at pH 7 is exhibited in Fig. 2 for three different temperatures, 4°, 25°

and 50°, from which the hyperchromicity can be calculated for all wavelengths.

In the case of poly-Me-rT, no change in absorption could be detected at 266 mμ even when the $MgCl_2$ concentration was increased to 0.1 M.

Following determination of the peak extinction of poly-rT at the temperature provoking collapse of secondary structure (see Fig. 2), the solution was brought to room temperature and the polymer subjected to the action of pancreatic ribonuclease. This resulted in an additional increase in absorption of 8%. Acid (1 N-HCl, 25°, 20 hr.) and alkaline (0.3 N-NaOH, 25°, 20 hr.) hydrolysis gave increases ranging from 7.5 to 9.5%. In all cases it was established by paper chromatography that hydrolysis to mononucleotides was complete.

Table 2

Synthesis of poly-ribothymidylic acid catalysed by polynucleotide phosphorylase

The amount of polymer formed was determined by spectrophotometry of a paper chromatogram eluate (solvent *B*)

Time of incubation (hr.)	Polymer formed (%)
13	traces
15	15
20	35
22	39
25	40.5

Hydrolysis to mononucleotide of poly-Me-rT by acid (1 N-HCl, 25°, 20 hr.) or snake venom (*Crotalus adamanteus*) resulted in an increase in extinction of 7%.

Complexes (1:1) with poly-A. These were formed under standard conditions [42, 5] at room temperature in 0.15 M-NaCl and 0.015 M-sodium citrate. Changes in optical density resulting from mixing of the polymers were measured at three wavelengths: 257, 266 and 262.5 mμ, which are the λ_{max} respectively for poly-A, poly-rT and the 1 : 1 complex of the two. Complex formation was followed by "titration" of poly-A by poly-rT, and conversely, until the molar ratio of the two components was 1 : 1; following which the temperature profile was determined. Since, under the conditions prevailing, each polymer exhibits its own secondary structure, the temperature profile of each was also determined for a concentration equal to that present in the complex (see Fig. 4).

The temperature profile for a complex consisting of poly-A and oligo-rT (i.e. poly-rT which had spontaneously degraded to give smaller

chains) is shown in Fig. 4. With a 1 : 1 ratio of the two components, such a complex exhibited a maximum hypochromicity of only 19%.

The absorption spectrum of the complex poly-A + poly-rT at room temperature and at 85° is presented in Fig. 5.

No complex formation could be observed between poly-A and poly-Me-rT under the above standard conditions. An increase in NaCl concentration to 1 M and a decrease in temperature to 4° gave only a simple mixture for which the optical density was always the arithmetic sum of the two components.

Instability of polymers. (a) To neutral solutions of yeast RNA (5 mg./ml.) were added, respectively, poly-rT and poly-Me-rT to a final concentration of 0.5 mg./ml. Aliquots of both solutions, as well as that of a control not containing synthetic polymer, were chromatographed in solvent *B* at intervals of several hours. Following 20 hr. incubation at room temperature, the test solution did not differ from the control. (b) Solutions of poly-rT and poly-Me-rT (each at a concentration of 5 mg./ml.) in sealed ampoules were heated on a water bath for 5, 30 and 65 min., left at room temperature for 18 hr., and then chromatographed in solvent *B*. The sample heated for 5 min. was found to have undergone the most extensive degradation. That which had been heated for 65 min. was practically identical chromatographically to a sample chromatographed immediately after heating. Poly-U under the foregoing conditions was unaffected. (c) Solutions of poly-rT and poly-U (5 mg./ml.) were brought to pH 2.5 and their rate of hydrolysis followed by chromatography in solvent *B*. After 4 hr. at room temperature poly-U was unchanged, i.e. no oligonucleotides could be observed; following 20 hr. oligonucleotides, including dinucleotides, made their appearance. Poly-rT showed signs of hydrolysis shortly after acidification; following 20 hr. the high molecular weight polymer had entirely disappeared, to be replaced by oligonucleotides, and some dinucleotide and monomer.

Table 3

Hydrolysis of poly-ribothymidylic acid catalysed by pancreatic ribonuclease

Details see text

Time of incubation (min.)	Hydrolysis products			
	Polymer	Oligonucleotides	Dinucleotide	Monomer
10	+ +	+ + +	trace	trace
20	+	+ + +	+ +	+
30	—	+ +	+ + +	+ +
60	—	+	+ + +	+ + +
120	—	trace	+ +	+ + +
480	—	—	—	+ + +

Metachromasia. Both poly-rT and its methylated analogue exhibited pronounced metachromasia against toluidine blue, using a dye concentration of 10^{-4} M and a polymer concentration of 7×10^{-5} M. Change of colour was observed visually against a control solution of the dye alone. For poly-rT the degree of metachromasia increased with decreasing temperature; and decreased, but did not disappear, on raising the temperature to 60°. The degree of metachromasia of poly-Me-rT was unaffected by modifications of temperature.

Enzymic tests. Pancreatic ribonuclease (Armour): (a) Poly-rT, 3 mg./ml. in 0.12 M-phosphate buffer, pH 7.2, containing 0.01 M-$MgCl_2$, was treated with ribonuclease at a concentration of 3 μg./ml. A control contained no enzyme and a third sample contained poly-U and enzyme. Incubation was at 22° where poly-rT is largely in the helical form. The course of hydrolysis was followed chromatographically in solvent *B*, the relative amounts of each component being estimated visually, with the results for poly-rT as shown in Table 3. The results for hydrolysis of poly-U were identical. In the control sample after 4 hr. traces of oligonucleotides were present. At considerably higher enzyme concentrations, used for determination of residual hyperchromicity of poly-rT, hydrolysis was very rapid. (b) A 1 : 1 complex of poly-rT with poly-A was prepared at a concentration of poly-rT of 3 mg./ml. in 0.15 M-phosphate buffer, pH 7.2. Enzyme was added to a concentration of 15 μg./ml. Traces of ribosethyminephosphate could be detected after 10 min. incubation at room temperature. Following 30 min. incubation the amount of monomer had increased to 16% and, after 6 hr. incubation, only monomer was present. Poly-A was unaffected and remained at the starting point on the chromatograms. (c) No hydrolysis of poly-Me-rT could be detected under the foregoing conditions even when the ratio of enzyme to substrate was increased to 1 : 10.

Snake venom (*Crotalus adamanteus*): In a typical experiment 3 - 5 mg./ml. polymer in 0.1 M-borate buffer, pH 8.9, was treated with lyophilized venom at a concentration of 0.3 - 0.5 mg./ml. Both poly-rT and poly-Me-rT were completely transformed to the nucleosides.

The authors are particularly indebted to Dr. M. Grundberg-Manago and Prof. S. Ochoa for gifts of polynucleotide phosphorylase.

SUMMARY

Poly-ribothymidylic acid (poly-rT) and poly-(*N*-methyl-ribothymidylic) acid (poly-Me-rT) have been prepared and their properties compared with those of poly-U and poly-MeU.

The residual hyperchromicities of all four polymers are similar, 7 - 9%. Poly-rT exhibits very high temperature hyperchromicity with a T_m of 36° in 0.01 M-$MgCl_2$ as compared to the much lower hypochromicity of poly-U with a T_m of 8.5°. At 25 - 30°, where poly-U is in the form of a random coil, poly-rT possesses a highly ordered configuration.

As previously observed for poly-MeU, poly-Me-rT exhibits no temperature hyperchromicity and hence no secondary structure under any conditions. Qualitative studies of metachromasia are in agreement with the foregoing and show that the metachromasia of polynucleotides against basic dyes is dependent both on the linear structure of a polymer and its secondary structure.

Like poly-MeU, poly-Me-rT is completely resistant to pancreatic ribonuclease.

The twin-stranded complex of poly-rT with poly-A is considerably more stable than that of poly-U with poly-A, the T_m being 20° higher. The base pair A-T is therefore not equivalent to the base pair A-U, a finding of some significance with respect to nucleic acid structure.

Poly-Me-rT is incapable of complexing with poly-A, in agreement with the Watson-Crick hypothesis according to which one of the hydrogen bonds in the base pair A-U involves the ring N_3 nitrogen as a hydrogen donor.

Both poly-rT and poly-Me-rT undergo slow, spontaneous, degradation in aqueous medium at room temperature. Evidence has been presented to show that this is due to the presence in polynucleotide phosphorylase of traces of a highly specific phosphodiesterase inactive against poly-U or yeast RNA.

The results, and their implications with respect to the structure of nucleic acids and synthetic polynucleotides, are discussed. In particular, it is concluded that the stability of polynucleotide helices is not due solely to the strength of the twin-strand hydrogen bonds, but must involve additional factors, the precise nature of which remains to be established.

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STRUKTURA DRUGORZĘDOWA KWASÓW POLI-URYDYLOWEGO I POLI-RYBOTYMIDYLOWEGO, ICH *N*-METYLOWANYCH ANALOGÓW ORAZ KOMPLEKSÓW Z KWASEM POLI-ADENYLOWYM

Streszczenie

Przeprowadzono syntezy kwasu poli-rybotymidylowego (poli-rT) i kwasu poli-*N*-metylorybotymidylowego (poli-Me-rT); porównano ich właściwości z właściwościami poli-U i poli-MeU.

Wartość hiperchromazji szczątkowej wszystkich czterech polirybonukleotydów jest podobna i wynosi 7 - 9%. Poli-rT wykazuje bardzo wysoki temperaturowy efekt hipochromowy i temperaturę połowicznego rozpadu struktury drugorzędowej (T_m) w 0.01 M-$MgCl_2$ 36° w odróżnieniu od poli-U, który posiada znacznie niższą hipochromazję temperaturową i T_m 8.5°. W temperaturach 25 - 30° łańcuch poli-U jest nieuporządkowany, natomiast poli-rT posiada wysoce uporządkowaną strukturę.

Poli-Me-rT, podobnie jak poli-MeU, nie wykazuje temperaturowej hipochromazji i wobec tego nie posiada drugorzędowej struktury w roztworach w najbardziej sprzyjających warunkach temperatury i siły jonowej. Ilościowe oznaczenia efektu metachromowego w stosunku do zasadowych barwników są zgodne z powyższym i wykazują, że metachromazja zależy od liniowej struktury polirybonukleotydu i od jego drugorzędowej struktury.

Poli-Me-rT jest całkowicie odporny na działanie rybonukleazy trzustki, podobnie jak poli-MeU.

Kompleks złożony z podwójnego łańcucha poli-rT i poli-A jest znacznie trwalszy od kompleksu poli-U i poli-A i posiada T_m o 20° wyższą niż ten ostatni. Para uzupełniających się zasad A-T nie jest więc równoważna parze A-U, jak przyjmowano dotychczas, co posiada określone znaczenie w strukturze kwasu nukleinowego.

Poli-Me-rT nie tworzy kompleksów z poli-A; jest to zgodne z modelem Watsona-Cricka, który przewiduje, że jedno z wiązań wodorowych w parze A-T zawiera pierścieniowy N_3 jako donor wodoru.

Poli-rT i poli-Me-rT w roztworach wodnych w temperaturze pokojowej ulegają powolnej, spontanicznej degradacji. Przedstawiono dowody, że jest to proces związany z obecnością w preparatach polinukleotydowej fosforylazy wysoko specyficznej fosfodwuesterazy, nieaktywnej w stosunku do RNA z drożdży i poli-U.

Otrzymane wyniki przedyskutowano w odniesieniu do struktury kwasów nukleinowych i polirybonukleotydów. W szczególności wyciągnięto wniosek, że trwałość podwójno-łańcuchowych kompleksów polinukleotydów jest uwarunkowana nie tylko siłami wiązań wodorowych pomiędzy uzupełniającymi się zasadami; wchodzą tu w grę dodatkowe czynniki stabilizujące, których charakter pozostaje do zbadania.

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CHANGES IN PHOSPHOLIPIDS DURING AUTOLYSIS OF RAT BRAIN AND LUNG

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Lysolecithin, a substance known for its lytic properties, has been found in lipid extracts of a number of mammalian tissues. The origin of lysolecithin found in the tissues *post-mortem* is not clear although there is evidence that it is present in living tissues [14, 11]. The majority of papers, as far as we are aware, were concerned only with phospholipids behaviour during *post-mortem* autolysis. Fairbairn [5] has found that immediately after death there is a rapid increase in free fatty acids in the tissues, which can be accounted for by partial hydrolysis of the phospholipids. However, much slower breakdown of phospholipids was observed by other authors [6, 17].

This paper is concerned with a study of the behaviour of lysolecithin and other phospholipids during autolysis of rat brain and lung.

EXPERIMENTAL

Animals and tissues. Albino rats of both sexes aged 3 - 6 months were killed by decapitation. The brain and lungs were removed as soon as possible with sterile instruments. Brain without cerebellum and lungs with removed trachea and main bronchial tubes, were used. For experiments with whole organs, brain and lungs were cut into two parts, left and right, one part being used as a control. The tissue homogenates were prepared in a Potter-Elvehjem homogenizer with M/15 phosphate buffer, pH 6.5, in proportion of 3 ml. of buffer per 1 g. of fresh tissue. The homogenates were squeezed through a double layer of gauze.

Incubation. All incubations were carried out at 25°. The intact tissues were incubated in sterile vessels with tight-fitting glass stoppers. The homogenates were incubated in 22×90 mm. test tubes. In some experiments the homogenate before incubation was heated on a water bath at 65°±2° for 15 min. The sterilization of vessels and instruments was carried out by 20 min. boiling in water and drying at 120 - 150°.

Extraction of lipids. These were extracted with chloroform - methanol (2:1, v/v) according to Folch *et al.* [7], 19 ml. of the solvent mixture being used per 1 g. of fresh tissue or 1 ml. of homogenate. The extrac was filtered and the solution washed with 1/5 of its volume of distilled water. After centrifuging, the upper phase was discarded and the lower phase washed three times with methanol - water - chloroform (48:47:5, by vol.). The washed extract was evaporated to dryness *in vacuo* at no more than 50°, and left overnight in a desiccator over $CaCl_2$ under reduced pressure. The dry residue was dissolved in a minimum volume of chloroform. Samples of chloroform solution equivalent to 2 - 5 mg. of fresh tissue were taken for the determination of total phospholipids phosphorus, and the remainder was applied quantitatively to a chromatographic column.

Chromatographic separation. The columns of silicic acid or alumina were used. The mixture of 2 g. of silicic acid (activated at 120° overnight) and 1 g. of Hyflo-Supercel was transferred to a column as a slurry in chloroform. The columns were about 9×130 mm. The chloroform solution of lipids equivalent to 500 - 800 mg. of fresh tissue was transferred quantitatively to a column which was then washed with 20 ml. of chloroform. The elution based on Hanahan *et al.* [8a] procedure was performed as follows: 50 ml. of chloroform - methanol (9:1, v/v) was used to elute cephalins; 150 ml. of chloroform - methanol (3:1, v/v) to elute lecithin and sphingomyelin; and 50 ml. of methanol to elute lysolecithin. Approximately half of the phosphorus of the last fraction corresponded to lysolecithin, and the rest to sphingomyelin. Fig. 1 illustrates the elution pattern of brain lipids.

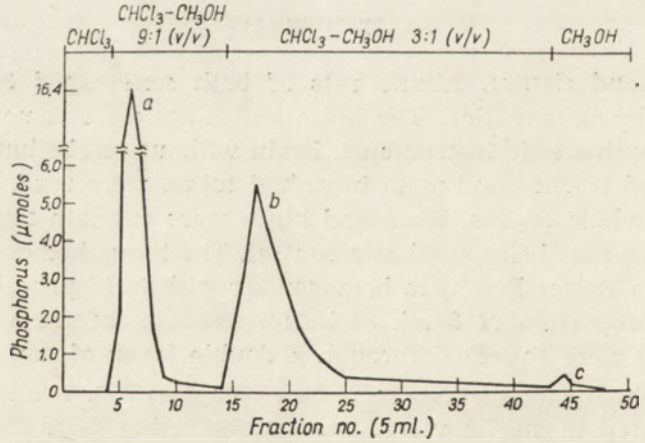

Fig. 1. The elution pattern of brain lipids separated on silicic acid - Hyflo - Supercel column. Peak (*a*) corresponds to cephalins (phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol); peak (*b*), to lecithin and sphingomyelin; peak (*c*), to lysolecithin

To separate lysolecithin, the last fraction was taken up to dryness under reduced pressure and dissolved in chloroform - methanol (2:1, v/v). Samples equivalent to 100 - 200 mg. of fresh tissue were spotted in duplicate onto the silicic acid impregnated paper and developed as described previously [13].

Some experiments were carried out on alumina columns loaded with 3 g. of Al_2O_3 as a slurry in chloroform. The chromatography was performed after Davison & Wajda [1a]. Lipid chloroform solution equivalent to 500 - 800 mg. of fresh tissue was transferred quantitatively to a column which was then washed with 30 ml. of chloroform. Next 30 ml. of chloroform - methanol (1:1, v/v) was used to elute choline-containing phospholipids, and 50 ml. of chloroform - ethanol - water (2:5:2, by vol.) to elute cephalins. To compare the elution pattern from silicic acid and alumina columns, the same amounts of lung lipid extract were applied to both columns. From the silicic acid column 6.3 μmoles of cephalins and 13.3 μmoles of choline-containing phospholipids were obtained, and from the alumina column 6.4 μmoles and 11.7 μmoles, respectively.

Analytical methods. Lipid phosphorus was determined by the method of Ernster, Zetterstrom & Lindberg [4] as modified by Stricland, Thompson & Webster [18]. Lysolecithin was determined as described previously [13].

Reagents. The organic solvents were of analytical grade. Silicic acid "for chromatography, 325 mesh" was purchased from Bio-Rad Laboratories, U.S.A. "Alumina", aluminium oxide, Hopkin Williams Ltd., England, 100 - 200 mesh, Brockman activity 1 - 2.

RESULTS AND DISCUSSION

Controls. The brain hemispheres as well as the lung lobes were examined separately for lipid content. The results shown in Table 1 indicate that no significant differences were found between the right and left parts of the organs tested.

According to Dawson [2] the analytical results are rather doubtful if, under non-sterile conditions, the time of autolysis is longer than 24 hr. In the present experiments several precautions were taken to avoid bacterial infection (sterilization of instruments and of incubation vessels, disinfection of hands). The surfaces of brain and lung were assayed for sterility on blood agar. The blood plates were incubated for 24 hr. at 38° and the colonies were counted. The brain just after removal, and kept for 4 hr. at 25° was practically sterile (0 to 2 colonies per plate); after 24 hr., however, there were 20 - 50 colonies per plate. The lungs even after 24 hr. incubation were practically sterile (0 to 1 colony per plate). It should be mentioned that the lungs were removed from the

Table 1

Content of phospholipids in the right and left brain hemispheres and lung lobes

The lipids were separated on alumina column. Details see text

Tissue	Part	Total lipids	Choline phosphatides	Cephalins
		(μmoles P/g. fresh tissue)		
Brain	right	63.7	27.9	23.7
	left	66.7	29.1	20.0
Brain	right	64.0	30.2	—
	left	63.0	30.1	—
Brain	right	67.4	33.8	—
	left	69.8	34.1	—
Brain	right	—	26.0	23.3
	left	—	26.6	24.7
Brain	right	72.6	27.9	24.4
	left	71.2	28.6	24.4
Lung	right	32.7	16.6	10.5
	left	32.1	15.6	10.2
Lung	right	31.0	14.9	10.2
	left	30.7	16.0	11.0

body through the abdominal cavity but while removing the brain it was difficult to avoid an infection. It seems, however, that microorganisms growing on brain surface have no influence on lipids in the tissue while it is kept at 25° for 24 hr. To examine the influence of bacterial infection on lipid metabolism in tissue homogenates, antibiotics were added before incubation. No difference in lipid content was found between lung homogenates incubated for 24 hr. with and without penicillin. The autolysis of tissue lipids in the presence of streptomycin was even faster than in the homogenate alone.

Phospholipids during autolysis. The high content of phospholipids and the low rate of their turnover are characteristic for brain; in lung the content of lipids is lower but there is an active phospholipids metabolism. Therefore these two tissues were chosen for experiments. Table 2 represents the amounts of phospholipids in intact brain and lung at 0 time and after autolysis for 24 hr. The rate of the breakdown of individual phospholipid fractions, except lysolecithin, was similar. There were no significant differences between the decomposition of phospholipids in intact brain and in brain homogenate. The rate of breakdown of lung lipids, except lysolecithin, was significantly greater than that of brain lipids. The breakdown of these substances in lung homogenate was much faster than in intact tissue. Table 3 presents the decomposition of total

Table 2

Phospholipids in rat brain and lung after 24 hr. autolysis at 25°

The lipids were separated on silicic acid column. Figures in brackets refer to the number of experiments. The mean values, ± S.D. are given.

Tissue	Incubation (hr.)	Total phospholipids			Lecithin and sphingomyelin			Cephalin			Lysolecithin		
		found	decomposed		found	decomposed		found	decomposed		found	formed (+) or decomposed (—)	
		(μmoles P/g. fresh tissue)		(%)	(μmoles P/g. fresh tissue)		(%)	(μmoles P/g. fresh tissue)		(%)	(μmoles P/g. fresh tissue)		(%)
Intact brain	0	75.0 (12)			36.0 (9)			36.0 (9)			0.30 (6)		
	24	67.0 (12)	8.0 ± 1.5	10.7	31.0 (9)	5.0 ± 3.2	13.9	31.8 (9)	4.2 ± 2.1	11.7	0.58 (6)	+0.28 ±0.11	+93.0
Brain homogenate	0	65.0 (8)			30.9 (5)			30.3 (6)			0.29 (5)		
	24	56.5 (6)	10.0 ± 2.5	15.4	26.3 (4)	5.3 ± 3.0	17.2	25.0 (4)	5.4 ± 2.4	17.8	0.60 (3)	+0.27 ±0.09	+93.0
Intact lung	0	33.0 (9)			23.5 (7)			10.9 (9)			0.49 (6)		
	24	25.9 (9)	7.1 ± 1.9	21.5	17.7 (7)	5.8 ± 1.8	24.7	7.6 (9)	3.3 ±1.2	30.2	0.68 (6)	+0.19 ±0.13	+38.8
Lung homogenate	0	34.2 (8)			23.9 (7)			7.2 (7)			0.73 (4)		
	24	12.4 (7)	21.3 ± 1.5	62.4	8.6 (7)	15.3 ± 1.1	64.0	2.4 (7)	4.8 ± 1.7	66.0	0.43 (4)	—0.30 ±0.19	—41.0

phospholipids during autolysis of different rat tissues, and the data of several authors are compared with the present results.

Lysolecithin in lung homogenate during 24 hr. autolysis behaved like other phospholipid fractions, i.e. its amount decreased at a similar rate. However, in intact lung and in both intact and homogenized brain, the amount of lysolecithin increased considerably after 24 hr. autolysis. Such a behaviour of lysolecithin does not agree with the supposition that lysolecithin is an artifact formed during the isolation procedure [1], but seems to indicate that lung and brain cells possess an enzymic system able to form and to break down lysolecithin. In other communications this possibility was also supposed [9, 19, 12]. However, no suggestions concerning the mechanism of the changes in lysolecithin content during prolonged autolysis can yet be made.

The effect of heating. It is known that animal phospholipase A from different sources is strongly resistant to heating especially at pH below 7, the only exception being phospholipase A from intestinal mucosa [3, 15, 10, 20]. To ascertain whether the increase in lysolecithin content during the autolysis was due to the action of phospholipase A, the effect of heating was examined. For this purpose the brain and lung homogenates adjusted to pH 6.5 were heated for 15 min. at 65°, and then incubated for 24 hr. at 25°. Fig. 2 illustrates the results of analysis (6 to 12 individual experiments); it can be seen that heating inhibited virtually completely the autolytic changes in the content of lysolecithin and other phospholipids. This heat-susceptibility seems to indicate that the system producing lysolecithin is different from phospholipase A.

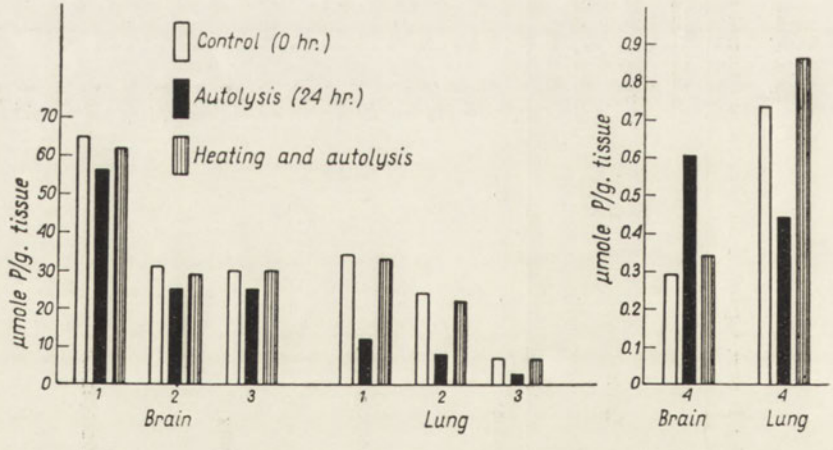

Fig. 2. Effect of heating for 15 min. at 65° on phospholipid fractions found in tissue homogenates autolysed for 24 hr. at 25°. The values are averages from 6 to 12 determinations. (*1*), P of total phospholipids; (*2*), P of choline phosphatides; (*3*), P of cephalins; (*4*), P of lysolecithin

The comparison of lysolecithin content in tissue not submitted to autolysis (control experiments) with the content after autolysis with and without heating, suggests that lysolecithin found in the tissue is not an artifact and that during autolysis of brain the increased amount of lysolecithin is formed enzymically.

Effect of the duration of autolysis. The time-rate of the breakdown of phospholipids is shown in Fig. 3. Phospholipid fractions were determined in intact tissues at 0 time and after autolysis for 1, 4 and 24 hr.

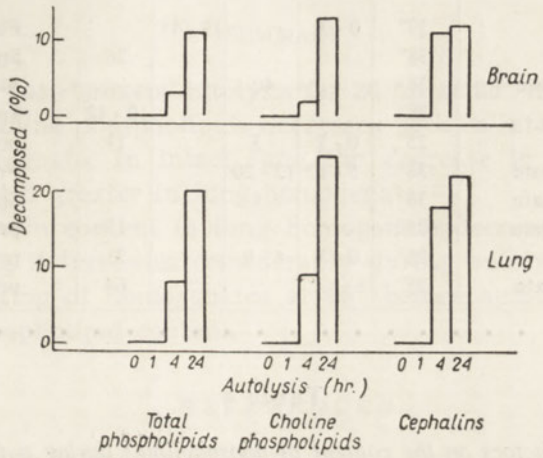

Fig. 3. The time-rate of the decomposition of phospholipids in intact brain and lung. The data representing mean values of phospholipids decomposed are expressed in percentages of the initial content

After 1 hr. incubation no significant breakdown of brain and lung phospholipids was observed. Later the decomposition of cephalins was faster than of choline phospholipids. The phospholipid-splitting mechanism in lung appeared to be more active that in brain.

Effect of various factors. At 0° the breakdown of phospholipids in intact brain was completely inhibited. In intact lung, low temperature (0°) did not prevent autolysis, and some breakdown of phospholipids was observed.

At pH 6.5 the decomposition of phospholipids in tissue homogenates was greater than at pH 7.4.

The addition of Hg^{2+} in 1 mM concentration reduced the autolysis in about 50% as compared with the control experiment. Mercuric ion in 2 mM concentration inhibited completely the autolytic changes in phospholipids, including lysolecithin.

Conclusion. The presented results indicate that in rat brain and lung a thermolabile system is present which is able to produce and decompose lysolecithin. This system seems to be responsible for the changes

Table 3

Total phospholipids decomposition during autolysis of rat tissues according to several authors

Tissue tested	Temperature of incubation	Time of incubation (hr.) 1	4	6	24	Author
		Phospholipids decomposed (%)				
Liver slices	37°	0 - 3		19 - 44		Fishler *et al.* [6]
Liver pieces	38°				26	Sperry *et al.* [17]
Liver homogenate	38°		40			Fairbairn [5]
Intact brain	38°				10 - 12	Sperry *et al.* [17]
Intact brain	25°	0 - 3	3		11	present results
Brain homogenate	38°	5 - 12	12 - 20			Fries *et al.* [8]
Brain homogenate	38°		8			Sperry [16]
Brain homogenate	25°				13	present results
Intact lung	25°	0 - 3	6 - 9		21	present results
Lung homogenate	25°				64	present results

Table 4

Effect of various factors on the content of phospholipids during autolysis of rat brain and lung

Typical conditions of autolysis: 25°, 24 hr., pH 6.5

Tissue	Factor applied	Time of incubation (hr.) 0	8	24
		Total lipids (μmoles P/g. fresh tissue)		
Intact brain	0°	67.6	—	65.8
Intact brain	0°	67.8	—	66.2
Intact lung	0°	33.2	—	30.5
Intact lung	0°	31.4	—	27.4
Lung homogenate	none	25.2	13.7	—
Lung homogenate	Hg^{2+}, 1 mM	24.6	18.7	—
Lung homogenate	Hg^{2+}, 2 mM	24.6	24.6	—
Lung homogenate	none	25.2	13.7	—
Lung homogenate	pH 7.4	26.0	18.2	—

in lysolecithin content occurring during autolysis of the tissue. In brain homogenates during 24 hr. autolysis the amount of lysolecithin increased, whereas in lung homogenates it diminished. The increase of lysolecithin was probably not due to phospholipase A action.

For quantitative studies of phospholipids, including lysolecithin, the tissues should be analysed within 1 hr. after the death of the animal. The storage of the tissue at 0° prevents the autolytic changes in phospholipids but only when the tissue is cooled immediately after its removal from the body.

The authors wish to thank Mrs. Melania Łabuda for expert technical assistance.

SUMMARY

1. During *post-mortem* autolysis for 24 hr. at 25° the content of cephalins and choline phospholipids decreases both in intact rat brain and in brain homogenate. In intact lung the decrease is greater than in brain, and is still greater in lung homogenate.

2. Lysolecithin content in lung homogenate decreases, but in brain and intact lung it increases considerably during autolysis.

3. The heating of homogenates at 65° before autolysis inhibits the changes in phospholipid content.

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ZMIANY POZIOMU FOSFOLIPIDÓW W CZASIE AUTOLIZY MÓZGU I PŁUC SZCZURA

Streszczenie

1. Podczas 24-godz. autolizy nienaruszonego mózgu i homogenatu mózgu szczura w temperaturze 25° ulegają rozpadowi kefaliny oraz fosfolipidy zawierające cholinę. Rozpad w płucach nienaruszonych jest większy niż w mózgu; największy rozpad obserwowano w homogenatach płuc.

2. Poziom lizolecytyny zmniejsza się w homogenatach płuc, natomiast znacznie wzrasta w mózgu i w nienaruszonych płucach.

3. Ogrzanie homogenatów do 65° przed autolizą hamuje niemal całkowicie rozpad fosfolipidów.

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NAD GLYCOHYDROLASE IN CARP LIVER

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Nicotinamide-adenine dinucleotides (NAD and NADP) are cleaved enzymically in the animal tissues either at the pyrophosphate linkage by pyrophosphatase or at the *N*-ribosyl-nicotinamide linkage by specific NAD glycohydrolases (NADases). The latter enzymes from various sources, except *Neurospora* enzyme, are universally inhibited by nicotinamide but show an interesting species-dependent specificity with respect to inhibition by isonicotinyl hydrazide (INH) [12]. This specificity was found to be associated with a second function of these enzymes i.e. the ability to catalyse the transfer reaction resulting in formation of NAD or/and NADP analogues. In the "INH-insensitive" systems this mode of action may dominate with complete suppresion of the hydrolytic function. Kaplan therefore postulated that NADases in animals are primary transglycosidases rather than hydrolytic enzymes [5].

Hydrolysis: $NAD^+ + HO \cdot H \rightarrow HO \cdot \text{ribose} \cdot ADP + \text{nicotinamide} + H^+$

Transfer reaction: $NAD^+ + INH \rightleftharpoons INH^+ \cdot \text{ribose} \cdot ADP + \text{nicotinamide}.$

In our studies on fishes we found that NADase in the extract from carp liver mitochondria was so active as to enable NAD-dependent deamination of glutamic acid. In connection with this high activity it appeared that carp liver mitochondria are a good object for studying specificity and function of NADases whose function in cell metabolism may be not limited to hydrolytic properties.

EXPERIMENTAL

Materials and methods

Preparation of the extracts from the subcellular fractions. All operations were conducted at 0° - 4°. Carp (*Cyprinus carpio*) liver, rat liver, and pig brain were homogenized in 10 vol. of 0.25 M-sucrose and were

fractionated as described by Hogeboom [3] in the Servall Superspeed centrifuge with SS-34 rotor. Microscopic examination of the nuclear fraction revealed only single undisrupted cells. Mitochondria were separated at 10 000 *g* and were washed twice. The microsomal fraction was spun down at 37 000 *g* and contained the bulk of microsomes. The isolated subcellular fractions suspended in 0.05 M-phosphate buffer, pH 7.6, were kept overnight in a refrigerator and were then disrupted in a glass Potter homogenizer. The extracts thus obtained were centrifuged for 15 min. at 4000 - 6000 *g*. The samples suitably diluted were used for enzymic assays.

The pig brain NADase was purified according to Zatman, Kaplan, Colowick & Ciotti [13]; the enzyme passed into supernatant upon a 10 min. treatment in the 20 kc. sonic oscillator. The activity was 5 units/ml. of the supernatant fluid.

Reagents. INH analogue of NAD was synthesized with the purified pig brain NADase after Zatman *et al.* [13], and its spectrum was compared with that given by the same authors. Nicotinamide-adenine dinucleotide (NAD) (C. F. Boehringer & Soehne, Mannheim); nicotinamide-adenine dinucleotide phosphate (NADP) (Carl Roth, Karlsruhe); isonicotinyl hydrazide (INH) was a gift from the Department of Organic Technology of the Warsaw Politechnic School. Nicotinamide (Hurtownia Farmaceutyczna, Katowice), alcohol dehydrogenase (Nutritional Biochemicals Corporation U.S.A.), tris (Sigma), potassium cyanide (Hurtownia Farmaceutyczna, Katowice), tannin (CeFarm, Gliwice).

The enzyme assay. Determination of the NADase and NADPase activity was based on measurements of the decrease in extinction at 325 mμ of NAD or NADP cyanide complex [2] and of NAD at 340 mμ with the yeast alcohol dehydrogenase [2]. In the first method both the nicotinamide mononucleotide and dinucleotide are measured while in the second one only the intact coenzyme is determined. The nicotinamide mononucleotide reacts with KCN, and cleavage of NAD or NADP at the pyrophosphate linkage is not accompanied by any change in the optical density of the cyanide complex. By combining both these methods it is possible to determine whether the destruction of pyridine dinucleotides is due to the cleavage of the *N*-glycoside linkage or of the pyrophosphate linkage, or both.

In both procedures the extract was incubated in 0.05 M-phosphate buffer, pH 7.6, and 0.5 μmoles of NAD for 10 min. at 37°. The volume of the incubation mixture was 1.8 ml. in the cyanide method and 0.7 ml. in the dehydrogenase method. The amount of protein in the incubated samples varied according to the activity of the extract, as follows: carp liver mitochondria 0.005 - 0.009 mg., nuclei 0.13 mg., microsomes and supernatant 0.35 mg. of protein; rat liver mitochondria 0.45 mg., nuclei 0.3 mg. and supernatant 4.5 mg. of protein. At the end of the incubation

period in the cyanide method 1.4 ml. of 2 M-KCN was added, while in the dehydrogenase method the alcohol dehydrogenase and 0.1 ml. of 3 M-ethanol were added and then tris buffer, pH 9.0, to final volume of 3.2 ml. Spectrophotometric readings (Unicam SP 500) with quartz cuvettes of 1 cm. light path were taken against blanks in which the coenzyme was omitted. Appropriate controls containing no enzyme accompanied the experimental vessel.

Decomposition of NAD by mitochondria was found to be linear for quantities of the enzyme which split up to 50% of the dinucleotide. No reduction of NAD by the extract from liver mitochondria in dilution used was observed during a 10 min. incubation period.

A unit of NADase or NADPase was defined according to Kaplan [4] as that amount which cleaves 1 μmole of NAD per hr. per mg. of protein. Calculation was based on activity measurement after a 10 min. incubation period.

Determination of protein. The protein content was determined by the Mejbaum-Katzenellenbogen tannin method [9].

RESULTS AND DISCUSSION

Most of the NADase activity in carp liver, as measured by the cyanide method, was found in the mitochondrial fraction (Table 1) with small only amounts remaining in microsomes and supernatant (about 9%) and in nuclei (about 0.5%). In the analogous experiments with the rat liver the highest activity of NADase was shown in nuclei, while the mitochondria were half as active.

Decomposition of NAD by the extract from carp liver mitochondria proceeded very rapidly and the activity of NADase therein was about

Table 1

Distribution of the NADase activity in the subcellular fractions of carp liver and rat liver

The composition of the samples and details see Methods. Incubation: 10 min., 37°. The activity was measured by the cyanide method and expressed in μmoles of NAD split/hr./mg. of protein. The figures are averages from 2-3 determinations and in case of carp liver mitochondria, from 7 determinations; in parentheses the limit values

Subcellular fraction	Carp liver	Rat liver
Nuclei (with cellular debris)	0.3	1.2
Mitochondria	75.5 (24.0-111.0)	0.6
Microsomes	4.2	0.18
Supernatant	3.0	

100 times higher than in rat mitochondria, about 40 times higher than in raw bull semen [1] and about 15 times higher than in the crude homogenate of beef spleen [4]. The activity of the extract from carp liver mitochondria was of the same order as that of the purified NADase preparations from bull semen, obtained by Leone & Bonaduce [6] by salting, acetone precipitation and passage through calcium phosphate gel, and concedes only to the preparation of Abdel-Latif & Alivisatos [1], which had approximately 1430 NADase units per mg. of protein.

Data reported in Table 2 include measurements of NAD decomposition as determined by the cyanide and dehydrogenase methods. The convergent results of measuring NAD decomposition by both methods (Table 2) in carp liver mitochondria indicate that NAD glycohydrolase was almost totally responsible for NAD decomposition; thus the results obtained by the dehydrogenase method can be regarded in this instance as a measure of NADase activity. On the contrary in rat liver mitochondria the measurements obtained by the alcohol dehydrogenase method were about three times higher than those obtained by the cyanide method, indicating a considerable pyrophosphatase activity.

Table 2

Splitting of NAD in the mitochondria of carp liver and rat liver, determined by the cyanide and alcohol dehydrogenase methods

Details see Methods. The activity is expressed as a decrease in extinction at 325 and 340 mμ after a 10 min. incubation period at 37°. Milimolar extinction coefficients of NAD-CN complex at 325 mμ and $NADH_2$ at 340 mμ are 6.3 and 6.22 respectively

Liver mitochondria	Cyanide method (NADase) ($-\Delta E_{325}$)	Alcohol dehydrogenase method (NADase + pyrophosphatase) ($-\Delta E_{340}$)
Carp	0.36	0.40
	0.37	0.39
	0.37	0.37
Rat	0.08	—
	0.08	0.26
	0.08	0.28

Substrate specificity. Data shown in Table 3 indicate that the activity of carp liver mitochondria towards NAD was about 6 times higher than towards NADP. Michaelis constants, calculated from the regression lines of 1/S against 1/V plots [7] were 2.2×10^{-4} for NAD and 1.1×10^{-3} for NADP. It was not certain whether these data should be related to one or to two enzymes as it was suggested by Leone & Bonaduce [6].

Inhibition by nicotinamide. Nicotinamide was found to inhibit NADase from carp liver mitochondria to the same extent as NADases from other tissues. The concentration of nicotinamide causing 50% inhibition was found to be 5.5×10^{-3} M (Fig. 1) which is of the same order

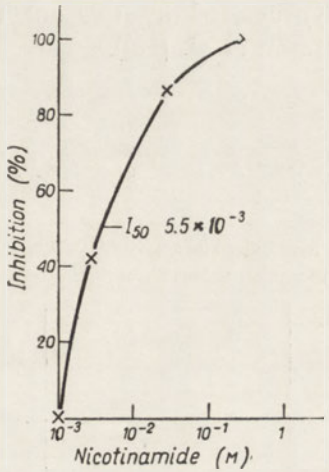

Fig. 1. The effect of nicotinamide on the activity of NADase from carp liver mitochondria. Incubation 10 min. at 37°. NAD assayed by the cyanide method. Details see text

as that found by Quastel & Zatman [10] for beef brain (1.75×10^{-3} M) and McIlwain [8] for guinea pig or sheep brain, and ox spinal cord (about 10^{-3} M). The only NADase which showed marked resistance to nicotinamide was that from *Neurospora crassa*, which is inhibited at concentration as high as 1.45×10^{-1} M [11].

The effect of INH. The activity of NADase from carp mitochondria remained unchanged at the concentration of 10^{-2} M-INH. These results indicate that NADase from carp mitochondria falls into the category of INH-insensitive enzymes such as those from *Neurospora crassa*, human spleen, rat and pig tissues, in contrast to "sensitive" NADases of birds and ruminants, which are inhibited by INH at 10^{-3} M [13].

Type of the NADase activity. According to Zatman, Kaplan, Colowick & Ciotti [12] insensitivity of NADases to INH is associated with a marked transglycosidase activity of these enzymes resulting in formation of an INH-analogue of NAD in which the INH moiety is substituted for nicotinamide. The analogue shows a distinct maximum of absorption in alkali at 385 mμ. No such compound was formed with INH-insensitive NADase from carp liver mitochondria, even with the concentration of the enzyme 4 times higher (3 units) than that used by Kaplan [5] and the incubation period prolonged to 22 hr. The concentration of INH used was 10^{-1} M or 7.7×10^{-2} M. NAD added was almost completely decomposed within 3 hr. but the repeated spectrophotometric readings during incubation revealed no increase in absorption at 385 mμ characteristic for the formation of the analogue. In the parallel experiments with the

INH-insensitive NADase from pig brain (0.2 units) and 10^{-1} M-INH an increase in absorption at 385 mμ was noticeable after a 10 min. incubation; during a 22 hr. incubation the mixture became distinctly yellow and 0.14 μmoles of the analogue was formed. NADase from carp liver mitochondria (10 units) was also inactive towards INH-analogue of NAD (synthesized by the enzyme of pig brain from NAD and INH after Zatman *et al.* [13]).

Table 3

The activity of NADase and NADPase in the mitochondria of carp liver and rat liver

Details see Methods. The activity is expressed in μmoles of NAD or NADP split/hr./mg. of protein; the mean values from 3 determinations with the accuracy of ±1.5% are given

Mitochondria	NADase		NADPase	
	activity	K_m	activity	K_m
Carp	108.0	2.2×10^{-4}	18.0	1.1×10^{-3}
Rat	0.6	—	0.6	—

It appears thus that NADase from carp liver mitochondria similarly to that from *Neurospora crassa* does not show any transglycosidic activity, despite its insensitivity to INH. The carp enzyme, however, in contrast to *Neurospora* NADase is sensitive to nicotinamide and as such is an exception among NADases known so far in various species. Thus if the mechanism of nicotinamide inhibition consists in competition between nicotinamide and water for the NAD-enzyme complex, as suggested by Zatman, Kaplan & Colowick [11] the results obtained might indicate that the enzyme from carp liver mitochondria is capable of catalysing only the exchange of nicotinamide bound in the NAD molecule for the added nicotinamide but can not catalyse transfer reaction with analogous compounds. It might be, however, that INH is not a suitable cosubstrate for carp NADases and that some other pyridine derivatives could serve as acceptors in transglycosidic reaction.

SUMMARY

A highly active NADase has been found in the carp liver mitochondria. The enzyme is easily extractable from the cell structure, it is inhibited by nicotinamide, is highly specific to NAD, insensitive to isonicotinyl hydrazide and does not exhibit transglycosidic action towards this compound.

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NAD GLIKOHYDROLAZA W WĄTROBIE KARPIA

Streszczenie

Stwierdzono wysoką aktywność NADazy w mitochondriach wątroby karpia. Enzym daje się łatwo ekstrahować, jest hamowany przez amid kwasu nikotynowego, jest specyficzny dla NAD, niewrażliwy na hydrazyd kwasu izonikotynowego i nie wykazuje własności transglikozydazowych w stosunku do tego związku.

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RECENZJE KSIĄŻEK

SCIENCE AND THE FUTURE OF MANKIND (Edited by Hugo Boyko). Dr. W. Junk Publishers — Den Haag. 1961, 380 str., 8 rys. Cena $ 9.50. Oprawa w płótno.

W grudniu 1960 roku powstała „Światowa Akademia Sztuki i Nauki" („World Academy of Art and Science"), a recenzowana książka jest jej pierwszym wydawnictwem. Dwa główne cele pragnie spełnić nowopowstała instytucja: 1) stopniowo tworzyć ponadnarodowe forum, na którym możnaby rozważać najżywotniejsze problemy ludzkości, oraz 2) działać jako bezstronny, niezależny od interesów grupowych doradca w służbie Dobra Ludzkości. Realizacja tych celów nie będzie łatwa; jedynymi siłami, które mogą tu być użyte, to wiedza, głębokość i siła uczuciowego nastawienia wobec nędzy materialnej i moralnej ludzkości, a także, oparta o nie, siła przekonywania i nadzieja, że ludzkość wreszcie zrozumie, że „świat jest obecnie zbyt niebezpieczny dla wszystkiego, co nie jest prawdą, i za mały dla czegokolwiek, co nie jest braterstwem" (str. 239). W swoich zadaniach chce Akademia pomóc w realizacji dezyderatu, który Einstein, jeden z duchowych ojców tej instytucji, ujął słowami: „Twory naszego rozumu powinny być błogosławieństwem, a nie przekleństwem ludzkości" (str. 368). O potrzebie, zamiarach i celach Akademii pisze w ocenianej książce dwudziestu autorów. Omawiane zagadnienia dotyczą bardzo szerokiego zakresu; widać to z załączonego do obecnej recenzji spisu rzeczy tej książki. Nie sposób streszczać poszczególnych artykułów; charakteryzuje je wielka zwięzłość, poczucie głębokiej odpowiedzialności za wyrażane poglądy, jasność myśli i staranna kontrola realności przedstawianych planów. To co łączy ze sobą artykuły niemal wszystkich autorów, to głębokie przekonanie, że obecny wstrząs cywilizacyjny, związany z potężnym rozwojem wiedzy ludzkiej, nie będzie mógł być inaczej opanowany jak przez głęboki istotny postęp w moralnej postawie Ludzkości. Recenzja książki tak pełnej głębokich myśli jak oceniane dzieło nie może niczego więcej dokonać, jak zwrócić uwagę czytelnikowi na to, że wydawnictwo to jest wydarzeniem wielkiej miary i że przestudiowanie tej książki dostarczy bardzo cennego materiału każdemu pracownikowi naukowemu, którego myśl nie ogranicza się tylko do jego wąskiej specjalności.

SCIENCE AND THE FUTURE OF MANKIND

Contents

R. Oppenheimer, Princeton, N.J.: Thoughts on Art and Science: 1. Science and Our Times, 2. Prospects in the Arts and Sciences
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The Means (a few examples by a few scientists)

H. J. Muller, Bloomington, Ind.: The Prospects of Genetic Progress
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S. W. Tromp, Leiden: The Significance of Border Sciences for the Future of Mankind
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I. Berenblum, Rehovot: Science and Modern Civilization
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World Academy of Art and Science

Manifesto
List of Co-Workers in the Preparatory Steps and of Charter Members
Informatory Notes

Włodzimierz Mozołowski

O. H. Warburg. WEITERENTWICKLUNG DER ZELLPHYSIOLOGISCHEN METHODEN. Angewandt auf Krebs, Photosynthese und Wirkungsweise der Röntgenstrahlen. Arbeiten aus den Jahren 1945 - 1961. (New Methods of Cell Physiology. Applied to Cancer, Photosynthesis and Mechanism of X-Ray Action. Developed 1945 - 1961). Georg Thieme Verlag, Stuttgart 1962, i Interscience Publishers, New York. Str. XVI+644, rys. 177, lex. Cena DM 138, $ 34.50. Oprawa w płótno. 8°.

Książka zaczyna się kilkuwierszową przedmową autora, następnie na 20 stronach podana jest nader zwięzła charakterystyka metod i najważniejszych wyników wraz z ich interpretacją. Przedmowa i ten rozdział powtórzone są w tłumaczeniu angielskim.

Następne 4 strony zajmuje przedrukowany z Biochimica et Biophysica Acta z r. 1953 artykuł Deana Burka pt. „Otto Warburg, artisan of cell chemistry", opatrzony podobiznami Warburga i Burka.

Z kolei mamy przedruk dłuższego ustępu o enzymach fermentacji alkoholowej z wyczerpanej książki Warburga z 1948 r. pt. „Wasserstoffübertragende Fermente". Przedruk zaopatrzył autor w notki aktualizujące przedstawione dane wedle stanu z r. 1961.

Główną część książki stanowią 73 przedruki prac oryginalnych Warburga i jego uczniów z lat 1946 - 1961, które ukazały się przeważnie w Zeitschrift für Naturfor-

schung, Science, Archives of Biochemistry, Biochimica et Biophysica Acta i innych. Tekst jest niemiecki lub angielski, zależnie od języka oryginalnej publikacji. I tu znajdujemy liczne notki jako „Zusatz 1961", aktualizujące tekst lub mające charakter polemiczny. Wreszcie ostatnią część książki stanowi 20 prac, dotąd nigdzie nie drukowanych, z których większość nie będzie gdzie indziej ogłoszona.

Wszystkie artykuły dotyczą rozwoju metod badawczych wprowadzonych, używanych i stale ulepszanych przez Warburga. Rozwój polega bądź na zwiększeniu czułości i dokładności, bądź na opartym na tych osiągnięciach uproszczeniu metod. Ważne miejsce zajmuje też wypracowanie optymalnych warunków hodowli badanych komórek.

Ten nacisk położony na metody stanowi charakterystyczny rys całego zbioru. Nowe doświadczenia opierają się na ulepszonych metodach lub na wprowadzeniu zasadniczych zmian w warunkach doświadczenia, np. podniesieniu ciśnienia CO_2 z 200 mm Brodie na 500 mm. Wyłaniają się w ten sposób nowe, nieoczekiwane fakty, których interpretacja prowadzi Warburga do nowych koncepcji teoretycznych, często sprzecznych z ogólnie przyjętymi poglądami.

Trzeba się zgodzić z autorem, kiedy w słowie wstępnym wyraża pogląd, że znajomość i opanowanie przedstawionych w książce metod są niezbędne dla każdego, kto chce w tej dziedzinie pracować. Dla tych osób książka posiada dużą wartość, bo oszczędza szukania szczegółów rozsianych w różnych czasopismach. Dla czytelnika szukającego ogólnej informacji o badanych zagadnieniach jest to lektura raczej ciężka. Już sama terminologia enzymów i koenzymów używana przez Warburga nasuwa duże trudności. Nie można odmówić prawa używania własnego słownictwa odkrywcy większości omawianych związków, ale szkoda, że utrudnia to zrozumienie młodym, którzy wzrośli już w słownictwie ogólnie przyjętym i stosowanym. Trudność nastręcza też zrozumienie poglądów Warburga na mechanizm fotosyntezy, gdyż są one zupełnie oderwane od ogólnie przyjętych schematów. Merytoryczną dyskusję na te temat postaram się podjąć na innym miejscu; tu chciałbym tylko stwierdzić, że omawiana książka jest bardzo pożyteczna, a jej lektura trudna lecz bardzo stymulująca.

Józef Heller

KLINISCHE ANWENDUNG DER ALDOSTERON-ANTAGONISTEN (herausgegeben von Priv.-Doz. dr F. Krück, dr Kh. R. Koczorek und dr G. Betzien). Georg Thieme Verlag. Stuttgart 1962. Str. VIII+118, 53 rys., 12 tabl. Cena 19,80 DM.

W maju 1961 r. odbyło się we Wiedniu sympozjum poświęcone klinicznemu zastosowaniu antagonistów aldosteronu. F. Krück, Kh. R. Koczorek i G. Betzien podjęli się trudu zebrania i opracowania materiałów tego sympozjum, dając monografię o spirolaktonach, nowych syntetycznych lekach wykazujących rewelacyjne efekty w leczeniu obrzęków. Książka poświęcona jest przede wszystkim zagadnieniom wiążącym się z wydzielaniem sodu i potasu w dystalnym odcinku kanalika nerkowego i z regulacją hormonalną tego etapu przemiany mineralnej i wodnej. Poruszono szereg aktualnych problemów grupujących się wokół mechanizmów regulujących transport i wydzielanie jonów sodowych i potasowych oraz ich wpływem na rozmieszczenie wody w przestrzeni wewnątrz- i zewnątrznaczyniowej.

Słowo wstępne napisane przez prof. dr H. P. Wolffa stanowi interesujące wprowadzenie w zakres poruszanych zagadnień. Wstęp i uwagi końcowe Krücka nadają książce ciągłość tematyczną. Dwa rozdziały Koczorka traktujące o fizjologii i patologii wydzielania aldosteronu przez korę nadnerczy oraz o podstawach farmakologicznego działania spirolaktonów ułatwiają zrozumienie dalszych rozdziałów pisanych przez różnych autorów a omawiających patomechanizm powstawania obrzęków oraz ich leczenie w świetle efektów uzyskiwanych przez stosowanie antagonistów aldosteronu. Rozdziały środkowe zawierają wiele obserwacji zebranych na bogatym i różnorodnym materiale klinicznym i posiadają niewątpliwe

znaczenie dla racjonalnego stosowania spirolaktonów w leczeniu obrzęków. W poszczególnych rozdziałach omówiono zastosowanie spirolaktonów w pierwotnym lub wtórnym hyperaldosteronizmie, a mianowicie w syndromie Conna, marskości wątroby, niewydolności krążenia, nerczycach, ciążach, i nadciśnieniu, tętniczym. Dla teoretyków przeznaczony jest artykuł Koczorka o pozanerkowym działaniu antagonistów aldosteronu wykazanym w eksperymentach przeprowadzonych na krwinkach czerwonych.

Bogata treść książki zajmującej się niewielkim wycinkiem przemiany mineralnej i wodnej, przejrzysty układ i bardzo staranna redakcja materiałów sympozjum dają w rezultacie wartościową monografię interesującą fizjologów, patologów, biochemików, endokrynologów, farmakologów i lekarzy.

Książka stanowi udaną próbę przedstawienia udziału aldosteronu w mechanizmie powstawania obrzęków. Przyczyni się też do podważania, przynajmniej na tym odcinku, przekonania o celowości zjawisk biologicznych, wykazując, że mechanizmy regulacyjne kształtujące się na różnych etapach rozwoju filogenetycznego, w tym przypadku zabezpieczające stałość składu jonowego i właściwą objętość płynu wewnątrznaczyniowego, stają się zgubne i prowadzą do głębokich zaburzeń w gospodarce wodnej w pewnych stanach patologicznych.

Książka o klinicznym zastosowaniu antagonistów aldosteronu jest pozycją w piśmiennictwie potrzebną i pożyteczną, tak jak pożyteczną jest każda praca torujące nowe drogi w rozwoju nauki, nawet jeżeli podawane fakty i propagowane teorie w miarę rozwoju wiedzy nie we wszystkim okażą się słuszne i prawdziwe.

Wanda Mejbaum-Katzenellenbogen

THE PLANT CELL WALL. International Series of Monographs on Pure and Applied Biology, vol. II by S. M. Siegel (group leader in physical biochemistry, Union Carbide Research Institute, Eastview, New York). Pergamon Press. 1962. Cena 42 s.

Monografia o objętości 123 stron druku składa się z krótkiego wstępu i czterech rozdziałów o konstytucji i budowie ścian komórkowych, ich dynamice, o chemii porównawczej wtórnych substancji komórkowych i ścian, i wreszcie o własnościach i użytkowaniu ich produktów.

Szeroki zakres informacji i próba syntezy w tak objętościowo ograniczonej monografii wskazują na to, że adresatem omawianej pracy mają być szerokie rzesze biologów z dziedzin pokrewnych, którzy nie mogą opanować całego ogromu piśmiennictwa specjalistycznego.

Fakty doświadczalne i teorie są przedstawione nader przejrzyście i jasno, do tego stopnia że czytelnik posiadający tylko podstawowe wiadomości z biochemii i biofizyki może bez trudu i z dużą dla siebie korzyścią opanować całość materiału.

Autor podkreśla uniwersalne znaczenie koncepcji organizowanej wielocukrowej makrocząsteczki, nie zapominając przy tym o heterogenności poszczególnych substancji budulcowych ścian komórkowych różnych organizmów.

Ostatni rozdział, omawiający drewno, włókno, tworzywa pochodzenia roślinnego i proces zwęglania w przyrodzie, odbiega tematycznie od całości. Informacje o włóknach przemysłowych, jak bawełna i len, technologia drewna, teorie bituminizacji są skondensowane na zaledwie 16 stronach druku i obejmują tylko fragmenty zagadnienia, bez szczegółów często niezbędnych. Może się wydawać, że autor nie bardzo się kwapił do napisania tego rozdziału i że jego miejsce w omawianej monografii wynikło z przyczyn od autora niezależnych. Ta wartościowa i pożyteczna książka, zaopatrzona kilkudziesięcioma cennymi pozycjami piśmiennictwa z ostatnich lat, zyskałaby znacznie a nie straciła na wadze, gdyby ten ostatni rozdział był pominięty.

Ignacy Reifer