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EFFECT OF MALEIC ACID ON THE KIDNEY

III. SUCCINATE AS HYDROGEN DONOR IN THE REDUCTIVE AMINATION IN RAT KIDNEY

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It is generally accepted that the synthesis of glutamate in the presence of ammonia is due to the dismutation of α-ketoglutarate [13]. As further hydrogen donors for reductive amination citrate and malate were recognized [13, 14]. It has been recently demonstrated that succinate can indirectly participate in this reaction [8, 11, 17]. The present paper describes a study of the role of succinate and other substrates in the synthesis of amino acids by rat kidney mitochondria.

MATERIALS AND METHODS

Rat kidney mitochondria were prepared in 0.25 M-sucrose according to Hogeboom [10]. Part of the experiments were performed not on mitochondria but on the suspensions of washed cell particles of rat kidney and liver which were prepared as previously described [15]. Respiration was measured by the conventional Warburg method, and the reaction was stopped by the addition of trichloroacetic acid or ethanol. The mixture was centrifuged and in the protein-free supernatant amino nitrogen (Yemm & Cocking [19]), ketoacids (Friedmann & Haugen [9]) and citrate (Beutler & Yeh [2]) were estimated. The results were calculated in μmoles per gram wet tissue per hour.

RESULTS

As shown in Table 1, the addition of ATP to washed kidney cell particles doubled the disappearance of ketoglutarate, and the addition of ammonia induced a further increase of ketoglutarate utilization corresponding to the amount of amino acids formed. Closer examination of the influence of ATP (Table 2) has shown that the relation between amino acid synthesis and ATP concentration was linear in the range from 1 to 5 μmoles ATP per 3 ml. of the reaction mixture, and 1 μmole of the added ATP corresponded to 2 μmoles of amino acids formed.

Table 1

Effect of ATP and ammonia on α-ketoglutarate consumption in rat kidney

Each vessel contained in a final volume of 3 ml.: 75 μmoles of tris buffer, pH 7.2; 100 μmoles KCl; 1.5 μmole $MgSO_4$; 1.5 μmole EDTA; 60 μmoles ketoglutarate and 1 ml. suspension of washed cell particles from 200 mg. of wet tissue wt. Besides where indicated 5 μmoles ATP, and/or 60 μmoles NH_4Cl were added. The incubation was carried out in an atmosphere of air at 38° for 60 min. with continuous shaking. The results are calculated per gram wet tissue. Mean values from 6 experiments, ± SD are given.

Addition	Ketoglutarate consumption (μmoles/g. tissue/hr.)			$N\text{-}NH_2$ formed (μmoles/g. tissue/hr.)
	Without NH_4^+	With NH_4^+	Δ	
None	95.5 ± 8.5	106.0 ± 7.0	10.5	9.5 ± 1.0
ATP	221.0 ± 9.5	270.5 ± 9.0	49.5	50.0 ± 3.5

The dependence, however, of ketoglutarate consumption on the amount of added ATP did not show linear correlation. As no direct relation between the increased ketoglutarate consumption and the formation of amino acids was found, it seems possible that ATP enables in some way the participation of the ketoglutarate oxidation products (succinate and malate) in reductive amination.

Table 2

Effect of the amount of ATP added on α-ketoglutarate consumption and amino acid synthesis in rat kidney

The experimental conditions as described in Table 1, except that ammonia was added to all samples. Mean values from 6 experiments, ± SD are given.

ATP (μmoles/sample)	Ketoglutarate consumption	$N\text{-}NH_2$ formed	Ratio: ketoglutarate / $N\text{-}NH_2$
	(μmoles/200 mg. wet tissue/hr.)		
0	21.0 ± 3.0	2.1 ± 0.2	10.0
1	38.0 ± 3.5	4.1 ± 0.3	9.25
3	48.0 ± 4.0	7.8 ± 0.5	6.15
5	53.5 ± 3.3	10.9 ± 1.1	4.9
10	58.0 ± 1.9	13.9 ± 1.2	4.15

To verify the above supposition, experiments were made in which malonate was used to suppress the oxidation of succinate and malate formed from α-ketoglutarate, thus preventing their possible participation in the synthesis of amino acids. In liver cell particles, ketoglutarate consumption was not changed in the presence of malonate. The synthesis of amino acids was by one half lower, and the addition of ATP was without any effect (Table 3), as contrasted with the system

without malonate where the addition of ATP increased the formation of amino acids and led to the lowering of the ratio of ketoglutarate consumption to the increase of amino nitrogen, from 1.75 to 1.25. In this case the bulk of ketoglutarate served as substrate for the reductive amination whereas hydrogen was derived from other sources. The reactions supplying hydrogen contributed also to the reductive amination even in the absence of ATP.

Table 3

Effect of ATP and malonate on amino acid synthesis from α-ketoglutarate in rat kidney and liver

The experimental conditions as described in Tables 1 and 2. Mean values from 5 - 7 experiments, ± SD are given.

Addition (μmoles/sample)	Kidney			Liver		
	Ketoglutarate consumption (μmoles/g. tissue/hr.)	$N\text{-}NH_2$ formed (μmoles/g. tissue/hr.)	Ratio: ketoglutarate / $N\text{-}NH_2$	Ketoglutarate consumption (μmoles/g. tissue/hr.)	$N\text{-}NH_2$ formed (μmoles/g. tissue/hr.)	Ratio: ketoglutarate / $N\text{-}NH_2$
None	84.0 ± 3.5	7.0 ± 0.9	12.0	298.0 ± 8.0	168.0 ± 15.0	1.75
Malonate (100)	93.0 ± 4.5	7.5 ± 1.0	12.4	289.0 ± 7.5	75.5 ± 10.0	3.8
ATP (5)	269.0 ± 17.0	59.0 ± 4.8	4.6	300.0 ± 2.0	237.0 ± 21.0	1.25
Malonate (100) + ATP (5)	206.0 ± 23.5	17.0 ± 1.5	12.1	296.0 ± 3.5	68.5 ± 4.5	4.4

Experiments with kidney gave a different picture (Table 3). In the presence of malonate the addition of ATP doubled both the disappearance of ketoglutarate and the formation of amino acids. Without malonate added, ATP caused fivefold or even greater increase of amino acid synthesis. The difference in the yield of the synthesized amino acids with and without malonate was about 42 μmoles; also the consumption of ketoglutarate was lowered by nearly the same value by the presence of malonate. Without ATP the consumption of ketoglutarate in the presence of malonate was the same as without ammonia, i. e. 93 μmoles (Tables 3 and 1). Therefore it seems justified to assume that in rat kidney, malonate inhibited the synthesis of amino acids from ketoglutarate and ammonia even in the presence of ATP.

As these results indicated that the effect of ATP on the synthesis of amino acids must indeed concern other reactions than the dismutation of ketoglutarate, the role of succinate and malate in the reductive amination of ketoglutarate in rat kidney was examined.

Succinate-linked NAD reduction in aerobic mitochondria had been originally described by Chance & Hollunger [5]. More recently Ernster [8], Klingenberg [11, 12] and Slater *et al.* [17] applied the reductive amination of ketoglutarate as $NADH_2$ trapping system. To prevent the

oxidation of ketoglutarate, Slater *et al.* [17] and Snoswell [18] used arsenite.

In our previous work [1] we have found that in rat kidney mitochondria, maleate at 3×10^{-3} M concentration inhibited completely ketoglutarate oxidation but had no effect on succinate oxidation and on glutamate dehydrogenase. Therefore in the experiments on the role of succinate and malate we have used maleate instead of arsenite.

Table 4

Succinate as hydrogen donor in the reductive amination in rat kidney mitochondria

Each Warburg vessel contained in a final volume of 3 ml:. 100 µmoles of tris buffer, pH 7.2, 30 µmoles KCl, 4 µmoles EDTA, 40 µmoles phosphate buffer, pH 7.2, 6 µmoles $MgCl_2$, 5 µmoles ATP, 60 µmoles NH_4Cl, 125 µmoles sucrose and 0.5 ml. of mitochondrial suspension from 500 mg. of tissue. Other additions were: α-ketoglutarate 60 µmoles, succinate 60 µmoles, maleate 9 µmoles, arsenite 3 µmoles. Incubation time 60 min. at 30° in an atmosphere of air. Mean values from 4 - 9 experiments, ± SD are given.

Addition	Oxygen uptake (µg. atoms)	Ketoglutarate consumption (µmoles/g. tissue/hr.)	N-NH_2 (µmoles/g. tissue/hr.)	Ratio: ketoglutarate / N-NH_2
None	5.4 ± 0.2	—	10.6 ± 2.6	
α-Ketoglutarate	75.0 ± 5.8	101.8 ± 13.0	38.2 ± 5.3	2.65
α-Ketoglutarate + maleate	12.4 ± 1.1	12.0 ± 1.5	10.3 ± 2.1	
Succinate	79.4 ± 3.0	—	12.2 ± 2.8	
Succinate + maleate	74.6 ± 5.5	—	12.0 ± 3.0	
α-Ketoglutarate + succinate	83.2 ± 7.0	97.2 ± 11.5	42.2 ± 4.5	2.3
α-Ketoglutarate + succinate + + maleate	73.0 ± 4.5	45.6 ± 7.8	36.8 ± 2.5	1.25
α-Ketoglutarate + arsenite*	7.0	14.8**	9.8	
α-Ketoglutarate + succinate + + arsenite	66.6 + 4.0	31.0 ± 3.9**	28.4 ± 3.2	

* One experiment.
** Enzymic determination [16]

As can be seen from Table 4 the addition of succinate together with ketoglutarate to kidney mitochondria had no significant effect on oxygen uptake, amino acid formation and ketoglutarate consumption. The addition of maleate to such a system had virtually no effect on oxygen uptake and amino acid synthesis but brought about a considerable decrease of ketoglutarate disappearance. The ratio of ketoglutarate consumption to N-NH_2 formation was 1.25 indicating that almost all metabolized ketoglutarate underwent reductive amination.

When in the presence of succinate, arsenite instead of maleate was used as inhibitor of ketoglutarate oxidation, the synthesis of amino

acids was definitely lower than with maleate. This indicates that in rat kidney mitochondria maleate better than arsenite can serve as a tool for the study of reductive amination of ketoglutarate in the presence of succinate or another hydrogen donor.

In experiments with arsenite the application of the enzymic method [16] for the determination of ketoglutarate was necessary. The used Friedmann-Haugen method is not specific for ketoglutarate as it determines the total amount of ketoacids. Arsenite, in contrast to maleate, inhibits the pyruvate oxidase to the same degree as the ketoglutarate oxidase. Thus, in the incubation mixture containing succinate, there may be an accumulation of pyruvate and even oxaloacetate, and the amount of ketoacids may increase even above the amount of ketoglutarate added. It should be pointed out that in all other types of experiments presented in Table 4, the amount of ketoacids determined by Friedmann-Haugen method agreed well with the amount of ketoglutarate determined enzymically.

Table 5

Malate as hydrogen donor in the reductive amination in rat kidney mitochondria

Experimental conditions as described in Table 4. Other additions: ketoglutarate 60 μmoles and malate 60 μmoles. Final concentrations of maleate as indicated in the Table. Mean values from 4 - 5 experiments, ± SD are given.

Addition	Oxygen uptake	$N\text{-}NH_2$
	μg. atoms per g. wet tissue/hr.	μmoles per g. wet tissue/hr.
None	6.0 ± 0.4	10.8 ± 2.0
Malate	74.6 ± 9.1	11.2 ± 0.9
Malate + maleate (3×10^{-3} M)	21.4 ± 2.5	10.2 ± 0.5
Malate + maleate (5×10^{-4} M)	63.5 ± 5.0	10.6 ± 0.9
α-Ketoglutarate + malate	86.6 ± 4.8	35.4 ± 5.2
α-Ketoglutarate + malate + maleate (3×10^{-3} M)	33.2 ± 2.1	16.0 ± 2.9
α-Ketoglutarate + malate + maleate (5×10^{-4} M)	69.3 ± 5.3	16.9 ± 2.0

Similarly as the addition of succinate, also the addition of malate (Table 5) to a system containing ketoglutarate and ammonia did not influence markedly the synthesis of amino acids. However, in the presence of maleate at the same concentration as in the experiments with succinate (3×10^{-3} M) a considerable decrease of amino acid formation was observed, but this effect may be interpreted as a result of decreased malate oxidation (about 68% inhibition). Therefore a smaller concentration of maleate was used (5×10^{-4} M) which still inhibited ketoglutarate oxidation but had virtually no effect on malate oxidation.

In this case the oxygen uptake was only slightly inhibited whereas the amino acid formation was small, like in experiments with higher concentration of maleate. This result indicates that in kidney, malate even in the presence of ammonia and ATP is preferentially oxidized *via* the respiratory chain.

By comparing the amounts of amino acids formed with succinate and with malate as hydrogen donors under conditions in which there was no decrease of the oxidation of these substrates we may estimate to what degree succinate on the one hand, and malate on the other contribute to the synthesis of amino acids (see Tables 4 and 5).

It seems that in rat kidney mitochondria the succinate-linked reduction of NAD can take a great part in the reductive amination of ketoglutarate, whereas the oxidation of malate is less important for this reaction. The participation of succinate, however, is possible only in the presence of added ATP.

Table 6

Synthesis of amino acids from citrate in rat kidney

The experimental conditions as described in Table 1 except that 60 μmoles of citrate, instead of ketoglutarate, and 30 μmoles of maleate were added. ATP was added in the amount of 5 μmoles/sample. Mean values from 5 experiments, ± SD are given.

Addition	Citrate consumption	Ketoglutarate found	Calc. consumption	$N\text{-}NH_2$ formed	Ratio: ketoglutarate / $N\text{-}NH_2$
	(μmoles/g. tissue/hr.)				
None	188.0 ± 19.1	137.0 ± 13.5	51.0	46.0 ± 5.2	1.1
ATP	226.5 ± 21.5	159.0 ± 13.1	67.0	56.0 ± 3.1	1.2

In kidney the oxidation of isocitrate is also an important source of hydrogen for the reductive amination of ketoglutarate as it was shown in the previous paper [14]. The amounts of amino acids formed from the added citrate were even greater than when ketoglutarate was used. Since those experiments were carried out in the presence of ATP, the question arose whether this addition was indispensable. The data reported in Table 6 indicate that the synthesis of amino acids from citrate may also proceed without the addition of ATP.

DISCUSSION

In this and other papers of this series [15, 14] an attempt was made to demonstrate the participation of various reactions providing hydrogen for the reductive amination of ketoglutarate in rat kidney and liver.

From the obtained results it can be concluded that in kidney, even in the presence of ammonia, the oxidation of ketoglutarate is for the

most part connected directly with the respiratory chain. Ketoglutarate may serve also as a source of the carbon skeleton for glutamate synthesis, but not as a hydrogen donor for reductive amination. For the last reaction, citrate should be named in the first place:

$$\text{Citrate} + NH_3 \longrightarrow \text{glutamate} + CO_2$$

In this reaction the glutamate synthesis does not require ATP, and proceeds efficiently due to the very active citrate metabolism in the kidney, and to the fact that oxidation of isocitrate gives ketoglutarate and $NADPH_2$ which both are substrates for the reductive amination.

In the controlled state of mitochondria (state 4 of Chance [7]) when sufficient ATP is supplied the addition of succinate to the mitochondrial suspension brings about the reduction of NAD [5, 5a, 6, 18]. This succinate-linked reduction of NAD is interpreted as a reversal of oxidative phosphorylation by ATP [4, cf. also 3] and may be coupled with the reductive amination of ketoglutarate (Ernster [8], Klingenberg [11, 12] and Slater *et al.* [17]).

$$\text{Ketoglutarate} + \text{succinate} + NH_3 \xrightarrow{ATP} \text{glutamate} + \text{fumarate}$$

The amount of amino acids formed in the kidney in this reaction may be rather considerable. Under certain conditions, e.g. in rats treated with maleate, this reaction can serve even as a basic reaction for the synthesis of amino acids.

The dismutation of ketoglutarate which is generally regarded as the main pathway of glutamate synthesis seems to play a minor role in rat kidney. The increased formation of amino acids from ketoglutarate in the presence of ATP may be explained by the fact that ATP enables the participation of succinate in the reductive amination.

Malate seems to have little significance in reductive amination. Its contribution to the synthesis of amino acids is rather to provide oxaloacetate or pyruvate as substrates for transamination with glutamate.

In rat liver, in contrast to the kidney, fundamental role in the reductive amination of ketoglutarate belongs to the dismutation of two molecules of ketoglutarate, although citrate and succinate may be also very important hydrogen donors for this reaction.

These findings may suggest that the fate of hydrogen in the reduced pyridine nucleotides can be more or less determined in advance. It could be expected that the pyridine nucleotide reduced by one particular enzyme system *in vivo* might be reoxidized preferentially in another particular system. This would mean a limitation to the generally accepted concept of an undetermined hydrogen pool.

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SUMMARY

In kidney mitochondria, in contrast to liver mitochondria, citrate is a major hydrogen donor for the reductive amination of ketoglutarate, whereas succinate may play an important role provided that ATP is added. The dismutation of ketoglutarate is of lesser importance for this reaction.

To prevent the oxidation of ketoglutarate, maleate was used instead of arsenite.

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DZIAŁANIE KWASU MALEINOWEGO NA NERKĘ

III. BURSZTYNIAN JAKO DONATOR WODORU DLA REDUKTYWNEJ AMINACJI W NERCE SZCZURA

Streszczenie

W mitochondriach nerki, przeciwnie niż w mitochondriach wątroby, cytrynian jest głównym donatorem wodoru dla reduktywnej aminacji ketoglutaranu. Bursztynian również może mieć duże znaczenie pod warunkiem dodania ATP. Natomiast reakcja dyzmutacji ketoglutaranu nie ma większego znaczenia.

Dla zahamowania utleniania ketoglutaranu zastosowano maleinian zamiast arseninu.

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J. ROGULSKI and S. ANGIELSKI

EFFECT OF MALEIC ACID ON THE KIDNEY

IV. SYNTHESIS OF AMINO ACIDS IN THE KIDNEY OF MALEATE-TREATED RATS

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It has previously been shown [1, 2] that in rat the intraperitoneal administration of maleate induced aminoaciduria, ketoaciduria and glycosuria. Further it was demonstrated *in vitro* [13, 11, 12] that maleate has a marked inhibitory effect on α-ketoglutarate oxidation and amino acid synthesis by rat kidney homogenate and mitochondria.

The present paper deals with the synthesis of amino acids by kidney homogenates of maleate-treated rat in the presence of ketoglutarate, ammonia and hydrogen donors. The obtained data are discussed on the assumption that the conditions for amino acid synthesis are more favourable in kidney of maleate-treated rats than in kidney of normal animals.

EXPERIMENTAL

White Wistar rats fed the lactose diet for two weeks were used [1]. The tissue homogenates were prepared as previously described [13]. The synthesis of amino acids in kidney homogenates of maleate-treated rats was tested 3 or 36 hr. after intraperitoneal injection of 300 mg. of maleate per kg. body weight. In order to determine the content of amino nitrogen, ketoacids and citrate in kidneys, they were removed as soon as possible, weighed and homogenized with hot ethanol or 10% trichloroacetic acid. In protein-free supernatant amino nitrogen [14], total ketoacids [6] and citrate [4] were determined. The quantitative estimation of particular ketoacids was carried out after paper chromatographic separation in a system of *n*-butanol - 0.5 N-NH_4OH - ethanol (7:2:1, by vol.) according to McArdle [9]. The urine was collected during 3 or 36 hr. and in this time the rats were also kept on the lactose diet. The reagents were the same as in the earlier experiments [3].

RESULTS

The glutamate dehydrogenase (L-glutamate:NAD and/or NADP oxidoreductase, EC 1.4.1.2 or 1.4.1.3) is found in many animal tissues, and the equilibrium of the reaction catalysed by this enzyme is shifted towards

the synthesis of glutamate [10]. From the yield of the synthesis of amino acids from α-ketoglutarate (Table 1) it can be seen that only three organs have an important part in this process: the liver, kidney and brain. The liver in the presence of ATP converted almost the whole of ketoglutarate added into the equivalent amount of glutamate. The kidney and brain gave a moderate yield of amino acids (5 and 10 times less, respectively, than the liver). The heart muscle showed a marked oxidative metabolism of ketoglutarate and a nearly complete lack of amino acid synthesis. This last observation was in full agreement with the data of other authors who have shown that the heart is almost completely devoid of glutamate dehydrogenase activity [7, 5]. It is in line with the character of this tissue performing continuous mechanical work which requires much of oxidative energy. Glutamate formation deviates ketoglutarate and so may handicap the energy production *via* the citrate acid cycle, but it does not occur in the heart. This is an example of tissue specificity which in some degree depends on the presence or absence of particular enzyme activity. The other tissues showed less active ketoglutarate metabolism and, like the heart, an almost complete lack of amino acid synthesis.

Table 1

The synthesis of amino acids from α-ketoglutarate and ammonia by various rat tissues

The reaction mixture contained in a final volume of 3 ml.: 75 μmoles of tris buffer, pH 7.2; 100 μmoles KCl; 1.5 μmole $MgSO_4$; 1.5 μmole EDTA; 60 μmoles ketoglutarate; 60 μmoles NH_4Cl; 5 μmoles of ATP, and 1 ml. of homogenate from 200 mg. tissue. Incubation: 60 min. at 37°. Mean values from 6 - 10 experiments, ± SD are given.

Tissue	Ketoglutarate consumption	N-NH_2 formed	Ratio: ketoglutarate / N-NH_2
	(μmoles/g. tissue/hr.)		
Liver	298.5 ± 15	237.0 ± 30	1.25
Kidney	267.5 ± 19	54.0 ± 7	4.95
Brain	130.5 ± 12	22.0 ± 4	5.9
Heart	297.0 ± 12	3.5 ± 0.4	85.0
Muscle	51.0 ± 6.1	5.5 ± 0.9	9.3
Spleen	32.5 ± 2.5	5.5 ± 0.4	5.9
Testis	30.5 ± 2.9	2.0 ± 0.1	15.2
Lung	31.0 ± 2.9	3.5 ± 0.3	10.3
Intestinal mucosa	25.5 ± 3.0	0.0	

It has previously been shown [3] that in maleate-treated rat the oxidation of ketoglutarate was inhibited in kidney but not in liver and brain. Therefore in kidney there should be an accumulation of ketoglutarate enhancing the synthesis of amino acids, and leading to a temporary increase of ketoacids in the blood and their increased excretion in the urine during the first 2 - 3 hr. following the administration of ma-

leate [2]. The changes in the excretion of particular ketoacids are shown in Table 2. In rats not treated with maleate (control), ketoglutarate formed about 94% of the excreted ketoacids whereas in the intoxicated rats it formed only 41%. The excretion of pyruvate, and especially the excretion of other, non identified ketoacids which normally form only a fraction of 1%, increased to a greater extent than the excretion of ketoglutarate. As it was shown previously [3] the oxidation of ketoglutarate in the kidney of maleate-treated rat was much more inhibited than the oxidation of pyruvate. However, the relatively smaller increase in the excretion of ketoglutarate as compared with that of pyruvate can be explained by the conversion of ketoglutarate into glutamate in the kidney. Other ketoacids including pyruvate do not undergo the amination, and are excreted.

Table 2

Excretion of ketoacids by maleate-intoxicated rats

Maleate was injected intraperitoneally in a dose of 300 mg. per kg. body wt. Urine from 24 hr. was collected and after chromatographic separation the ketoacids were estimated. On the second day after injection of maleate only the total amount of ketoacids was assayed. Mean values from 4 experiments are given; in parentheses the limit values.

Compound	Control	Day after injection	
		1	2
	(μmoles/24 hr.)		
α-Ketoglutarate	36.2 (32.5 — 41.0)	82.2 (75.1 — 92.3)	
Pyruvate	2.16 (5.8 — 1.15)	60.5 (53.4 — 70.6)	
Other ketoacids	0.24 (0.15 — 0.41)	56.1 (49.5 — 62.3)	
Total	38.6 (34.8 — 44.3)	198.8 (159.3 — 207.4)	41.50 (34.5 — 46.7)

It seems that *in vivo* in kidney of maleate-treated rats the formation of amino acids due to the dismutation of ketoglutarate is inhibited as it was demonstrated *in vitro*. The accumulated ketoglutarate may serve chiefly as a substrate for the reductive amination but hydrogen must be provided from reactions which are not inhibited by maleate. The experiments *in vitro* [11, 12] point first of all to citrate and succinate, and to a smaller extent to malate and perhaps to glucose-6-phosphate as possible hydrogen donors.

The experiments presented in Table 3 show that in kidney homogenate three hours after the injection of maleate almost no amino acids were synthesized from ketoglutarate. Even 36 hr. after the injection the synthesis was by one half smaller than normally. When citrate was

Table 3

The synthesis of amino acids by kidney homogenates of maleate-treated rats

Each vessel contained in a final volume of 3 ml.: 100 μmoles of tris buffer, pH 7.2; 6 μmoles $MgSO_4$; 4 μmoles EDTA; 5 μmoles ATP; 60 μmoles NH_4Cl; where indicated in the Table, other additions were: 60 μmoles ketoglutarate or citrate, 30 μmoles succinate, 30 μmoles malate, or 30 μmoles glucose-6-phosphate and 0.5 μmole NADP. To each sample 1 ml. of homogenate from 200 mg. wet tissue was added. Incubation: 60 min. at 37°.

Maleate was injected intraperitoneally in a dose of 300 mg. per kg. of body wt., and rats were killed 3 or 36 hr. after the injection. Mean values from 6 - 10 experiments, ± SD are given.

Substrate	$N\text{-}NH_2$ formed (μmoles/g. tissue wet wt./hr.)		
	Non treated rats	After injection of maleate	
		3 hr.	36 hr.
Ketoglutarate	51.0 ± 3.0	5.5 ± 0.5	28.0 ± 4.0
Citrate	68.0 ± 3.5	55.5 ± 3.5	48.5 ± 2.5
Ketoglutarate + succinate	46.0 ± 2.5	32.5 ± 2.0	45.0 ± 3.0
Ketoglutarate + malate	46.0 ± 13.5	25.0 ± 2.5	29.0 ± 2.5
Ketoglutarate + G-6-P + NADP	67.5 ± 17.0	24.0 ± 3.0	52.0 ± 17.0

used as substrate the amount of amino acids formed was nearly the same as that formed in homogenates of normal rat. Also after the addition of succinate together with ketoglutarate the quantity of amino acids synthesized was close to that in the control rat. With malate the effect was smaller but, as it was earlier described [3], the oxidation of malate may be partly inhibited by maleate. The contribution of G-6-P is rather large but, as it has been discussed in the previous paper [11] this coupling has probably less significance *in vivo*.

Table 4

The concentration of amino nitrogen, ketoacids and citrate in rat kidney after the administration of maleate

Maleate was injected intraperitoneally in a dose of 300 mg. per kg. body wt., and rats were killed 3 or 36 hr. after the injection. Mean values from 10 experiments, ± SD are given.

Time after the injection of maleate	Ketoacids	Citrate	$N\text{-}NH_2$
	(μmoles/g. of tissue wet wt.)		
Control	0.19 ± 0.01	0.40 ± 0.06	20.5 ± 2.8
3 hr.	1.62 ± 0.90	0.90 ± 0.47	15.1 ± 3.8
36 hr.	0.28 ± 0.05	0.69 ± 0.11	26.6 ± 1.6

In rat kidney, 3 hr. after maleate injection (Table 4) the amount of ketoacids per g. of tissue was 8 - 9 times greater than normally, and in the course of these 3 hr. the amount of excreted ketoacids was 12 times

Table 5

Effect of maleate on the excretion of ketoacids, citrate and amino nitrogen

Urine was collected from 0 to 3 hr. and from 0 to 36 hr. following administration of maleate in a dose of 300 mg. per kg. body weight. Results are expressed in μmoles per 3 or 36 hr. Mean values from 10 experiments, ± SD are given.

Rat	Time after injection of maleate	Ketoacids	Citrate	N-NH_2
		(μmoles/3 hr.)		
Control	3 hr.	9.0 ± 1.2	14.7 ± 2.7	15.4 ± 3.0
Intoxicated		115.3 ± 26.0	35.3 ± 9.0	82.2 ± 21.0
		(μmoles/36 hr.)		
Control	36 hr.	108.0 ± 14	186.0 ± 17	199.0 ± 39
Intoxicated		182.4 ± 21	192.9 ± 24	1530.0 ± 272

greater than normally (Table 5). Similarly the excretion of citrate was more than doubled at that time corresponding to the doubled level of this compound in the kidney. The amount of ketoacids and citrate in the kidney decreased gradually but even 36 hr. after intoxication it was by 60 - 70% higher than in the control rats. However, the examination of ketoacids and citrate in the urine indicated that between the 3rd and the 36th hour following the injection, the maleate-treated rats excreted less of those substances than the normal rats. The differences in excretion were about 70 μmoles of ketoacids (182 - 115 = 67) and about 160 μmoles of citrate (193 - 35 = 158) in maleate-treated rats whereas in the controls they were 100 and 170 μmoles, respectively. This shows that in spite of the increased level of those substances in the kidney they are not totally excreted but are in part utilized, probably for the synthesis of glutamate. The content of amino acids in the kidney of intoxicated rats showed distinct changes; 3 hr. after maleate injection it was by 25% less and after 36 hr. by about 30% greater than normally. The origin of these changes is not quite clear. The excretion of amino acids was increased about 5 - 7 times above the normal level both during 3 and during 36 hr. following the administration of maleate.

DISCUSSION

It is generally known that under normal conditions there is no accumulation of intermediates of the tricarboxylic acids cycle in the tissues. Therefore the results of experiments in which to the homogenates an excess of ketoglutarate was added (Table 3) are to be regarded as indication of the potential possibilities of amino acid synthesis by the kidney of normal and maleate-treated rats. However, the data concerning the homogenates of intoxicated rat correspond more closely to

the values *in vivo* because under the conditions of maleate intoxication there is an accumulation of ketoglutarate in the kidney (Table 4).

According to Krebs [8], in rat kidney every 10 seconds about 0.2 μmole of ketoglutarate is formed per gram of tissue. The weight of the kidneys of an adult rat is on average 1.5 - 2 g. Assuming that the inhibition of ketoglutarate oxidation in the kidneys in the first 24 hr. following maleate injection amounts to about 50% we can calculate that during this time 1300 - 1700 μmoles of ketoglutarate accumulates and may undergo reductive amination. Similarly, assuming that the inhibition of ketoglutarate oxidation during the next 24 hr. after the injection averages 20 - 25% we can calculate that during this time the kidney may synthesize about 600 - 800 μmoles of amino acids more than normally. Those are very large quantities and they correspond approximately to the intensity of aminoaciduria observed in maleate intoxication. It should be pointed out that in the first 36 hr. following the administration of maleate, the rat excretes only about 180 μmoles of ketoacids and as much as nine times that amount of amino acids.

Are the kidneys of maleate-treated rat able to synthesize such a great amount of amino acids? From the data presented in Table 2 it can be seen that kidney homogenates of rats killed 3 hr. after the injection of maleate may synthesize from an excess of citrate as much as 60 μmoles of amino acids per gram of tissue and hour. In relation to the weight of the kidneys of an adult rat it corresponds to about 2100 - 2900 μmoles per 24 hr. This indicates that the activity of the enzymes involved in amino acid synthesis in the kidneys of maleate-treated rat is sufficiently great to convert into amino acids the whole of the accumulating ketoglutarate.

As it was previously stated [3] the level of amino nitrogen in the blood of maleate-treated rat is not increased, therefore it may be concluded that the whole excess of amino acids produced by the kidneys is excreted in the urine. From the above calculation it follows that the increased synthesis of amino acids in the kidneys takes place mainly during the first two days following the injection of maleate.

It seems that our experiments allow to explain the renal aminoaciduria in maleate intoxication by an inhibition of ketoglutarate oxidation in the kidneys enhancing the synthesis of amino acids.

SUMMARY

1. Of 9 rat tissues tested, only liver, kidney and brain synthesize considerable amounts of amino acids from α-ketoglutarate and ammonia.

2. In the kidney of maleate-treated rat ketoacids are accumulated.

3. Kidney homogenates of maleate-treated rats synthesize the amino acids with good yield, utilizing for ketoglutarate reduction the hydrogen derived from isocitrate, succinate and to a smaller degree from malate.

4. The renal aminoaciduria in maleate intoxication may be explained by enhanced synthesis of amino acids in kidney due to inhibition of ketoglutarate oxidation.

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DZIAŁANIE KWASU MALEINOWEGO NA NERKĘ

IV. SYNTEZA AMINOKWASÓW W NERKACH SZCZURÓW ZATRUTYCH MALEINIANEM

Streszczenie

1. Spośród 9 badanych tkanek szczura tylko wątroba, nerka i mózg syntetyzują znaczne ilości aminokwasów z ketoglutaranu i amoniaku.

2. U szczurów zatrutych maleinianem gromadzą się w nerce duże ilości ketokwasów.

3. Homogenaty nerek szczurów zatrutych maleinianem mogą syntetyzować aminokwasy z dużą wydajnością wykorzystując do redukcji ketoglutaranu wodór pochodzący z izocytrynianu, bursztynianu i w mniejszym stopniu z jabłczanu.

4. Aminoacyduria występująca w zatruciu maleinianem może być wynikiem wzmożonej syntezy aminokwasów, spowodowanej zahamowaniem utleniania ketoglutaranu w nerce.

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STUDIES ON HYDROLASES OF DIGESTIVE JUICES

VIII. CHOLINESTERASE OF DOG PANCREATIC JUICE*

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Many animal tissues including blood serum contain the so-called non-specific or pseudo-cholinesterase (acylcholine acyl-hydrolase, EC 3.1.1.8), an enzyme which hydrolyses other choline esters more rapidly than acetylcholine. The properties of the enzyme, especially that originating from blood serum, have been studied and several attempts at its purification have been undertaken [22, 16, 12].

Among mammalian tissues studied, dog pancreas was found to contain a large amount of cholinesterase and in 1943 Mendel & Mundell [18] carried out a partial purification of the enzyme starting from this material. Later cholinesterase was also found in dog pancreatic juice [17, 10], the properties of this enzyme, however, have not been thoroughly studied.

In the course of some earlier studies carried out in this laboratory several esterases including cholinesterase have been observed in dog pancreatic juice [23]. In the present study pancreatic juice cholinesterase has been separated from other esterases and some properties of the partly purified enzyme have been investigated.

MATERIALS AND METHODS

Abbreviations used. AcCh, acetylcholine; PrCh, propionylcholine; BuCh, butyrylcholine; BzCh, benzoylcholine; MCh, acetyl-β-methylcholine; DFP, diisopropylfluorophosphate; PCMB, *p*-chloromercuribenzoic acid.

Enzyme preparation. Dog pancreatic juice freshly collected from a permanent fistula (single portion about 150 ml.) was acidified to pH 5 with 2 N-phosphoric acid and the formed precipitate centrifuged for 15 min. at 3000 r.p.m. To the clear supernatant, solid ammonium sulphate was added, with stirring, to 0.4 saturation. In order to reduce the coprecipitation of cholinesterase, ammonium sulphate was added in two por-

* Previous communication of this series [24].

tions, the first to 0.3 and the second to 0.4 saturation, and both precipitates were centrifuged as above. To the supernatant, ammonium sulphate was added to 0.8 saturation, the mixture left for 1 hr. and centrifuged for 20 min. at 3000 r.p.m. The collected precipitate was dissolved in 0.033 M-phosphate buffer of pH 7.0 (1/10 of the original volume of pancreatic juice) and dialysed against two successive 500 ml. portions of the same buffer for 16 hr. All steps of the procedure were carried out at 0 - 4°. The dialysed solution could be stored at 2° for a month with only negligible loss of activity. The preparation was more stable in lyophilized form, the process of lyophilization caused, however, some additional inactivation. The above procedure was repeated five times in order to obtain sufficient amount of the enzyme preparation necessary for completion of experiments. Four preparations were used in the form of dialysed solution and discarded after a month. One preparation was lyophilized and used during four months.

Substrates. Acetylcholine bromide and acetyl-β-methylcholine chloride (Light, England); acetylcholine perchlorate was prepared from bromide according to Bell & Carr [6]; propionylcholine perchlorate and butyrylcholine perchlorate were prepared as described by Aldridge [2]; butyrylthiocholine iodide and benzoylcholine chloride (Nutritional Biochemicals Co., U.S.A.); tributyrin (Lachema, Czechoslovakia); *p*-nitrophenyl acetate was prepared according to Huggins & Lapides [15].

Inhibitors and other reagents. Eserine sulphate and sodium arsenite (Merck, Germany); diisopropylfluorophosphate, EDTA, tetraethylammonium bromide and *p*-chloromercuribenzoic acid (Light, England); iodine (resublimed, Malinckrodt, U.S.A.); *N*-ethylmaleimide (Schuchardt, Germany). Phosphate buffer was prepared from analytical grade reagents.

Assay of cholinesterase activity. Enzyme solution was incubated with 2.0 ml. of 0.066 M-phosphate buffer, pH 7.0, and substrate solution (final concentration 6×10^{-3} M) in the total volume of 5.0 ml. at 37°. Immediately after the addition of the substrate and later in 10 min. intervals, duplicate samples of 0.5 ml. were taken and the amount of the unchanged ester determined by the Hestrin hydroxamic method [13]. When *p*-nitrophenyl acetate was used as a substrate, its concentration was 10^{-4} M and hydrolysis was assayed by the Huggins & Lapides method [15]. All extinction measurements were carried out in the Coleman Junior spectrophotometer.

Protein determination. The Folin & Ciocalteu method [9] modified as described earlier [14] was used.

RESULTS

The 0.4 - 0.6 ammonium sulphate saturation fraction of dog pancreatic juice contained about 1/3 of the cholinesterase activity originally present and only 1/30 of the original amount of protein. Thus about 10-fold

Table 1

Relative activity of cholinesterase preparation from dog pancreatic juice towards some choline and non-choline esters

Substrate	Relative activity
Butyrylcholine	100.0
Butyrylthiocholine	94.6
Propionylcholine	59.4
Acetylcholine	39.6
Benzoylcholine	28.3
Acetyl-β-methylcholine	0.2
Tributyrin	5.3*
p-Nitrophenyl acetate	0.4*

* For non-choline esters the values were calculated by subtracting from the whole activity the activity not inhibited by 10^{-5} M-eserine (about 15% of the whole activity).

purification was obtained with 35% yield. The procedure used made possible the separation of cholinesterase from other esterases present in dog pancreatic juice, i.e. cholesterol esterase and the esterase insensitive to organophosphorus compounds, not attained formerly with the use of zone electrophoresis [23]. The preliminary experiments demonstrated that other enzymes present in pancreatic juice, i.e. amylase, proteases and lipase, are precipitated mainly at lower ammonium sulphate concentration.

The values of relative activity of the cholinesterase preparation towards several choline and non-choline esters are shown in Table 1. The rate of hydrolysis was the highest for butyrylcholine and decreased for other choline esters in the order: BuCh > PrCh > AcCh > BzCh ≫ MCh. Non-choline esters were hydrolysed much slower. No significant difference in rates of hydrolysis between butyrylcholine and its thioester analogue was observed. It was also found in preliminary experiments that the kind of anion associated with choline ester (chloride, bromide or perchlorate) had no effect on enzymic activity.

Table 2

Ratio of activity found in the pancreatic juice and in partly purified cholinesterase preparation

The values are the means, ± S.E.M. from twelve determinations in the case of non-fractionated juice and six determinations in the case of the purified preparation.

Material	Ratio of activities	
	BuCh/AcCh	BzCh/AcCh
Non-fractionated pancreatic juice	2.45 ± 0.17	0.74 ± 0.07
Cholinesterase preparation	2.64 ± 0.21	0.77 ± 0.08

The specificity pattern of cholinesterase did not change during the course of purification since the ratios BuCh/AcCh and BzCh/AcCh for the pancreatic juice and for the purified preparation showed no signi-

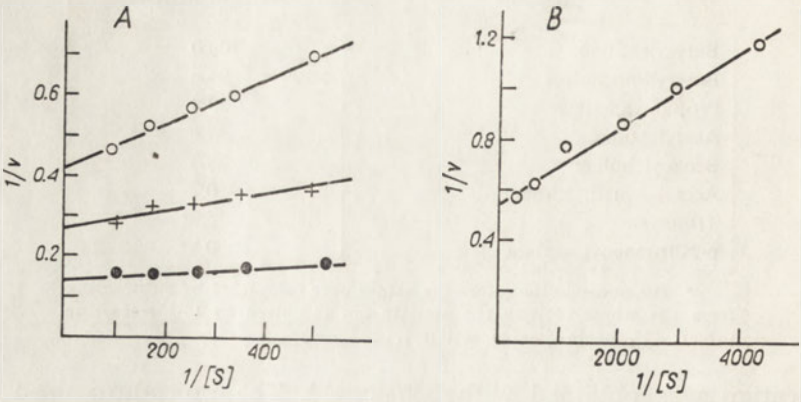

Fig. 1. Lineweaver & Burk plots for hydrolysis of choline esters by cholinesterase preparation from dog pancreatic juice. All curves were constructed by the least squares method. Reaction velocity v is expressed in μmoles of hydrolysed substrate per minute and mg. of protein, substrate concentration in moles per liter. A, (○), acetylcholine; (+), propionylcholine; (●), butyrylcholine; B, (○), benzoylcholine.

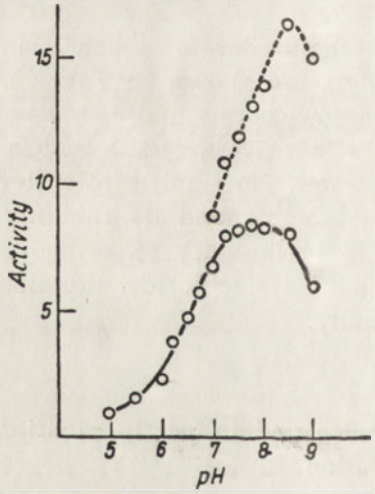

Fig. 2. Effect of pH on cholinesterase activity. All activity measurements were carried out with 0.066 M-phosphate buffer and butyrylcholine as a substrate. The activity is expressed in μmoles of the split ester per minute and mg. of protein. The non-enzymic hydrolysis was checked for every pH value used. Solid line, actual activity measurements; dotted line, values calculated on the basis of enzyme inactivation in alkaline medium.

ficant differences (Table 2). It seems therefore that only one enzyme hydrolysing choline esters is present in dog pancreatic juice.

Figure 1 gives the Lineweaver & Burk plots for hydrolysis of four choline esters. The values of Michaelis constant and maximum velocity are collected in Table 3. The enzymic hydrolysis of these esters seems to agree with the Michaelis-Menten equation and within the concen-

tration range used no inhibitory effect of the increasing amount of substrate was observed.

The maximum rate of butyrylcholine hydrolysis was observed at pH about 7.5 (Fig. 2). This value, however, did not represent the true pH optimum because of the progressive inactivation of the enzyme in alkal-

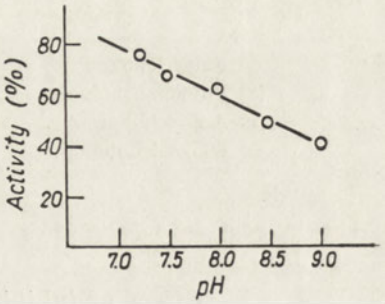

Fig. 3. Cholinesterase inactivation in alkaline medium. The enzyme solution was preincubated with 0.066 M-phosphate buffer of appropriate pH for 30 min. at 37°, adjusted to pH 7.0 with calculated amount of KH_2PO_4 solution and the activity towards butyrylcholine was determined. The activities are expressed as percentages of the activity measured in standard conditions (pH 7.0, without preincubation)

ine medium (Fig. 3). When the pH - activity curve taking into account this inactivation was constructed (Fig. 2, dotted line), the maximum activity was found to be at pH about 8.6.

The cholinesterase preparation was highly sensitive to the action of the well known esterase inhibitors, eserine and DFP (Table 4 and Fig. 4). Also some thiol reagents had a well pronounced inhibitory effect. No inhibition, however, was produced by *N*-ethylmaleimide,

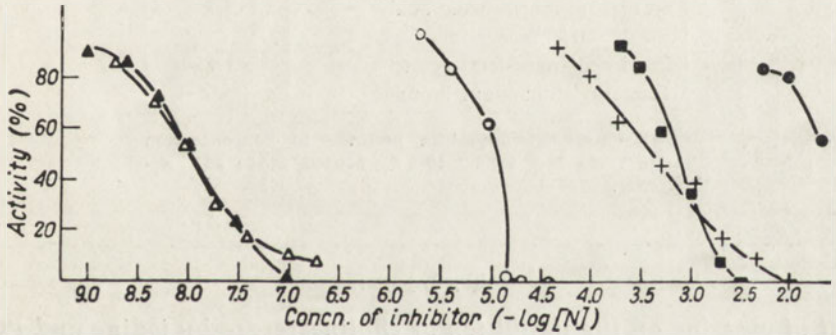

Fig. 4. Effect of inhibitor concentration on cholinesterase activity. The activity was measured in standard assay conditions with butyrylcholine as a substrate. The buffered enzyme solutions were preincubated with the appropriate amount of inhibitor for 20 min. at 37°. The activities are expressed as percentages of the activity measured in the absence of inhibitor. (△), Eserine; (▲), DFP; (○), iodine; (+), arsenite ion; (■), PCMB; (●), EDTA.

a very potent thiol reagent, even at the concentration as high as 3×10^{-2} M. Cysteine protected the enzyme against the inhibitory action of iodine and PCMB, but no other effect of this agent was observed (Table 5). A slight inhibition by EDTA was observed, tetraethylammonium bromide, on the other hand, caused some activation.

Table 3

Michaelis constant and maximum velocity values for hydrolysis of choline esters by cholinesterase preparation from dog pancreatic juice

The values of K_m and V_{max} calculated from Lineweaver & Burk plots with the least squares method are the means, ± S.E.M. from four series of experiments.

Substrate	K_m	$V_{max.}$
Butyrylcholine	$6.3 \pm 0.3 \times 10^{-4}$	6.39 ± 0.71
Propionylcholine	$8.3 \pm 0.3 \times 10^{-4}$	3.79 ± 0.38
Acetylcholine	$1.6 \pm 0.1 \times 10^{-3}$	2.53 ± 0.26
Benzoylcholine	$2.2 \pm 0.2 \times 10^{-4}$	1.80 ± 0.18

Table 4

Effect of inhibitors on cholinesterase activity

The values of pI_{50} represent the negative logarithm of the concentration of inhibitor producing 50% inhibition.

Inhibitor	pI_{50}
Diisopropylfluorophosphate	8.0
Eserine sulphate	8.0
Iodine	5.0
Sodium arsenite	3.8
p-Chloromercuribenzoic acid	3.1
N-Ethylmaleimide	—
Ethylenediamine-tetraacetate	1.5
Tetraethylammonium bromide	—*

* Tetraethylammonium bromide in concentration range from 10^{-3} to 2×10^{-2} M caused 13 to 20% activation.

Table 5

Effect of cysteine on the inhibition of cholinesterase by iodine and PCMB

To the buffered enzyme solution with or without cysteine, inhibitor solution was added and the mixture preincubated for 20 min. at 37° before the enzymic activity was determined.

Addition	Relative activity
None	100
Iodine, 2×10^{-5} M	0
Cysteine, 10^{-4} M + iodine, 2×10^{-5} M	97
PCMB, 10^{-3} M	36
Cysteine, 10^{-3} M + PCMB, 10^{-3} M	100
Cysteine, 10^{-3} M	100

DISCUSSION

An appreciable lability of cholinesterase and its sensitivity to proteolytic enzymes cause considerable difficulties in the purification of this enzyme. The best cholinesterase preparations are certainly far from being pure and no crystallization of the enzyme has been accomplished as yet. The procedure used in this study gave only 10-fold purification, yet it made possible the separation of cholinesterase from other esterases present in dog pancreatic juice.

The data collected so far suggest that only one enzyme of the cholinesterase group occurs in pancreatic juice obtained from a permanent fistula. This view is supported by the fact that the ratio of activities towards various substrates was practically unchanged in the course of purification of the enzyme. Some earlier results suggesting the presence of two cholinesterases in dog pancreatic juice [23] have not been confirmed. The appearance of cholinesterase activity in the two electrophoretically separated fractions which was then observed, was probably due to the partial degradation of the enzyme by proteases or to the formation of cholinesterase-protein complex exhibiting different electrophoretic mobility.

The specificity pattern of the enzyme was typical for cholinesterase. The enzyme hydrolysed butyrylcholine with maximum rate, showed relatively high activity towards benzoylcholine and only negligible activity towards acetyl-β-methylcholine. This proves that the preparation was virtually free of acetylcholinesterase since this enzyme, in contrast to cholinesterase, hydrolyses acetyl-β-methylcholine with moderate activity and displays no activity towards benzoylcholine [4].

The value of Michaelis constant was the highest for acetylcholine and decreased through propionylcholine to butyrylcholine. The maximum velocity changed in the opposite direction showing the maximum value for butyrylcholine. This can be interpreted as a gradual increase of the enzyme affinity to the substrate from acetylcholine to butyrylcholine or the rise of the velocity constant k_2 relating to the decomposition of enzyme-substrate complex in the direction of product formation. Benzoylcholine showed the smallest K_m value and at the same time the smallest velocity as compared with the other substrates. This may be connected with still greater affinity to the substrate or much smaller k_2 value.

The cholinesterase preparation showed a marked sensitivity to alkaline medium and the optimum pH could be determined only indirectly. At this pH (about 8.6) more than 50% inactivation of the enzyme took place during the incubation of the reaction mixture. For this reason in

the determinations of cholinesterase activity a lower pH value had to be used, such in which the activity was high enough and the inactivation not so marked.

The effect of eserine and DFP was typical for the enzymes of the cholinesterase group. A slight inhibition caused by EDTA was probably due to the disappearance of divalent cations from the incubation mixture, for it has been reported that Ca^{2+} and Mg^{2+} ions enhance the activity of cholinesterase [4]. The activity of the enzyme was slightly enhanced by tetraethylammonium bromide. This effect, observed also in the case of serum cholinesterase [4], may be connected with the presence of only one anionic site in the activity centre of cholinesterase molecule [7, 4]. Also the observed lack of inhibition by the excess of substrate may be explained in this manner. The inhibition by some thiol reagents observed here has been also reported for serum cholinesterase [20, 11]. The relatively high concentration of these inhibitors necessary for 50% inhibition suggests that their action is rather unspecific, and no essential thiol group is present in the activity centre of the enzyme.

It is difficult to compare the enzyme investigated in the present work with cholinesterase preparations obtained by other authors because of marked differences in experimental conditions and the scope of the studied properties. The specificity pattern of the dog pancreatic juice cholinesterase was similar in some details but different in others, from cholinesterases obtained from other sources. The ratio BzCh/AcCh, for example, was much higher than that reported for cholinesterase from dog pancreas [19] but approached the value found for dog serum cholinesterase [21]. Also the BuCh/AcCh ratio was very similar to that reported for cholinesterase from dog serum [21] but deviated from the values characteristic for cholinesterase from sera of man [1, 3], horse [2] and other species [5, 21]. The comparison made above supports to some degree the view assuming the existence of species-specific but not organ-specific cholinesterases [21, 8].

The differences in specificity patterns between cholinesterases originating from various animal species led to the distinction between propionylcholinesterases, butyrylcholinesterases etc. according to the substrate hydrolysed with maximum rate [4]. On the basis of the studied properties, the enzyme present in dog pancreatic juice can be classified as butyrylcholinesterase.

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SUMMARY

Cholinesterase from dog pancreatic juice has been partly purified by ammonium sulphate fractionation. The enzyme hydrolysed butyrylcholine most actively, and propionylcholine, acetylcholine and benzoylcholine at a slower rate; acetyl-β-methylcholine, tributyrin and *p*-nitrophenyl acetate were hydrolysed much slower. The optimum pH was found to be about 8.6 but at this pH the enzyme was unstable. Some kinetic properties of the enzyme and its sensitivity towards inhibitors have been investigated. The cholinesterase studied has been compared with cholinesterases from other sources and was classified as butyrylcholinesterase.

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BADANIA NAD HYDROLAZAMI SOKÓW TRAWIENNYCH

VIII. ESTERAZA CHOLINOWA Z SOKU TRZUSTKOWEGO PSA

Streszczenie

Przeprowadzono częściowe oczyszczenie esterazy cholinowej z soku trzustkowego psa przy zastosowaniu frakcjonowania siarczanem amonowym. Enzym hydrolizuje najaktywniej butyrylocholinę, słabiej propionylocholinę, acetylocholinę i benzoilocholinę. Acetylo-β-metylocholina, trójbutyryna i octan p-nitrofenolu ulegają hydrolizie tylko w małym stopniu. Enzym wykazuje optimum aktywności przy pH około 8,6, ale jest w tym środowisku nietrwały. Zbadano niektóre własności kinetyczne enzymu i jego wrażliwość na działanie inhibitorów. Przeprowadzono porównanie badanego enzymu z esterazami cholinowymi pochodzącymi z innych źródeł i sklasyfikowano go jako esterazę butyrylocholinową.

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ORNITHINE CARBAMOYLTRANSFERASE IN HIGHER PLANTS

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The operation of the ornithine cycle in higher plants may now be regarded as an established fact. In several papers from this laboratory published in the course of the last 5 years we have reported the presence of ornithine carbamoyltransferase (carbamoylphosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3.) in green peas, beans, soya beans, white and blue lupins as well as in the young seedlings of wheat and perennial ryegrass [4, 8, 9, 10]. At the same time it was established that homogenates from wheat seedlings in the presence of ATP and L-aspartic acid are capable of converting citrulline into arginine [2], and in acetone powders from green pea seedlings argininosuccinate lyase was found to be present [1]. Finally the presence of arginase in 30 plants belonging to 12 different botanical families was reported [7].

In this paper we report on the presence of ornithine carbamoyltransferase in 46 plants belonging to 19 botanical families, as well as on the activity of the enzyme throughout the full life cycle of wheat and the green pea.

MATERIALS AND METHODS

Young plant seedlings (1 - 5 g.) were frozed hard in a mortar placed in a flat vessel containing dry ice and alcohol, for about 15 min. The plant material was then finely powderized, transferred into a small beaker to which 5 - 25 ml. (5 times the vol. of the wet wt. of the sample) of 0.1 M-phosphate buffer solution of pH 7.2 was added. After 10 - 15 min. of standing at room temperature with frequent stirring, the liquid was filtered off through a soft filter paper into a test tube immersed in an ice-water bath. Extracts from seeds were prepared in a similar manner after grinding the seeds in the Junior laboratory mill to a fine powder. In order to ascertain optimum enzyme activity in the extracts, the incubation with ornithine and carbamoylphosphate (CP) was generally conducted at three levels. (1), 1 ml. extract, 0.1 ml. ornithine (20 μmoles of DL-ornithine - HCl), 0.1 ml. CP (20 μmoles); (2), 0.5 ml. extract, 0.5 ml. phosphate buffer, pH 7.2, 0.1 ml. ornithine, 0.1 ml. CP; (3), 0.2 ml. extract, 0.8 ml. phosphate buffer, pH 7.2, 0.1 ml. ornithine, 0.1 ml. CP.

Control determinations were carried out in the same way except

that the extract was inactivated with 0.1 ml. of 60% $HClO_4$ prior to the addition of ornithine and CP. All the samples were incubated at 30° for 30 min., and those with the active enzyme were inactivated with 0.1 ml. of 60% $HClO_4$. The mixture was then made up with distilled water in small cylinders to a volume of 10 ml. and the precipitated proteins were filtered off.

According to the amounts of citrulline to be expected, 1 - 3 ml. of the filtrate was used for the determination of citrulline [5]. The activity was expressed in μmoles of synthesized citrulline per 1 g. of protein, on incubation for 30 min. at 30°. Protein determinations were carried out with the Folin phenol reagent according to the method described by Lowry *et al.* [6].

Activity of ornithine carbamoyltransferase during the life-cycle of the plants was studied on the green pea, variety "cukrowy-karłowy", and on spring wheat, variety "Opolska oryginalna". Representative samples were prepared from 7 plants of wheat or pea, respectively.

The dilithium salt of carbamoylphosphate was prepared according to the method of Jones, Spector & Lipmann [3]. Solutions of CP are relatively unstable, therefore they had to be always prepared freshly just before use.

RESULTS AND DISCUSSION

The results concerning ornithine carbamoyltransferase activity in dry seeds and young seedlings of 46 plants belonging to 19 botanical families are reported in Table 1. It can be seen that all the investigated plants contained the enzyme, and its activity was invariably higher in seedlings than in seeds. The activity in seeds varied from 0.06 to 0.43, whereas in seedlings it varied from 0.1 to 1.44 μmole citrulline/1 g. protein. The differences between the various species within a botanical

Table 1

Activity of ornithine carbamoyltransferase in higher plants

The activity is expressed in μmole of citrulline formed/1 g. protein/30 min. incubation at 30°.

Family	Species	Activity in seeds	Age of seedlings (days)	Activity in seedlings
1	2	3	4	5
Graminae	Wheat	0.43	8	1.37
	Rye	0.21	7	0.63
	Sweet corn	0.22	8	0.34
	Barley	0.25	6	0.60
	Oats	0.13	7	0.68
	Per. ryegrass	0.15	7	0.25

1	2	3	4	5
Cucurbitaceae	Cucumber	0.22	8	1.20
	Pumpkin	0.10	10	0.45
Liliaceae	Onion	0.11	12	0.67
	Leek	0.27	14	0.57
Solanaceae	Tomato	0.26	9	0.31
	Tobacco (white Bedder)	0.074	10	0.12
Cruciferae	Cabbage	0.11	6	0.35
	Mustard	0.10	5	0.23
	Radish	0.40	5	0.45
	Spring rape	0.33	6	0.54
	Alyssum comp.	0.04	10	0.18
	Mathiola bicormis	0.08	10	0.35
Papilionaceae	Green pea	0.19	8	1.44
	Bean	0.06	8	0.08
	White clover	0.10	9	0.28
	Lucerne	0.21	7	0.57
	Lupin (Russel)	0.09	9	0.10
Linaceae	Hemp	0.26	10	0.83
	Flax	0.10	11	0.22
Umbelliferae	Carrot (Amager)	0.09	15	0.32
	Celery	0.10	13	0.17
	Parsley	0.12	15	0.28
	Dill	0.06	14	0.17
Chenopodiaceae	Beet-root	0.12	8	0.28
	Spinage (Gaudry)	0.20	9	0.68
	Kochia scoparia	0.07	12	0.19
Polygonaceae	Sorrel	0.25	9	0.35
Compositae	Sunflower	0.16	7	0.45
	Lettuce	0.13	5	0.22
	Ageratum mexicanum	0.11	9	0.25
	Chrysanthemum leucan.	0.07	10	0.10
	Callisthephus chin.	0.09	12	0.12
Papaveraceae	Poppy	0.29	9	0.37
Violaceae	Pansy	0.17	14	0.15
Tropheolaceae	Nasturtium	0.17	10	0.20
Labiatae	Antirrhinum majus hijac.	0.17	9	0.19
Caryophyllaceae	Carnation	0.17	12	0.60
Aceraceae	Maple tree	—	leaves 20-days	0.54
Tilliaceae	Lime tree	—	,, ,,	1.60
Hippocastanaceae	Horse chesnut	—	,, ,,	0.56

family were just as great as between the various families themselves. The highest carbamoyltransferase activity was found in wheat seeds as well as in the wheat and pea seedlings. Unexpectedly high enzyme activity was observed in very young tree leaves, particularly

of the lime tree, whereas old leaves collected in the early autumn have shown about 10 times lower activity (Table 2).

It is interesting to note that the content of the endogenous ureide compounds found in the controls and expressed as citrulline, accumulated in considerable quantities in older leaves thus confirming earlier observations on the alder tree [11].

Table 2

Activity of ornithine carbamoyltransferase and content of ureides in relation to the age of plant material

Family	Species	Activity (μmoles citrulline/1 g. protein)		Ureides (mg. citrulline/1 g. fresh wt.)	
		Leaves collected			
		May 13	Oct. 5	May 13	Oct. 5
Aceraceae	Maple tree	0.54	0.06	1.80	4.37
Tiliaceae	Lime tree	1.60	0.02	1.03	2.15
Hippocastanaceae	Horse chestnut	0.56	0.04	1.46	3.00

The results of experiments presented in Figs. 1 and 2 show that ornithine carbamoyltransferase is present in pea and in wheat during the entire course of growth and development.

At the early stages of plant growth a distinct increase in the enzymic activity was observed, followed by a continuous drop lasting till bud formation in the pea, and till shooting into ears in the wheat, when again the activity was markedly increasing. When flowers in the pea and ears in the wheat were fully formed, a rapid decrease of activity was noted. Then followed another peak in the pea marking the formation of pods, after which a steady decline of activity in the ageing stalks and leaves, analysed separately without the pods, was observed. Flowers analysed together with their stems have shown an activity of 2.7 μmole citrulline/1 g. protein, which was the highest observed at any time or in any organ of the plant. In the young pods there was again a rise in activity, then leading to a sharp and continuous decline back to the starting point in the mature seeds.

Similarly in the wheat there was a rise in activity up to the moment of bloom fall, followed by a decline lasting till the stage of early seed formation. Another peak was reached at the stage of "milky" maturity, followed by a continuous decline through "waxy" and „horny" maturity back to the starting point in the mature seeds.

The curves for ornithine carbamoyltransferase were virtually the same for the pea as for the wheat suggesting that the increases of activity were closely linked up with the particularly important changes

in plant growth and development. Rises in enzymic activity were observed in young seedlings, at the stage just preceding blooming or shooting into ears, and finally in the very early period of formation of the reproductive organs of plants.

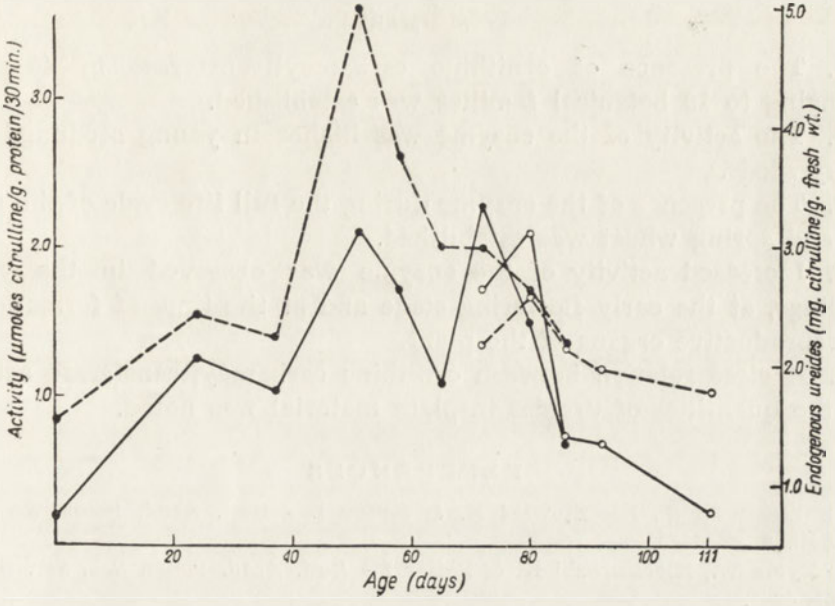

Fig. 1. Ornithine carbamoyltransferase activity during the life cycle of the pea plant. (———), Activity; (— — —), ureides; (●), leaves and stalks; (○), pods.

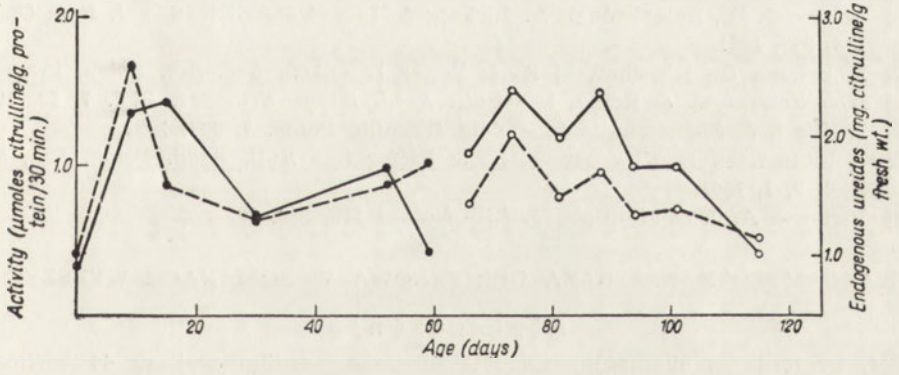

Fig. 2. Ornithine carbamoyltransferase activity during the life cycle of wheat. (———), Activity; (— — —), ureides; (●), leaves and stalks; (○), wheat ears.

It may be interesting to note that the curves for the quantities of endogenous ureide compounds expressed in mg. citrulline/1 g. fresh weight, in the pea as well as in the wheat run almost parallel to the curves for ornithine carbamoyltransferase activity (expressed in μmoles citrulline/1 g. protein). This observation applies to the entire life cycle

of the two investigated plants suggesting that the ornithine carbamoyltransferase activity and the product of its catalytic action may play a much more important role in the non-protein nitrogen metabolism in higher plants than thus far recognized.

SUMMARY

1. The presence of ornithine carbamoyltransferase in 46 plants belonging to 19 botanical families was established.

2. The activity of the enzyme was higher in young seedlings than in dry seeds.

3. The presence of the enzyme during the full life cycle of the green pea and spring wheat was established.

4. Increased activity of the enzyme was observed in the young seedlings, at the early flowering stage and at the time of formation of the reproductive organs of the plant.

5. A close relation between ornithine carbamoyltransferase activity and the quantities of ureides in plant material was noted.

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KARBAMYLOTRANSFERAZA ORNITYNOWA W ROŚLINACH WYŻSZYCH

Streszczenie

1. Stwierdzono obecność karbamylotransferazy ornitynowej w 46 roślinach należących do 19 rodzin botanicznych.

2. Aktywność karbamylotransferazy ornitynowej była zawsze wyższa w kiełkach aniżeli w nasionach.

3. Wykazano enzym we wszystkich okresach rozwoju grochu i pszenicy.

4. Aktywność enzymu rośnie w pierwszej fazie wzrostu kiełków, w okresie butonizacji i we wczesnej fazie tworzenia się narządów reprodukcyjnych u roślin.

5. Między aktywnością karbamylotransferazy ornitynowej i zawartością związków ureidowych w roślinach istnieje ścisła zależność.

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[^{14}C]CARBAMOYL-β-ALANINE AS PRECURSOR OF PYRIMIDINES IN HIGHER PLANTS

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Biosynthesis of pyrimidine nucleotides has been systematically studied on a variety of biological material for over ten years. The pioneering research of Reichard, Kornberg, Wright and others has successfully established the enzyme system and the intermediates of this process, called in short "The orotic acid path". However, more recent work has shown that this mechanism need not be the only source of pyrimidine derivatives in microorganisms and various animal organs. It has been pointed out that the free bases may play the part of intermediates in an alternative path of pyrimidine nucleotide biosynthesis, quite independent from the metabolism of orotic acid. Research on quickly growing animal cells (regenerating tissue and tumorous growth) has been particularly stimulating and this new path has been described as "Salvage mechanism" of nucleotide biosynthesis (see review by P. Reichard [14]).

It has been shown recently in our laboratory that uracil may also be an intermediate of pyrimidine derivative synthesis in higher plants [4]. In this connection our attention has been drawn to *N*-carbamoyl-β-alanine [1] as a possible precursor of uracil in plant tissue particularly as the part played by β-amino acids and carbamoyl-β-amino acids in the anabolic path has already been established in animal organs [7, 10, 11] as well as in microorganisms [6, 9]. The only reference we found concerning the part of CBA in higher plants was the work by Barnes & Naylor [1], who suggest that CBA undergoes exclusively catabolic changes in the embryos of longleaf and slash pines.

MATERIAL AND METHODS

Radioactive CBA labelled with ^{14}C in the carbamoyl group has been synthesized from β-alanine and [^{14}C]KCNO according to the method of Nyc & Mitchell [13]. The specific activity of the [^{14}C]CBA was

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[1] The following abbreviations are used: CBA, carbamoyl-β-alanine; 5′-UMP, uridine-5′-phosphate; 5′-CMP, cytidine-5′-phosphate; 2′(3′)-UMP, uridine-2′(3′)-phosphate; 2′(3′)-CMP, cytidine-2′(3′)-phosphate.

2,200 counts/sec./μmole. [^{14}C]KCNO was obtained by oxidation of [^{14}C]KCN [12]. All other reagents were obtained from commercial sources.

All experiments were carried out on 5 days old excised blades of winter wheat, variety "Dańkowska 40", prepared as already described [15]; 2 g. samples of freshly cut off blades were immersed in 2 ml. of 20 mM solution of [^{14}C]CBA and incubated for 0.5, 2, 8 and 24 hr., respectively, at 35°.

The determinations of the total intake of [^{14}C]CBA and of the excess recovered from the plant material were performed as in the case of feeding carbamoylaspartic acid [15], except that the CBA not metabolized by the plant has been separated by paper chromatography. For the chromatography of CBA a mixture of butanol and 3% ammonia (3:1, v/v) was employed [16], in which this compound moves with an R_F 0.20.

Extraction, chromatographic separation, identification and quantitative determinations of the pyrimidine derivatives as well as measurements of radioactivity were carried out as already described [3, 2].

RESULTS

The intensity of intake of CBA and the metabolic changes in the excised wheat blades are illustrated in Table 1. In the first 8 hr. the intake into the plants was directly proportional to the time of incubation. Afterwards the speed of absorption has considerably diminished. The absorbed [^{14}C]CBA has undergone rapid changes in plant tissue and only a part not exceeding 40% of the intake has been recovered as non-metabolized CBA. The specific activity of the [^{14}C]CBA isolated from the plant tissue has remained virtually on the same level as that of the substrate employed for feeding.

Table 1

Ingestion and metabolism of [^{14}C]carbamoyl-β-alanine by excised wheat blades

In each experiment 2 g. sample of plants was incubated at 35° with 2 ml. of 20 mM solution of [^{14}C]CBA with specific activity 2,200 counts/sec./μmole.

Time of feeding (hr.)	Uptake (μmole)	Recovered		Metabolized (μmole)
		(μmole)	(counts/sec./μmole)	
0.5	2.9	1.3	2,110	1.6
2	9.0	3.8	2,200	5.1
8	26.7	10.8	2,130	15.9
24	36.6	13.7	2,160	22.9

Results of the quantitative determinations of pyrimidine derivatives isolated from plants fed with [^{14}C]CBA for different periods of time as well as the radioactivity of the various products are reported in Table 2. After 30 min. of incubation the carbon label was detected in uracil, uridine and 5′-UMP. The specific activity of uracil has been 40 times greater than that of uridine, which was twice as active as 5′-UMP. No activity could be detected in 5′-CMP and in the products of hydrolysis of the acid insoluble fraction. After 2 hr. of incubation a further sharp increase in the activity of uracil, uridine and 5′-UMP has been observed and also 5′-CMP and the pyrimidine nucleotides of the acid insoluble fraction have shown some small but measurable incorporations of ^{14}C. Further extention of time of incubation with [^{14}C]CBA led to additional increases in activities of the investigated compounds and the specific activity of uracil approached after 8 hr. the level of [^{14}C]CBA employed in the feeding experiment.

Table 2

Amounts and specific activities of pyrimidine derivatives in wheat blades fed with [^{14}C]carbamoyl-β-alanine

Amounts expressed in μmoles/2 g. of fresh weight, specific activity in counts/sec./μmole. Conditions of incubation as for Table 1.

Pyrimidine compounds	Time of feeding (hr.)							
	0.5		2		8		24	
	μmoles	sp. act.	μmoles	sp. act.	μmoles	sp. act.	μmoles	sp. act.
Acid-soluble fraction								
Uracil	0.08	145	0.08	469	0.09	1090	0.07	1830
Uridine	0.33	3.7	0.35	11.6	0.35	43.1	0.30	89.8
5′-UMP	0.14	1.7	0.13	8.6	0.17	20.1	0.11	50.1
5′-CMP	0.09	0.0	0.07	4.5	0.09	12.1	0.11	43.7
Acid-insoluble fraction								
2′(3′)-UMP	1.14	0.0	1.18	0.6	1.16	1.5	1.04	3.3
2′(3′)-CMP	1.33	0.0	1.37	0.4	1.30	1.2	1.18	3.1

The sum of radioactivity recovered from all the pyrimidine derivatives in experiments with varying times of incubation, amounted to about 0.5% of the labelled carbon introduced with the [^{14}C]CBA.

The experiment reported in Table 2 has been repeated twice. In all 3 cases the intake of [^{14}C]CBA, the metabolic changes and the level of specific activities of the investigated pyrimidine compounds has been essentially the same. No radioactivity could be detected in either the free purine bases or in the purine derivatives of the acid soluble and acid insoluble fractions up to 8 hr. of feeding.

DISCUSSION

[^{14}C]CBA absorbed by the plant tissue is rapidly metabolized. Thus is should be assumed that the plant contains a complete enzyme system catalysing CBA metabolism. Yet the normal tissue contains practically no CBA as the radioactive CBA introduced remained virtually undiluted during the experiment (Table 1).

Following the introduction of [^{14}C]CBA into the plant tissue, considerable radioactivity could be detected in uracil, uridine, 5′-UMP and 5′-CMP. Very small but measurable amounts of radioactivity were also found in the products of hydrolysis of the acid insoluble fraction (Table 2). The incorporation of the label of CBA into the above mentioned compounds proves that CBA is capable of anabolic changes in the plant tissue, despite the fact that degradative changes constitute the main path of CBA metabolism, as previously pointed out by Barnes & Naylor [1].

The incorporation of CBA into pyrimidines and their derivatives proceeds at a considerably lower rate than that of carbamoylaspartic acid or orotic acid, which under similar conditions have shown about the same rate of anabolic and catabolic changes. Furthermore it must be strongly pointed out that the introduction of carbamoylaspartic acid or orotic acid always leads to considerable increases in the absolute quantities of the investigated pyrimidines in plant material [15, 3, 2], whereas the introduction of CBA even in prolonged experiments leaves the levels of the recovered pyrimidines virtually unchanged. The intensity of anabolic changes of CBA resembles closely the results obtained on introduction of uracil into excised wheat blades [4].

The specificity of experimentation *in vivo* is necessarily limiting the chances of explanation of the mechanism of metabolic changes within the tissue. Nevertheless in view of the results reported in this paper we believe that it is justified to assume that uracil is the first product of CBA anabolism in plant material. This observation seems to be particularly interesting in view of the fact that in animal organs [11] as well as in microorganisms [9] the formation of the pyrimidine ring is reported to be preceded by reactions of ribosidation and ribotidation. The suggestion that CBA is directly converted into uracil is supported by following observations: (1), ^{14}C was rapidly incorporated into uracil already after 30 min. of feeding with [^{14}C]CBA; (2), uracil showed specific activity 40 times higher than uridine, which in turn was twice as radioactive as 5′-UMP, the only pyrimidine nucleotide showing radioactivity after 30 min. of incubation; (3), after 8 hr. of incubation with [^{14}C]CBA, the specific activity of uracil has nearly equalled that of the CBA used for the feeding of the plant.

The only possible intermediate in the synthesis of uracil from CBA could be dihydrouracil. We were unable to detect the presence of this metabolite in our investigations, but the work of Mokrasch & Grisolia [10] seems to support this view.

The metabolism of CBA into uracil, followed by further anabolic changes reported previously [4] may constitute an alternative way of the pyrimidine nucleotide synthesis in higher plants, independent of the orotic acid path. This alternative path could not be limited to the role of "salvage mechanism" as proposed for the anabolic changes of free pyrimidine bases in animal cells [14], because the plant material used in no way resembles regenerating tissue or tumorous growth.

Furthermore the mechanism controlling the intensity of the observed changes in plant material seems to be different from the one in animal tissue. According to the suggestion of Canellakis [5] supported recently by Fritzson [8] the intensity of pyrimidine synthesis in animal tissue is regulated by the activity of the enzymes catalysing their degradation. Although in the experiments reported in this paper the degradative changes are also incomparably more intense than anabolic changes, yet there remains in the plant tissue a considerable quantity of non-metabolized CBA (Table 1). For this reason the low rate of anabolic changes of CBA into pyrimidines cannot be explained by the exhaustion of the substrate owing to the catalytic activity of the degradative enzymes.

The results obtained indicate that there are marked differences in the utilisation of CBA in plant material on one hand and in animal tissues and microorganisms on the other. Particular attention is drawn to the fact that uracil is an intermediate in the synthesis of pyrimidine nucleotides in higher plants.

SUMMARY

In excized wheat blades fed with [^{14}C]carbamoyl-β-alanine a rapid incorporation of radioactivity into uracil has been observed. Considerable activity was also detected in uridine, 5′-UMP and 5′-CMP, and some activity in the pyrimidine nucleotides of the acid-insoluble fraction. The total radioactivity recovered from the pyrimidine derivatives amounted to about 0.5% of the metabolized [^{14}C]carbamoyl-β-alanine.

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[^{14}C]KARBAMYLO-β-ALANINA JAKO PREKURSOR PIRYMIDYN U ROŚLIN WYŻSZYCH

Streszczenie

Odcięte źdźbła pszenicy dokarmiano [^{14}C]karbamylo-β-alaniną. Stwierdzono jej szybką przemianę do uracylu oraz włączanie ^{14}C do urydyny, 5′-UMP i 5′-CMP. Niewielką radioaktywność stwierdzono także w nukleotydach pirymidynowych frakcji kwasonierozpuszczalnej. Radioaktywność odnaleziona w pochodnych pirymidynowych stanowi około 0,5% aktywności karbamylo-β-alaniny, która uległa przemianie.

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THE METABOLISM OF MOUSE EMBRYO CELLS GROWN *IN VITRO* AND INFECTED WITH THE ONCOGENIC VIRUS SE POLYOMA*

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Penetration of virus into the host cells produces profound metabolic changes, as a result of which the basic processes in the cell serve the synthesis of virus particles. The character of these metabolic changes has been extensively studied on bacteriophage-infected *Escherichia coli* cultures by Cohen *et al.* [5, 6, 16], Hershey & Chase [9], Pardee *et al.* [18, 19], and recently by Kornberg *et al.* [13]; infection by bacteriophages was found to activate certain enzymes, inhibit others and initiate production of new enzymes [21]. Synthetic processes leading to the formation of new DNA molecules, proteins and pyrimidine bases predominate. It is generally assumed that the energy for the increased syntheses is supplied by ATP, although it is still insufficiently elucidated which processes leading to ATP generation are utilized by the cells for the synthesis of bacteriophages. According to Cohen [21], normal oxidation processes are the source of energy; although after the infection of the cell by bacteriophage they are not intensified, the ATP produced is utilized for the synthesis of new non-physiological components. However, some data indicating the inhibition of some respiratory enzymes in bacteriophage-infected cells have been also published [11].

The metabolic changes in virus-infected animal cells are still more complicated [12, 14]. The experimental results point to a great diversity of the metabolic effects of infection, especially those affecting respiration and glycolysis. Some experiments indicated no increase in respiration of the infected cells, and even the possibility of virus reproduction under anaerobic conditions. Other experiments demonstrated dependence of viral reproduction on oxidative phosphorylation. While some authors have observed a marked increase of glycolysis in infected tissues, other did not mention such changes or even noted diminished anaerobic glycolysis.

The experiments on the metabolism of animal cells infected by various viruses were carried out under widely differing conditions (on

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organs, slices, tissue homogenates, tissue cultures) but the metabolic effects of infection by oncogenic viruses have not yet been studied. The possible changes in respiration and glycolysis could be of special value, since the transformation of a normal cell into a neoplastic one is associated with changes in the metabolism of glucose described in the classical work of Warburg. Therefore we have undertaken a study of the respiratory metabolism of cell cultures infected with the SE polyoma virus, which had been maintained at the Department of Medical Microbiology, Medical School, Kraków, and described by Porwit-Bóbr *et al.* [20] and Chłap *et al.* [4].

The polyoma virus described in 1933 by Stewart *et al.* [22], when introduced into newborn mice, rats or hamsters causes after a certain time neoplastic lesions of various histological character and localization. The virus multiplies easily in cultures of mouse embryo cells. Little is known up to now about its chemical properties; it probably contains deoxyribonucleic acid and is of relatively small size [22]. Its structure and physical properties have been described on the basis of electron-microscopic studies [15].

MATERIALS AND METHODS

Tissue cultures. Embryos of the Porton strain mice were used for the experiments. The membranes and placenta were removed and the tissue was chopped with scissors and washed. The cell suspension was prepared in 0.25% trypsin solution (Difco) and mixed with a magnetic stirrer. The centrifuged cells were added with a volume of the medium to obtain a suspension containing 10^5 cells per ml. The growth medium consisted of Earle's salt solution, 0.5% lactoalbumin hydrolysate (Nutritional Biochemicals Corp., Cleveland, Ohio) and 10% calf serum. The cells grew on the walls of Roux flasks as a monolayer.

Virus. The original SE polyoma virus had been obtained from the G. Roussy Institute in Villejuif thanks to the courtesy of Dr. G. Barski, and a strain derived from the mouse embryo cell culture passage was used for the experiments.

Infection of cultures with virus. In young 48-hr. cultures of mouse embryo cells the medium with 10% calf serum was replaced by one containing 3% serum. Immediately thereafter 1 ml. of virus, infectious titer 10^5 TCID per ml. (tissue culture infectious dosis), was added to each Roux flask containing on the average 70×10^5 cells.

Infectivity titration. Virus culture fluid, diluted 10^{-1} to 10^{-10} with Earle's salt solution, was added to the cultures of mouse embryo cells in amount of 0.1 ml., 5 tubes of culture being used for each dilution. The tissue culture infectious dosis was calculated after Kärber [10] by studying the cytopathogenic effect.

Preparation of the material for biochemical studies. Two series were prepared simultaneously: cultures of cells infected with the virus and uninfected cultures (controls). The cells were examined before the infection and 1, 3, 5, 6, 7, and 9 days after the infection. On the 7th day after infection many cells showed signs of degeneration and became detached from the walls of the Roux flasks. The material taken on the 9th day was composed only of the cells still attached to the glass. The medium from 12 flasks was decanted and discarded together with the detached cells present in the fluid. The flasks were then washed with Krebs-Ringer solution, pH 7.4, the cells remaining on the glass were detached by scrubbing with a brush, washed twice, centrifuged, and suspended in a small volume of Krebs-Ringer buffer solution without glucose. The amount of cells in the suspensions was estimated by determining their dry weight.

Estimation of oxygen consumption. Endogenous respiration was measured by the method of Warburg [23] at 38° in Krebs-Ringer phosphate buffer, pH 7.4, with KOH being placed in the central well. Respiration in the presence of glucose was estimated after adding to the vessel glucose to final concentration of 10 mM.

Aerobic glycolysis. Lactic acid was assayed by the Barker & Summerson method [1] after incubation of the cells with Krebs-Ringer solution added with glucose to 10 mM concentration.

RESULTS

The results of determinations of increase of virus titers in the culture medium are presented in Fig. 1. The earliest detectable amounts of the virus in the medium appeared 48 hr. after infection, followed by an

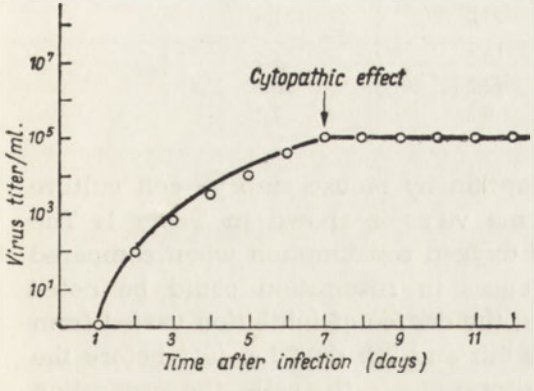

Fig. 1

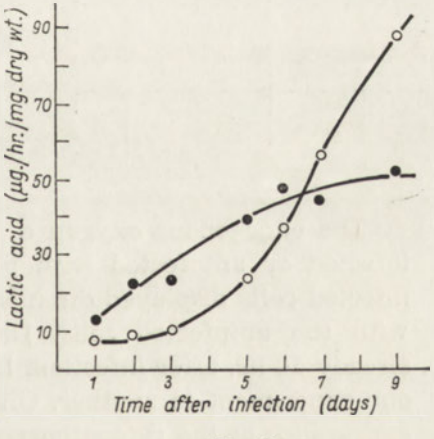

Fig. 2

Fig. 1. The SE polyoma virus infectivity titer in mouse embryo cell culture medium.
Fig. 2. Aerobic glycolysis in mouse embryo cell culture (○), normal and (●), infected with SE polyoma virus.

increase leading to maximum steady values on the 7th day which coincided with the beginning of the cytopathogenic effect. In earlier stages no cytopathogenic effect was observed microscopically.

Table 1

Endogenous respiration of mouse embryo cell cultures uninfected and infected with polyoma virus

Day after infection	Expt. no.	Oxygen consumption during first 20 min. of experiment (μl./hr./mg. dry wt.)	
		Uninfected control cells	Infected cells
—	1	11.1	—
	2	11.4	—
1	1	15.8	10.8
	2	11.3	6.8
3	1	13.0	11.3
	2	13.0	7.0
5	1	6.7	5.2
	2	9.5	3.4
	3	8.9	6.3
6	1	11.9	9.2
	2	8.8	6.1
	3	8.5	3.4
	4	15.6	6.6
	5	7.5	1.6
	6	10.5	2.7
7	1	7.2	0.8
	2	9.3	0.9
	3	10.0	1.4
9	1	10.5	4.9
	2	12.2	10.3
	3	9.3	7.2

The endogenous oxygen consumption by mouse embryo cell culture infected or uninfected with polyoma virus is shown in Table 1. The infected cells displayed diminished oxygen consumption when compared with the uninfected cells. Differences in respiration could be noted already 24 hr. after infection. Later, the degree of inhibition varied from one experiment to another. On the 6th and 7th day, i.e. just before the appearance of the cytopathogenic alterations in the cells, the respiration was the smallest.

These experiments showed that the first release of the virus into the medium was preceded by the reduction of oxygen consumption

observed after 24 hr. The lowest oxygen consumption coincided in time with the presence in the medium of the largest amount of virus and the appearance of necrotic changes in the cells. Oxygen consumption on the 9th day after infection was due to the cells remaining on the glass after the detached, i.e. necrotic cells had been discarded with the medium. Endogenous respiration of the cells selected in this way was much higher than in the whole material before the appearance of cytopathogenic changes.

Table 2

The effect of 10 mM-glucose on oxygen consumption by mouse embryo cell cultures uninfected and infected with polyoma virus

Mean values of 3 experiments are given.

Days after infection	Cells	Mean values of oxygen consumption during 1st hour of experiment (μl./hr./mg. dry wt.)		Changes in oxygen consumption
		endogenous	in presence of glucose	(%)
—	From 24-hr. uninfected culture			
	Attached to glass	8.1	6.5	—20
	Free in medium	12.0	14.0	+17
1	Uninfected	11.5	9.8	—15
	Infected	6.2	4.1	—34
3	Uninfected	8.9	6.6	—26
	Infected	6.3	4.1	—35
5	Uninfected	6.5	5.2	—20
	Infected	3.7	1.7	—54
6	Uninfected	10.4	6.2	—40
	Infected	8.1	4.5	—44
7	Uninfected	7.6	5.1	—33
	Infected	3.6	2.0	—45
9	Uninfected	15.2	12.2	—20
	Infected	11.2	9.4	—16

Oxygen consumption in the presence of glucose both by infected mouse embryo cells and by uninfected ones, was lower than the endogenous oxygen consumption (Table 2). Inhibition of oxygen consumption by glucose, i.e. the Crabtree effect, was always greater in the cells infected with polyoma virus except in those cells which had survived the period of cytopathogenic alterations and were examined on the 9th day. In 24-hr. uninfected cultures, the morphologically homogeneous cells attached to glass showed differences in oxygen consumption in the presence of glucose. This could be anticipated, as the Crabtree effect

had been previously observed [8] in primary cultures soon after their initiation. The remaining, morphologically different types of cells, which were suspended in the medium, did not display the Crabtree effect.

The characteristic feature of the metabolism of neoplasmas and of many cell lines cultivated *in vitro*, the aerobic glycolysis, was found also in the non-infected mouse embryo cells. The infection augmented the production of lactic acid in infected cells (Fig. 2) the increase being proportional to the increase in virus formation. In the uninfected cultures, aerobic glycolysis also increased with time but this increase was slower and was probably due to the long period of cultivation on unchanged medium. Aerobic glycolysis in the infected material was inhibited on the 7th day after infection when it became even lower than in the control material. The reduction in lactic acid formation was correlated in time with the decrease in oxygen uptake, detachment of the cells from the glass and the appearance of cytopathogenic changes.

DISCUSSION

Two approaches may be distinguished in the studies on the metabolism of cells infected with viruses. One of them is concerned with the synthesis of the virus in the cells and optimum conditions for this process, and the second one with the damaging effect of animal viruses on cell metabolism after the termination of the virus developmental cycle. The former studies were usually performed with an excess of the virus as compared with the amount of cells, and were not extended beyond the period of one developmental cycle of the virus. In order to ascertain whether the energy for animal virus synthesis is provided by the oxidative processes or by glycolysis, experiments with inhibitors of the Krebs cycle or respiratory chain, determinations of respiration and of glycolytic activity were carried out [12, 14]. The stimulation of the respiratory activity of the cells during multiplication of viruses, expected by some authors, was not observed; in most cases the oxygen consumption by infected material examined during the ecliptic phase did not differ from the oxygen consumption by control material. Reduction of oxygen uptake occurred simultaneously with the release of virus into the medium.

On the other hand, increased aerobic glycolysis was found as a rule in the infected material. This might indicate participation of the energy of glycolysis in the virus synthesis or might be due to non-specific injury to the cells. The variety of the viral and tissue materials studied was certainly responsible for the discrepancy of obtained results.

The studies on the damaging effect of virus infection are of special importance in the case of cells infected with oncogenic viruses. While the majority of these cells undergo necrosis, those which survive develop

properties which, according to Warburg [24], are characteristic for neoplastic metabolism. As far as we are aware, studies on the metabolic changes in cells infected with oncogenic viruses, in particular with the polyoma virus, have not been reported.

In our experiments, during the developmental cycle and before the appearance of the maximum amount of virus in the medium, the effect of the virus on the cells steadily increased. The decrease in endogenous oxygen consumption and the increase in aerobic glycolysis observed already 24 hr. after infection, i.e. before the appearance of virus in the medium, might constitute evidence for alterations in the metabolism of the cells. The fluctuations in the decrease in oxygen consumption between the lst and 5th day of observation were probably due to changes in the proportions of the cells already damaged and in the stage of secondary infections, and of still intact cells. The curve of increasing aerobic glycolysis has an analogous shape to that of increasing virus infectious titer. On the critical 7th day after infection, when necrotic changes in the cells were first observed, the amount of the virus reached its maximum and it did not rise further either because of the exhaustion of cells necessary for the synthesis of virus, or as the result of development of defense mechanisms in the still undamaged cells.

The curve of increase of SE polyoma infectious titer in the medium resembles that obtained by Negroni with the Mill-Hill virus [17]. When this author infected cultures of mouse embryo cells with an excess of the virus, the cytopathogenic effect as well as the release of the largest numbers of virus infective particles occurred on the 5th day after infection, i.e. only two days earlier than in our experiments. These data indicate that both types of the polyoma virus develop at a slower rate than other animal viruses. Our experiments performed on the 9th day after infection pertain only to cells which survived the period of infection, constituting therefore selected material with a metabolism different from that of the original material. The surviving cells were more resistant, or able to develop defense mechanisms, possibly leading to transformation into neoplastic cells. These cells may correspond to the fibroblast-type cells described by Negroni [17], more resistant to the virus and capable of surviving the period of cytopathogenic changes manifested mainly in sensitive embryo cells of the epithelial type.

The Crabtree effect is characteristic for the metabolism of cells cultivated *in vitro* [8] as well as for aerobically glycolysing neoplastic cells. The increased Crabtree effect in cells infected with the polyoma virus is connected with the increased aerobic glycolysis. According to present views, the Crabtree effect is due to a lack of ADP and inorganic phosphate (P_i) which are essential for activation of the respiratory chain, and to exhaustion of the ATP pool available to hexokinase [2, 3]. In a previous study [7] the importance of phosphorylation of glucose for

the initiation of the Crabtree effect was demonstrated. It seems that the synthesis of virus in the cells not only directly exhausts the energetic reserves of the cell but also affects the equilibrium in the metabolism of phosphorus compounds. Nucleotides and P_i which are essential for electron transfer coupled with oxidative phosphorylation in the respiratory chain, are also required for the synthesis of new nucleic acids. Nucleotides incorporated into viral nucleic acid are lost to the cell. The uncontrolled and rapid synthesis of viral nucleic acids does not stimulate the reactions connected with energy production. Competition for P_i and nucleotides and energy for the synthesis of the nucleic acids disturbs the equilibrium in the cell and leads to its death. Further studies concerning these assumptions have been undertaken.

SUMMARY

Endogenous respiration and aerobic glycolysis in mouse embryo cell culture uninfected and infected with SE polyoma virus were estimated. The release of virus to the medium was preceded by a decrease of oxygen consumption and an increase of lactic acid production. The greatest decrease of oxygen uptake and an inhibition of glycolysis were found on the 6th and 7th day after infection, just before the cytopathogenic effect appeared. The surviving cells examined on the 9th day showed greater oxygen consumption and glycolysis than during the cytopathogenic process.

The Crabtree effect was greater in the infected cells than in uninfected ones; this may be due to a shunt of phosphorus compounds which activate the respiration, to the synthesis of viral nucleic acids.

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METABOLIZM EMBRIONALNYCH KOMÓREK MYSICH, HODOWANYCH *IN VITRO* I ZAKAŻONYCH ONKOTWÓRCZYM WIRUSEM SE POLYOMA

Streszczenie

W embrionalnych komórkach myszy, hodowanych *in vitro*, niezakażonych i zakażonych wirusem SE polyoma badano oddychanie endogenne i glikolizę tlenową. Uwalnianie wirusa do środowiska było poprzedzone zmniejszeniem zużycia tlenu i wzrostem produkcji kwasu mlekowego. Największe zahamowanie zużycia tlenu oraz ograniczenie glikolizy występowało w 6 i 7 dniu po zakażeniu, tuż przed ujawnieniem się zmian cytopatogennych. Komórki, które przetrwały okres zmian cytopatogennych, badane w 9 dniu, wykazywały wyższe wartości zużycia tlenu i zwiększenie glikolizy.

Komórki zakażone wykazywały większy efekt Crabtree niż komórki niezakażone. Fakt ten autorzy tłumaczą zużywaniem do syntezy kwasów nukleinowych wirusa związków fosforowych, niezbędnych dla aktywacji łańcucha oddechowego.

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INCORPORATION OF [^{32}P]ORTHOPHOSPHATE INTO PHOSPHOLIPIDS OF FROG TISSUES DURING FEEDING AND STARVATION

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The role of phospholipids as intermediate products formed during the absorption of fat still remains controversial. Experimental evidence has been put forward both for and against the assumption of phospholipids being involved in fat absorption [cf. 15]. However, most studies concerning the digestion and absorption of lipids have been performed on mammals and our knowledge of these processes in lower animals is by far insufficient. It seems that evidence gained in experiments on lower animals may contribute to elucidation of some questions of general importance.

In the experiments reported here the frog (*Rana esculenta*) has been used, as this animal has been found previously [4] to be able to absorb appreciable amounts of fat given in the diet. It has been shown also by histochemical methods that in frogs the absorption of fat is accompanied by formation of phospholipids in the intestine [4].

In the present study the effect of fat and protein feeding on the incorporation of ^{32}P-labelled orthophosphate into the phospholipids of various frog tissues has been investigated.

METHODS

The frogs had been caught in September and kept without food at 7° - 10°. The experiments were performed on animals of both sexes, from October to April. Before each experiment a certain number of animals, weighing about 40 g. each, were transferred to and kept at room temperature for 2 to 3 weeks. The fasted frogs were given orally or subcutaneously single doses of $Na_2H^{32}PO_4$. Each animal received 5 to 10 mg. orthophosphate containing from 50 to 80 μc ^{32}P, in 0.2 ml. aqueous solution.

Three types of experiment were performed: (1), frogs were given both [^{32}P]orthophosphate and 0.2 ml. olive oil orally; (2), the animals were given $Na_2H^{32}PO_4$ subcutaneously and 0.2 ml. olive oil orally; (3), the oral administration of $Na_2H^{32}PO_4$ was followed by 100 mg. egg white, given orally. In each experiment two equal groups of about 10 animals

were used. One group received only the radioactive orthophosphate solution and served as control, whereas the other group was treated as described above. The animals were killed at intervals of one to six days after the treatment. Each type of experiment was repeated several times.

The liver, stomach, small intestine and, in some cases, the muscles (*gastrocnemii*) and brain were quickly excised; blood was taken by heart puncture. The stomach and intestine were opened and thoroughly washed for 5 min. under a stream of tap water. The weighed tissues were cut into small pieces, ground with sand in a mortar, and the lipids were repeatedly extracted with 50 volumes of boiling chloroform - methanol (2:1, v/v). The combined lipid extracts were filtered and the solvent was evaporated under reduced pressure almost to dryness. To the resulting residue a few drops of water were added and the lipids were re-extracted with petroleum ether - chloroform (2:1, v/v). The extract was dried over anhydrous Na_2SO_4, filtered and made up to a known volume.

The radioactivity of the lipids was measured in suitable samples with the use of a thin mica end-window Geiger-Müller counter.

Part of the lipid extract was taken for phosphorus determination. This was carried out according to Fiske & Subbarow [6], after digestion with concentrated nitric and sulphuric acids. The amount of phospholipids was calculated by multiplying the phosphorus value by 25.

Radioactive orthophosphate was purchased from the Institute for Nuclear Research, Warszawa.

RESULTS

The weights of frog organs and the phospholipid contents in various tissues are recorded in Table 1. As is seen, the phospholipid contents in the muscles, stomach and intestine are more or less stable, varying widely only in the liver.

Table 1

Phospholipid content of various frog tissues

The mean values, ± S.E. are given; in parentheses the limit values. The amount of phospholipids was calculated by multiplying the phosphorus value by 25.

Tissue	No. of frogs analysed	Organ wt. (g.)	Phospholipid content (% of fresh tissue)
Liver	75	1.17 (0.45—2.56)	2.79±0.130
Stomach	75	0.76 (0.40—1.65)	0.90±0.019
Intestine	75	0.70 (0.30—1.64)	0.98±0.025
Muscles *(gastrocnemii)*	60	1.40 (0.60—2.10)	0.53±0.019

Table 2

The time course of ^{32}P incorporation and the distribution of ^{32}P in phospholipids of various tissues of control and olive oil-fed frogs

The nos. refer to successive frogs tested. Each animal received orally 0.2 ml. aqueous solution containing 10 mg. $Na_2H^{32}PO_4$ (13.5×10^5 counts/min./mg.). In frogs nos. 41, 43, 45, 47, 49 the administration of orthophosphate was followed by 0.2 ml. olive oil given orally. Other details in the text.

Tissue	Days after ^{32}P administration									
	1		2		3		4		6	
	No. 40, control	No. 41, oil-fed	No. 42, control	No. 43, oil-fed	No. 44, control	No. 45, oil-fed	No. 46, control	No. 47, oil-fed	No. 48, control	No. 49, oil-fed
	Total activity (counts/min.)									
Liver	395	3,160	15,200	20,000	12,250	28,800	19,100	83,500	17,800	65,500
Stomach	605	1,300	2,220	3,440	2,470	3,830	2,630	11,450	5,000	12,500
Small intestine	13,000	137,000	53,500	244,000	51,800	425,000	40,000	382,000	14,700	240,000
Muscles (2 *gastrocnemii*)	80	120	320	420	340	420	620	1,300	560	940
	Specific activity (counts/min./mg.P)									
Blood	950	7,770	3,930	38,000	8,500	140,000	7,100	250,000	7,000	135,000
Liver	250	1,820	5,500	7,000	6,450	20,600	8,300	39,000	7,500	34,600
Stomach	2,000	3,420	6,200	8,000	8,000	11,000	10,800	27,400	12,700	29,000
Small intestine	62,000	364,000	138,000	715,000	207,000	760,000	165,000	715,000	105,000	480,000
Muscles (2 *gastrocnemii*)	175	182	940	1,510	920	1,200	2,080	5,200	2,800	3,570
Brain	360	500	750	720	800	760	1,750	1,860	2,470	3,160

Olive oil feeding. Following the ^{32}P administration, labelled phospholipids appeared in all tissues examined both of the fasted and the oil-fed frogs. Table 2 presents the results of a typical experiment on frogs which received both olive oil and $Na_2H^{32}PO_4$ orally. Five experiments of this kind were performed and gave quite similar results. No differences were found between males and females. It can be seen from Table 2 that the incorporation proceeded in the frog rather slowly and did not reach its maximum until the third (in the intestine) or the fourth day (in blood and liver) after the test meal. In the stomach, muscles and brain, the radioactivity of phospholipids showed a continuous rise within the time limit of the experiment.

The amounts of ^{32}P incorporated into the phospholipids varied widely from one tissue to another. The highest radioactivity was revealed by the intestinal phospholipids. Considerable amounts of

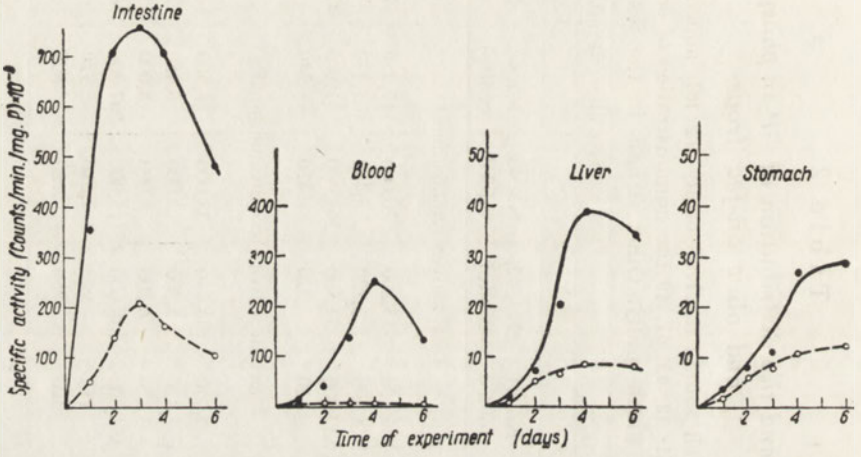

Fig. 1. Effect of olive oil feeding on the incorporation of ^{32}P into phospholipids of frog tissues. (- - -), Control; (——), olive oil-fed animals. Details as in Table 2.

radioactive phospholipids appeared in the liver, smaller but still significant amounts were found in the stomach and blood, whereas only minute labelling of the muscle and brain phospholipids occurred throughout the experiment.

The specific activity of the intestinal phospholipids exceeded many times that of all other tissues examined.

The effect of olive oil feeding on the incorporation of ^{32}P into phospholipids of various tissues is shown in Fig. 1. The most striking response was observed in the small intestine and in the blood. The specific activity of the intestinal phospholipids was several times higher, and that of the blood phospholipids even up to 20 times higher, in the fed animals than in the fasted ones.

A sharp increase in the specific activity of liver phospholipids was also found to follow the olive oil feeding (Fig. 1). The difference between the corresponding values for the control and fed animals increased with time and was highest on the fourth day after the test meal. But even

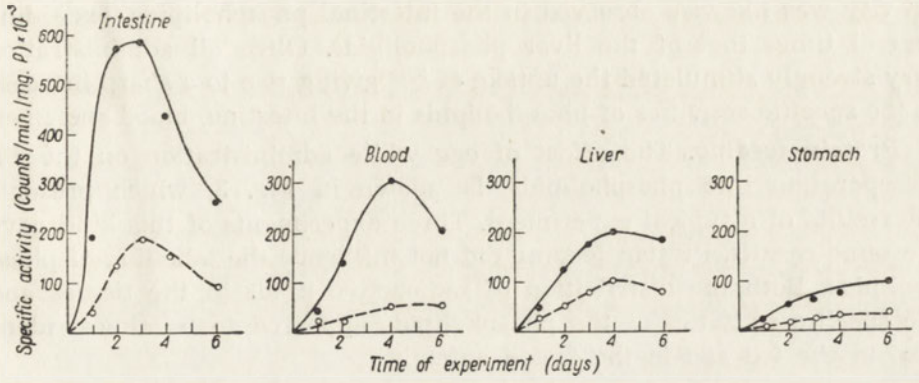

Fig. 2. Effect of olive oil feeding on the incorporation of ^{32}P into phospholipids of frog tissues. Each animal was given subcutaneously a single dose of 5 mg. $Na_2H^{32}PO_4$ (2.7×10^6 counts/min./mg.) in 0.2 ml. aqueous solution. (- - -), Control; (——), olive oil-fed animals (single doses of 0.2 ml.)

at the maximum of incorporation the specific activity of liver phospholipids was about 16 times lower than that of the small intestine and about 5 times lower than that of the blood phospholipids.

A smaller but still significant increase in the specific activity of the stomach phospholipids in response to olive oil administration was

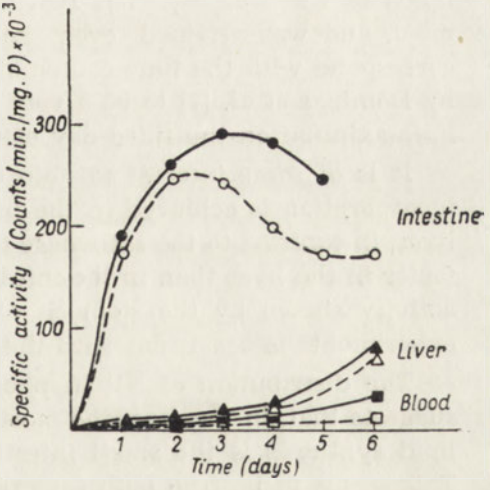

Fig. 3. Incorporation of ^{32}P into phospholipids of frog tissues, in fasted and protein-fed animals. Each animal was given orally 5 mg. $Na_2H^{32}PO_4$ (2.5×10^6 counts/min./mg.) in 0.2 ml. aqueous solution. (- - -), Control; (——), protein-fed animals (single doses of 100 mg. egg white)

revealed on the fourth and subsequent days of experiment. The labelling of muscle and brain phospholipids was not markedly influenced by fat feeding.

The second type of experiment was carried out on frogs which received radioactive orthophosphate subcutaneously and olive oil orally (Fig. 2). The distribution of ^{32}P showed generally the same pattern, and the rate of incorporation into various tissues followed a similar time course, as in frogs given the radioisotope orally. The highest specific activity was likewise observed in the intestinal phospholipids, exceeding several times that of the liver phospholipids. Olive oil administration very strongly stimulated the uptake of ^{32}P giving rise to a sharp increase in the specific activities of phospholipids in the intestine, blood and liver.

Protein feeding. The effect of egg white administration on the ^{32}P incorporation into phospholipids is shown in Fig. 3, which presents the results of a typical experiment. Three experiments of this kind gave the same results. Protein feeding did not influence the labelling of phospholipids. Both the distribution of radioactive lipids in the tissues and the specific activities of the phospholipids appeared to be almost identical in the fed and in the fasted animals.

DISCUSSION

The incorporation of ^{32}P into phospholipids of various frog tissues has been found to proceed at a very slow rate as compared with mammals so far investigated by other authors. In the rat, the highest incorporation (in liver and intestine) has been observed at about 10 to 12 hr. [13] or even at about 6 hr. [1] after administration of the radioisotope, and in the dog it was noted after about 18 hr. [5, 19]. In the present study, maximum incorporation of ^{32}P into the intestinal phospholipids of the frog was not reached until the third day after the test meal, and was attained even later in other tissues. This seems to correspond with the time course of fat absorption which has been shown by Dominas *et al.* [4] to be a very slow process in the frog and to attain its maximum on the third day after feeding.

It is of some interest to note that in the frog the maximum of ^{32}P incorporation is achieved in the intestine at an earlier time than in the liver, in contrast to the rat where the labelling has been found to proceed faster in the liver than in the intestine [13]. The continuous rise of radioactivity shown by the stomach phospholipids within the time limit of experiments is consistent with the observation of Fries *et al.* [7].

The distribution of ^{32}P in phospholipids of various tissues strongly suggests that, in the frog, the most active organ with regard to phospholipid synthesis is the small intestine, and to a lesser extent the liver. This seems to be true both for oral and subcutaneous administration of radioactive orthophosphate, thus presenting a marked contrast with the findings of other authors in experiments on mammals. With rats or dogs, numerous data point to the liver as the most active tissue with

respect to phospholipid metabolism [13, 7, 1, 8, 20]. The discrepancy between our results and those of other authors may be due to species differences and may be indicative of somewhat different routes of phospholipid formation. The intestinal wall seems to be the chief site of phospholipid synthesis in the frog, while in the rat or dog the intestine is less active than the liver in phospholipid formation.

As already mentioned, no unanimity has been so far achieved as to the participation of phospholipids in fat absorption. Since the early work of Sinclair [16, 17], a number of studies [cf. 15] provided evidence for increased phospholipid synthesis associated with fat absorption. By use of either ^{32}P or ^{14}C, appreciable increase of labelling of the intestinal and liver phospholipids during fat feeding has been repeatedly observed. In some cases even an increase of the lipid phosphorus value in the intestinal tissue has been found to accompany fat absorption. Thus, a statistically significant increase in phospholipid content has been observed by Artom [1] in the intestine of olive oil-fed rats. In the waxmoth, an insect which utilizes beeswax, rapid and strongly pronounced phospholipid synthesis has been found to occur during digestion and absorption of wax [18]. On the other hand, several studies suggest that phospholipids are not obligatory participants in fat absorption [19, 9]. This discrepancy may be, partly at least, explained by assumption that only a small part of the phospholipid fraction participates in fat absorption. This would be in line with the scheme of lipid synthesis as proposed by Kennedy [12], and supported by the results of Johnston & Bearden [11] and of Hübscher & Clark [10].

In the present study, no increase in lipid phosphorus values upon olive oil feeding could be observed in the intestine of the frog. However, using histochemical methods, Dominas *et al.* [4] noted the appearance of phospholipids in the mucosal epithelium of this animal during fat absorption. It seems most likely that the amount of newly formed phospholipids which are clearly visible histochemically is too small to be demonstrated by biochemical determinations on the whole intestinal tissue. Nevertheless, administration of [^{32}P]orthophosphate seems to offer evidence for increased turnover of phospholipids during absorption of olive oil by the frog. This may denote the involvement of at least some phospholipids in absorption of fat in frogs. The failure to find any effect of protein administration on the uptake of ^{32}P may indicate that the participation of phospholipids is confined to absorption of fat.

It seems that the increased formation of phospholipids in response to oil feeding is more pronounced in the frog than in the rat or dog. No explanation for it can be given as yet. On the basis of the present experiments, as well as of the previous ones on the waxmoth [18], one may suppose that the role played by intestinal phospholipids in fat absorption is more important in lower animals than in mammals.

However, this assumption is by far not proved and needs further evidence.

Another result of the present investigation which seems to be noteworthy is the very high specific activity of blood phospholipids observed after oil feeding. At the maximum absorption it exceeded several times the specific activity of liver phospholipids. This seems to indicate that the liver is not the only source of blood phospholipids, thus supporting the view shared by several authors [2, 3, 20, 14] that during fat ingestion the small intestine contributes appreciable amounts of phospholipids to the plasma.

The authors wish to express their thanks to Professor Dr. W. Niemierko for his continuous interest and helpful discussions. The skillful technical assistance of Miss B. Rudzisz is also gratefully acknowledged.

SUMMARY

1. The effect of olive oil and protein feeding on the incorporation of [^{32}P]orthophosphate into phospholipids of various frog tissues was studied.

2. Olive oil feeding strongly stimulates phospholipid synthesis in the blood, intestine and liver.

3. Protein feeding does not influence the incorporation of ^{32}P into phospholipids.

4. The conclusion is drawn that in the frog the phospholipids of the small intestine, liver and blood are involved in fat absorption. The most active synthesis of phospholipids is observed in the small intestine, which contributes to the blood some amounts of phospholipids newly formed during fat absorption.

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INKORPORACJA PROMIENIOTWÓRCZEGO FOSFORANU W FOSFOLIPIDY TKANEK ŻABY W CZASIE GŁODU I KARMIENIA

Streszczenie

1. Badano wpływ karmienia oliwą i białkiem na inkorporację [^{32}P]ortofosforanu w fosfolipidy różnych tkanek żaby (*Rana esculenta*).

2. Podanie oliwy znacznie wzmaga biosyntezę fosfolipidów, zwłaszcza w jelicie, wątrobie i we krwi.

3. Podanie białka jaja kurzego nie zwiększa inkorporacji ^{32}P w fosfolipidy tkanek.

4. Podczas wchłaniania oliwy aktywność właściwa fosfolipidów krwi jest kilkakrotnie wyższa od aktywności fosfolipidów wątroby, co nasuwa przypuszczenie, że pewna ilość nowopowstałych fosfolipidów przedostaje się ze ścianek jelitowych do krwi. Miejscem najintensywniejszej syntezy fosfolipidów u żaby jest tkanka jelitowa.

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PYRIMIDINE NUCLEOSIDE HYDROLASE IN *THERMOBACTERIUM ACIDOPHILUM* **

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Nucleosides may be split either by phosphorolysis giving rise to ribose-1-phosphate, or by hydrolysis with liberation of ribose. Phosphorolysis seems to be more common, at least in mammals where, in fact, hydrolysis has not been observed. In microorganisms both hydrolysis and phosphorolysis take place.

The nucleoside hydrolases active towards pyrimidine nucleosides have been found in yeast by Carter [1], in *Lactobacillus pentosus* by Lampen & Wang [4], in marine fishes by Tarr [11] and in *Lactobacillus delbrueckii* by Takagi & Horecker [10]. In the present paper evidence is given for the presence in *Thermobacterium acidophilum* R 26 Orla Jensen of a pyrimidine nucleoside hydrolase, which in several respects differs from the hydrolases previously described.

EXPERIMENTAL

Preparation of bacterial extracts. Th. acidophilum was grown in 250 ml. portions as described by Løvtrup & Roos [6]. After 22 hr. of incubation the cells were centrifuged and washed 3 times with distilled water. The yield was 300 - 400 mg. wet weight of bacteria per portion. After the last centrifugation the tube containing the bacterial pellet was placed in a freeze box. The bacteria may be stored in this state, and besides, freezing facilitates the removal of the bacteria from the tube.

Dry powders were prepared in two ways. (1) The bacteria were dried in a thin layer above P_2O_5 overnight in a vacuum desiccator in the cold room. Then they were ground with a glass rod and extracted with 0.01 M-phosphate buffer, pH 6.0, with 0.65% NaCl, or with water. The extraction medium was found to have no influence on the enzyme activity. (2) More active extracts were obtained from acetone-dried

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powders. Cold acetone (-15°) was added to a suspension of bacteria, and after 2 - 3 min. the precipitate was collected on a Buchner funnel, washed with cold acetone and dried in air. The powders were extracted with 1 ml. extraction medium per 100 mg. powder.

Determination of nucleoside hydrolase activity. The hydrolysis of cytidine, uridine, ribosylthymine was estimated by the concomitant changes in extinction. The activity of the enzyme towards 5-ribosyluracil was ascertained chromatographically. A test tube containing 20 µl. 0.16 M-nucleoside solution, 60 µl. 0.1 M appropriate buffer, and 20 µl. bacterial extracts, diluted with water when necessary, was incubated at 37°. At zero time and after various time intervals 7 µl. samples were withdrawn. In the case of cytidine each sample was added to a tube containing 2.5 ml. phosphate buffer, pH 7.2; when the cleavage of the other nucleosides was estimated 0.1 N-NaOH was used.

When in 1 ml. of solution at pH 7.2, one µmole cytosine is formed from cytidine a drop of extinction at 270 mµ occurs; $\Delta E_{270} = -3.1$. Similarly, when 1 µmole uridine is converted to uracil the extinction at 290 mµ in alkaline solution increases: $\Delta E_{290} = +5.37$. The corresponding change in ribosylthymine is $\Delta E_{300} = +3.6$.

Example of calculation: µmoles uracil formed $= \frac{\Delta E_{290} \times 35.7}{5.37}$

ΔE_{290} expresses the change in extinction at 290 mµ in alkaline solution; 35.7 is the dilution coefficient, and 5.37 is the change in extinction mentioned above, when uridine is hydrolysed. The reaction products were also checked chromatographically. For uracil-uridine and cytosine--cytidine separation butanol saturated with water or 0.2 N-HCl was used. This solvent was also used for separation of 5-ribosyluracil from uracil, whereas the upper phase of the mixture benzene - ethanol - water

Table 1

R_F *values for some pyrimidine derivatives*

Ascending chromatography on Whatman no. 1 paper in various solvents.

Compound	Butanol saturated with water	Butanol saturated with 0.2 N-HCl	Benzene-ethanol-water (169:45:15, by vol.)
Uracil	0.36	0.36	
Cytosine	0.23	0.09	
Thymine	—	—	0.13
Uridine	—	0.24	
Cytidine	0.11	0.03	
Ribosylthymine	—	—	0.02
5-Ribosyluracil	0.08	—	

(169:45:15, by vol.) was used for thymine - ribosylthymine separation (Table 1). When labelled uracil was used, the radioactivity was estimated with a windowless counter directly on cut-out spots localized by UV.

Substrates. Cytidine and uridine were obtained from Sigma (U.S.A.); [2-^{14}C]uracil (specific activity 5 μc/μmole) from California Corporation for Biochemical Research. Ribosylthymine and 5-ribosyluracil were kindly supplied by Professor David Shugar.

RESULTS

The chemical nature of the reaction. During the work upon the metabolism of pyrimidine derivatives in *Th. acidophilum* it was found that the bacterial extracts would split pyrimidine nucleosides at a high rate. Initial experiments with uridine showed that the rate of cleavage is the same in phosphate, tris, tris-arsenate, and acetate buffer. This fact suggested the reaction to be hydrolytic rather than phosphorolytic. However, it was possible that the extract contained a small amount of phosphate, sufficient for phosphorolysis to proceed. This possibility was excluded by the fact that after prolonged dialysis against distilled water (in the cold) the rate of reaction was unchanged for all substrates used.

Experiments with labelled uracil furnished additional evidence. Due to the reversibility of nucleoside phosphorolysis, an exchange may occur between free and bound pyrimidine base [9]. Incubation with a labelled base may thus give rise to formation of a labelled nucleoside. No such exchange was observed when 0.3 μc uracil was added to the normal reaction mixture. This indicates that the nucleosides are split by hydrolysis.

Table 2

The rate of cytidine and uridine hydrolysis by various extracts of Th. acidophilum

Rate of cleavage (μmoles/100 mg. dry wt./hr.)		Ratio of: cytidine / uridine
Cytidine	Uridine	
360*	31	11.6
338	40	8.4
200	16.6	12
787	66	11.8

* With this extract the rate of thymine liberation from ribosylthymine was 103.

Enzyme specificity. The enzyme did not act upon deoxyribosyl--nucleosides. Only C-N bonds can be split, as shown by the fact that no action was observed with 5-ribosyluracil. Cytidine, ribosylthymine, and uridine were all hydrolysed, the relative rates of reaction being approxi-

mately 11:3:1, respectively. The activity of some extracts towards cytidine and uridine is shown in Table 2.

The pH optimum. The optimum of the enzyme was close to pH 6 for both uridine and cytidine hydrolysis (Fig. 1). There were slight differences between the rate of activity decrease at both sides of the optimum for these two substrates. The activity towards cytidine decreased more rapidly on the acid side than on the alkaline side, while the activity decrease with uridine was steeper on the alkaline side. In spite of this it is probable that only one enzyme is involved; the slight differences in the pH optimum curves may only reflect differences between the substrates. The pH optimum for ribosylthymine was about 5.9 and the relative rates at pH 5.0 and 7.0 were 88 and 50, respectively.

Correlation between enzyme concentration and reaction rate. For determination of the enzyme activity, samples were taken at different time intervals. In the presence of excess substrate, and provided that the reaction products exert no inhibitory effects, the reaction rate should be constant. Plots of the extinction change against time would thus be straight lines. This expectation was satisfactorily met by uridine (Fig. 2) but not by cytidine (Fig. 3). The method employed does not allow for any substantial increases in cytidine concentration, because it is necessary for the sake of accuracy to work within a rather limited range of extinction. However, the slope of the straight lines indicated in the figures shows a linear relationship between reaction rate and enzyme concentration for uridine, and even for cytidine, when the reaction rate is not too high (Fig. 4).

Table 3

Thermostability of enzyme

After heating at various temperatures the water extracts of dried bacteria were immediately placed in an ice bath, and buffer and substrates were added.

Heating temperature	Time of heating (min.)	Loss of activity (%)	
		Cytidine	Uridine
50°	15	0	0
60°	30	0	0
70°	5	17	20
70°	10	33	30

Enzyme stability. The bacterial extracts did not show any loss of activity when stored at -20° for several months. Freezing and thawing had no influence upon the activity. For water extracts it was found that heat inactivation begins at 70° and the disappearance of the activity towards cytidine and uridine runs parallel, which again suggests that only one enzyme is involved (Table 3).

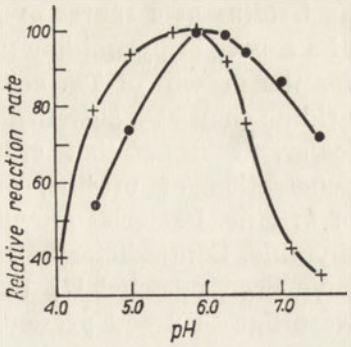

Fig. 1. Effect of pH on the relative reaction rate in citrate - phosphate buffer. (+), Uridine; (●), cytidine.

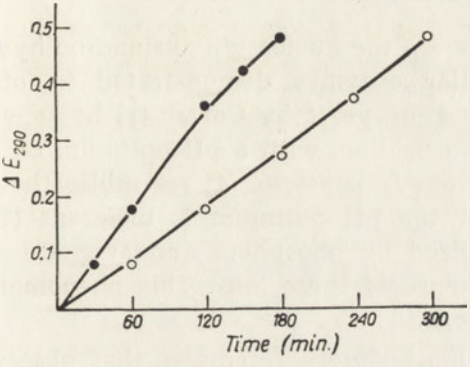

Fig. 2

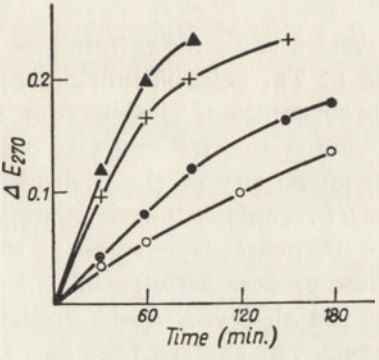

Fig. 3

Fig. 2. Rate of uridine hydrolysis versus reaction time. (○), 20 µl.; (●), 40 µl. enzyme solution.

Fig. 3. Rate of cytidine hydrolysis versus reaction time. (○), 1.6 µl.; (●), 3.3 µl.; (+), 6.6 µl.; (▲), 10 µl. enzyme solution.

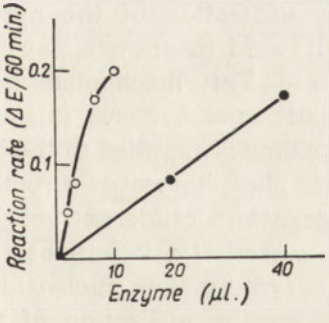

Fig. 4

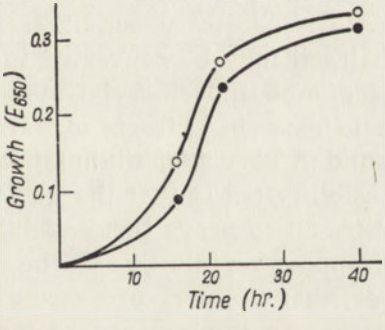

Fig. 5

Fig. 4. Relation between (○), cytidine, and (●), uridine hydrolysis and enzyme concentration.

Fig. 5. Growth of *Th. acidophilum* on (○), uracil or (●), uridine (concentration 0.36 µmole/ml.) as sole source of pyrimidine. Bacteria cultivated as described in ref. [6].

Uridine as a source of pyrimidine for bacterial growth. The addition of a source of pyrimidine (uracil or the precursor orotic acid) is necessary for the growth of *Th. acidophilum* [7]. As can be seen from Fig. 5 uridine may also sustain the growth, but the rate and extent is slightly lower. It seems that either the conversion of uridine to uracil, or penetration of uridine, or both, are limiting steps in the utilization of uridine. Bacterial growth was not supported by 5-ribosyluracil or by thymine. Contradictory observations with respect to the latter substance have been reported [7], but we have now been able to show that they were due to uracil present as an impurity in the thymine preparation.

DISCUSSION

There are some differences between the nucleoside pyrimidine hydrolase in *Th. acidophilum* and similar enzymes demonstrated in other microorganisms. The enzyme found in yeast by Carter [1] has a very narrow specificity and acts only on uridine, with a pH optimum of 7.0. The specificity of the hydrolase from *L. pentosus* [4] resembles that of the *Th. acidophilum* enzyme, but the pH optimum is different (7.5). The *L. pentosus* enzyme is stabilized by phosphate and arsenate and shows greater activity in the presence of these ions. This phenomenon was not observed in our preparations.

The *Th. acidophilum* enzyme most closely resembles that observed by Takagi & Horecker [10]. However, there is a certain difference in pH optimum with uridine as substrate, and thymine riboside is more readily split by *Th. acidophilum* extracts.

As far as the metabolism of RNA is concerned, it is difficult to suggest a function for the enzyme if uracil is supplied in the medium. With uridine as the pyrimidine source, matters are different. Nucleotide formation is a necessary condition for the utilization of the pyrimidine bases. Uracil may be converted to uridylic acid in one step by reaction involving 5-phosphoribosyl-1-pyrophosphate. This mechanism has been shown to exist in extracts of various lactic acid bacteria [2], and was also found in *Th. acidophilum* [3]. When uridine is supplied to the bacteria the possibility exists that the nucleoside is phosphorylated directly. It is very difficult to prove this possibility, because in crude extracts uridine will disappear rapidly due to the hydrolase activity. It will be realized, however, that the very presence of this enzyme makes nucleoside phosphorylation an unnecessary requirement for the utilization of uridine.

Another function of the enzyme may be envisaged. Cytosine which is present in both RNA and DNA is not required in the basal medium, and can be formed by the bacteria. We may presume that in *Th. acidophilum* as in other organisms CTP is formed from UTP by amination [5]. Two mechanisms are possible for the formation of the corresponding

deoxyribose derivatives. The first one is that the reaction deUTP⟶ deCTP may occur. The second possibility is that cytosine is liberated from CTP formed as mentioned above, in reactions in which the hydrolase may take part, and then may be converted in turn to deoxycytidine by the action of nucleoside deoxyribosyltransferase [8].

SUMMARY

The presence of a pyrimidine nucleoside hydrolase has been demonstrated in *Th. acidophilum*. Some properties of the enzyme were studied, and it was found that in certain respects it differs from similar enzymes demonstrated in other microorganisms.

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HYDROLAZA NUKLEOZYDÓW PIRYMIDYNOWYCH W *THERMOBACTERIUM ACIDOPHILUM*

Streszczenie

Stwierdzono obecność hydrolazy nukleozydów pirymidynowych w *Th. acidophilum* i zbadano niektóre właściwości tego enzymu. Różni się on niektórymi właściwościami od podobnych enzymów występujących w innych mikroorganizmach.

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FORMATION OF URACIL NUCLEOTIDES IN *THERMOBACTERIUM ACIDOPHILUM* **

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Two pathways are known by which uracil is converted to UMP. This may occur either in two steps: uracil ——→ uridine ——→ UMP, the first step being catalysed by uridine phosphorylase and the second one by uridine kinase; *or* UMP may arise by a one step reaction catalysed by UMP pyrophosphorylase with 5-phosphoribosyl-1-pyrophosphate (PRPP) as a donor of the phosphoribosyl moiety [18, 5]. Since extracts of *Thermobacterium acidophilum* R 26 Orla Jensen possess a strong pyrimidine nucleoside hydrolase activity whereas pyrimidine nucleoside phosphorylase activity is absent [10], it appears that uracil supplied to the medium for normal bacterial growth must be converted to UMP by the second reaction. The mononucleotides for various metabolic purposes must be further converted to di- or triphosphonucleotides by various kinase systems which show high specificity as regards the base of the nucleotide, the phosphate donor, and the manner of action.

In this work some features of the uracil metabolism in *Th. acidophilum* were investigated, viz. the pathway of conversion of uracil to UMP, UDP and UTP, and some of the properties of the enzymes involved. Attempts were also made to demonstrate polynucleotide phosphorylase activity in the bacterial extracts.

EXPERIMENTAL

Materials. [2-^{14}C]Uracil and [^{32}P]orthophosphate were purchased from The Radiochemical Centre, Amersham, Buckinghamshire, England. PRPP, ADP, UDP, UTP, phospho(enol)pyruvate and pyruvate kinase type II were obtained from Sigma Chemical Company, U.S.A. Bacterial acetone dry powders and water extracts were prepared as described previously [10].

Estimation of UMP pyrophosphorylase. The composition of the reaction mixture was similar to that used by Crawford, Kornberg & Simms

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[5]. Test tubes containing 5 µl. 0.02 M-[2-^{14}C]uracil (specific activity 4 mc/m-mole), 25 µl. 0.006 M-PRPP (Mg salt), 30 µl. 0.01 M-phosphate buffer pH 7.5, and 10 µl. bacterial extract, equivalent to 0.8 - 1.5 mg. bacterial dry powder, were incubated at 37°. At zero time, and after various periods of incubation, 10 µl. of the reaction mixture was withdrawn and placed on Whatman no. 1 paper. To stop the reaction the micropipette with the withdrawn sample was placed in a tube immersed in ice. The reaction occurring during drying on the chromatographic paper was negligible as judged from the samples taken at zero time. The chromatograms were developed in butanol saturated with 0.2 N-HCl, the spots localized with UV were cut out and the radioactivity estimated directly on the paper. In the experiments where greater amounts of PRPP were to be used, PRPP was dissolved in the phosphate buffer in order to avoid changing the final volume of the reaction mixture. The use of phosphate buffer has the advantage of overcoming the PRPP decomposition catalysed by Mg^{2+} [12]. UMP pyrophosphorylase is activated by the Mg^{2+} ion, which is supplied together with PRPP. Simultaneously with the increase of PRPP, the magnesium ion concentration was also increased in the reaction mixture. This is of no consequence because it was found that supplementary addition of magnesium ions up to the concentration of 7×10^{-3} M, which is the one reached with the highest concentration of PRPP used, does not change the reaction rate. The degree of conversion of uracil to UMP was calculated as percentage of radioactivity present in the UMP spot.

Assay of UMP and UDP kinases. Further phosphorylation of UMP was achieved in the following way. Labelled UMP was prepared in the manner described above, with the exception that the ratio uracil:PRPP was 1:2.5, and incubation 1 hr. During this time uracil was quantitatively converted to UMP. To 40 µl. of the mixture containing 0.057 µmoles UMP, 5 µl. ATP solution containing amounts ranging from 0.05 to 2 mg. was added. In some experiments 5 µl. 0.1 M-phosphopyruvate and 1 µl. 10-fold diluted pyruvate kinase were added. The tubes were incubated at 37° and after various periods of time 10 µl. samples were withdrawn and placed on Whatman no. 1 paper. After development by the descending method in the solvent system: isobutyric acid - 1 M-ammonium hydroxide - 0.1 M-sodium versenate (100:60:1.6, by vol.) [13] the spots were cut out and the radioactivity estimated. The degree of conversion was calculated as percentage of radioactivity present in the UDP and UTP spots.

Assay of polynucleotide phosphorylase. The presence of polynucleotide phosphorylase activity was checked in two ways: from the ability to form polynucleotide and from the exchange reaction with ^{32}P [7]. For polynucleotide formation the composition of the reaction mixture was as follows: 1 - 2 mg. UDP or ADP, 3 µl. 0.1 M-$MgCl_2$, 10 µl. 0.2 M-tris buffer,

pH 8.2, and bacterial extract equivalent to 2 - 4 mg. dry weight of bacteria, in a final volume of 30 µl. The mixture was incubated at 32°. At zero time and after different times up to 18 hr. a small sample was taken and checked for polynucleotide formation on Whatman no. 1 paper with 1 M-ammonium acetate - ethanol (3:2, v/v) as a solvent. For the exchange reaction 10 µl. 0.015 M-ADP, 5 µl. 0.1 M-phosphate buffer, pH 7.5, containing [^{32}P]orthophosphate with an activity of about 60,000 counts/min., 5 µl. 0.1 M-$MgCl_2$, 40 µl. 0.2 M-tris buffer, pH 8.2, and bacterial extract (5 - 10 mg. dry wt. of bacteria) with or without 1 µl. of 0.1 M-versene in a final volume of 100 µl., was incubated at 32° or 39°. After various incubation periods up to 2 hr., samples were taken and labelled phosphate was separated from ATP and ADP on Whatman no. 1 paper with 1% ammonium sulphate - isopropanol (1:2, v/v). The paper was soaked in advance with 1% ammonium sulphate; the ascending technique was employed. The appearance of ATP on the chromatograms indicated the presence of adenylate kinase activity.

RESULTS

Conversion of uracil to UMP. Bacterial extracts possess UMP pyrophosphorylase activity as UMP formation was observed from uracil and PRPP in the presence of magnesium ion. PRPP could not be replaced by ribose-5-phosphate and ATP. The rate of reaction decreased with time. However, with excess PRPP and sufficient amounts of extract it was possible to obtain almost 100% conversion of uracil to UMP. The decline in the reaction rate could be the result of decomposition of PRPP or of enzyme during incubation, of decline in the substrate concentration, or of an inhibitory effect of the UMP formed. These possibilities were tested as follows: PRPP was preincubated under the normal standard conditions without uracil for 10, 20 and 40 min., and after this time uracil was added to initiate the reaction. The rate was compared with experiments without PRPP preincubation. As can be seen from Table 1, there was no essential difference between the results in the two series of experiments. We can thus conclude that decomposition of PRPP or enzyme was not responsible for the decline in the reaction rate.

With the method used, it was difficult to show the influence of changes in uracil concentration, whereas we were more free to change the PRPP concentration. The reaction required an excess of PRPP, and the rate increased with increasing PRPP concentration (Table 2). In the Table are also given the results of some experiments, in which after 40 min. of incubation further quantities of PRPP were added. The reaction rate was increased in all cases, while in the controls the addition of the same quantity of buffer did not induce any change. It is obvious that at the concentration used the enzyme was not saturated

Table 1

Influence of preincubation of bacterial extract with 5-phosphoribosyl-1-pyrophosphate on UMP formation

Two different extracts were used in these experiments.

Preincubation (min.)		Uracil converted to UMP (%) Incubation (min.)				
		10	20	30	40	50
Expt. I	0	15.0	27.1	34.2	40.4	
	10	18.0	30.1	36.7	40.7	
	20	18.5	26.8	33.7	39.0	
Expt. II	0	12.2	15.3	19.4	21.5	25.5
	0	8.0	14.1	20.1	21.3	25.4
	40	8.7	13.3	19.1	21.2	25.1
	40	7.8	14.6	17.7	21.8	23.5

with respect to PRPP, but the PRPP addition could also act by changing the equilibrium of the reaction.

We tested the influence of several nucleotides on the reaction. Only UMP had some inhibitory effect, and this was exerted also in the concentration range corresponding to that arising during the reaction (Fig. 1). The inhibitory effect was quite strong, as the highest UMP concentration was only twice the quantity of uracil present at the beginning of the experiment. Cytidylic acid and adenylic acid (2.8×10^{-3} M), corresponding to the highest UMP concentration in Fig. 1 had no influence upon the reaction rate. In the experiments where UMP

Table 2

Influence of 5-phosphoribosyl-1-pyrophosphate concentration on UMP formation

PRPP added (μmoles)	Uracil converted to UMP (%) Incubation (min.)					
	10	20	30	40	50	50*
0.17	18.4	30.4	42.5	49.2	—	—
0.25	25.1	45.0	56.9	68.8	78.0	90.0
0.34	27.1	46.8	61.1	76.5	83.3	95.0
0.25	19.8	30.4	43.8	54.1	58.1	68.3
0.34	19.9	33.4	49.4	61.5	70.1	75.0
0.28	17.5	32.3	46.8	50.9	59.0	66.0
0.34	18.7	33.4	46.9	57.7	68.0	74.0
0.34**	17.3	31.7	44.2	50.2	61.0	67.0
0.67**	17.2	36.3	49.5	60.8	69.7	74.7

* After 40 minutes 10 μl. 5.6 m M-PRPP solution was added to 10 μl. of the reaction mixture.

** Because of the poor solubility of PRPP the final volume was 90 μl. in these experiments.

was added, the same amounts of UMP were added to the control spots on the chromatographic paper.

Contrary to the observation reported by Crawford *et al.* [5], the addition of glutathione had no influence on the reaction. This discrepancy is probably due to the fact that these authors used partially purified enzyme. The reaction occurred also in tris buffer but at a somewhat lower rate. The addition of inorganic pyrophosphate in quantities equivalent to the concentration of uracil did not change the rate of the reaction. NaF in a final concentration of 0.1 M lowered the reaction rate by about 35%.

Formation of UDP and UTP. When ATP was added to the reaction mixture the transfer of a phosphate group from ATP to [^{14}C]UMP occurred and labelled UDP was formed. Sometimes a slight activity was observed on the chromatograms in the region of UTP. When tested

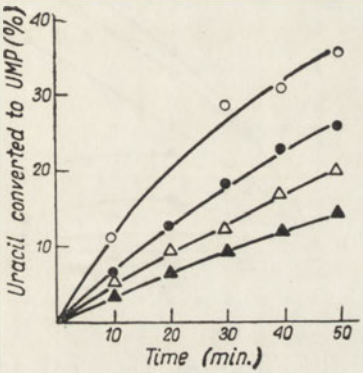

Fig. 1

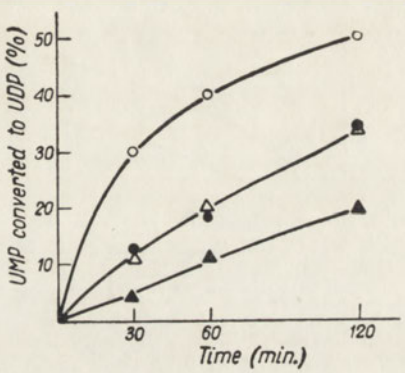

Fig. 2

Fig. 1. Influence of added UMP on the reaction rate of UMP formation from uracil. (○), Control without added UMP; (●), with 0.05 μmole; (△), 0.1 μmole; (▲), 0.2 μmole UMP.

Fig. 2. Influence of UDP and UTP on UDP formation from UMP. (○), Control; (●), with 0.02 μmole UDP; (△), 0.02 μmole UTP; (▲), 0.04 μmole UTP added.

with UDP the extracts did not show any uridylate kinase activity according to equation: 2 UDP → UTP + UMP. Likewise, the reverse reaction did not occur as no UDP formation could be observed when extracts were incubated with UMP and UTP. UDP was not changed during incubation, but UTP was dephosphorylated by the bacterial extracts. The possibility therefore exists that UDP formation occurs *via* UTP. The results of Bianchi, Butler, Crathorn & Shooter [2] suggest that the phosphorylation of TMP to TTP in supernatants of leucaemic spleen and lymph nodes from mice occurs by a one step reaction without TDP being formed as an intermediate, the appearance of TDP thus being the result of dephosphorylation. To test whether phosphorylation of UMP occurs in a similar manner, UTP was added to the

reaction tube in order to reduce the degradation of any radioactive UTP formed. Although after incubation UTP spots were always seen on the chromatograms, we never observed any radioactivity in these spots. Both UDP and UTP were found to exert a considerable inhibitory action on UDP formation (Fig. 2).

The dependence of the reaction rate upon the amount of ATP present in the mixture is shown in Fig. 3. Because of the presence of adenylate kinase in the extracts, the values do not indicate the actual amount of ATP in the reaction tubes. However, it is seen from the Figure that the rate of the reaction did not increase when the addition of ATP was increased above 0.2 mg. (0.3 μmoles). When the incubation time was

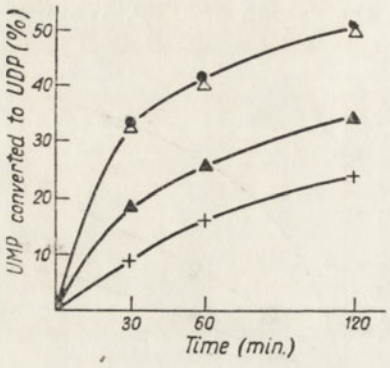

Fig. 3.

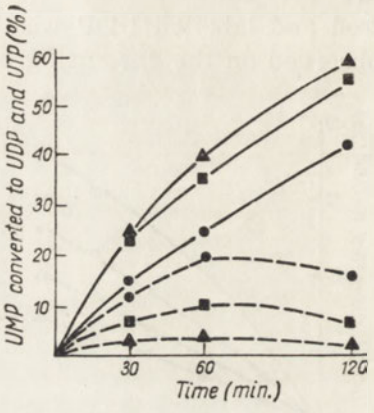

Fig. 4

Fig. 3. Influence of ATP on the conversion of UMP to UDP. (●), 1 mg.; (△), 0.2 mg.; (▲), 0.1 mg.; (+), 0.05 mg. ATP added per reaction tube.

Fig. 4. Relation between conversion of UMP to UDP and UTP and quantity of pyruvate kinase added. (▲), Undiluted pyruvate kinase; (■), dilution 1 : 10; (●), dilution 1 : 100. Broken line represents UDP formation, full line UTP formation.

prolonged, or supplementary amounts of extract added to the reaction mixture, the reaction might proceed as far as 70 - 80% conversion of UMP to UDP.

When phosphopyruvate and pyruvate kinase were added to the reaction mixture the conversion ADP → ATP occurred, but at the same time the reaction UDP → UTP took place (Fig. 4). The addition of phosphopyruvate alone could not entail a shift from UDP to UTP formation. Thus, no pyruvate kinase activity was observed in the bacterial extracts, even after replacing the sodium in the buffer with potassium, which is an activator of the enzyme [11]. A slight radioactivity which was sometimes observed in the UTP region without addition of phosphopyruvate and pyruvate kinase was probably due to the presence in the bacterial extracts of UDP kinase. This enzyme must be very labile because the extract lost this activity after storage at -15°. We never

were able to observe radioactivity in the region corresponding to di- or oligonucleotides, even when the reaction was carried out in tris buffer.

Polynucleotide phosphorylase. A considerable effort was made to demonstrate the presence of this enzyme. It was even attempted to fractionate the bacterial extracts with ammonium sulphate, but we were unable to observe any indication of the presence of polynucleotide phosphorylase.

DISCUSSION

The main route for uridine-5′-phosphate formation from uracil in *Th. acidophilum* seems to be as follows:

$$\text{Uracil} + \text{PRPP} \rightleftharpoons \text{UMP} + \text{pyrophosphate}$$

This reaction has been found before in some lactic acid bacteria by Crawford *et al.* [5], and in *Lactobacillus bulgaricus* by Canellakis [4]. The reaction is catalysed by UMP pyrophosphorylase (UMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.9), an enzyme analogous to orotidine-5′-phosphate pyrophosphorylase [14]. Both of these enzymes may be found in certain strains of lactic acid bacteria, in which case uracil as well as orotic acid may sustain growth. This seems to hold also for the investigated bacterial strain [16]. UDP may be formed from UMP by a transfer of a phosphate residue from a high energy phosphate compound like UTP or ATP, according to the equations:

$$\text{UMP} + \text{UTP} \rightleftharpoons 2\ \text{UDP} \quad (1)$$

$$\text{UMP} + \text{ATP} \rightleftharpoons \text{UDP} + \text{ADP} \quad (2)$$

The first reaction is similar in its action to adenylate kinase (myokinase); it was found in brewers yeast by Lieberman, Kornberg & Simms [15]. The second one has been demonstrated in yeast, rat liver, calf liver, and in *Ascaris* (17, 9, 8, 6). In the bacterial extracts examined by us only the second reaction took place. In freshly prepared extracts we were sometimes able to observe formation of UTP; this probably arose from phosphorylation of UDP by ATP, catalysed by the enzyme nucleoside-diphosphate kinase [1, 3]. However, this enzyme seems to be very labile. Both UMP pyrophosphorylase (Fig. 1) and nucleosidemonophosphate kinase (Fig. 2) were inhibited by the compounds formed by their action. With respect to the former enzyme this inhibitory effect may possibly be explained as a change in the position of the equilibrium, but more likely the effect arises by competitive inhibition. The equal inhibitory effect exhibited by UDP and UTP in the reaction catalysed by the second enzyme indicates that this effect results from a competition. The failure to demonstrate the presence of polynucleotide phosphorylase activity in the extracts, in spite of the wide distribution of this enzyme in microorganisms, seems to indicate a different metabolic pathway for RNA synthesis in the investigated strain.

It may finally be mentioned that the extracts afford an excellent means for obtaining labelled uracil compounds at various levels of phosphorylation.

SUMMARY

Extracts from *Th. acidophilum* exhibit activity of UMP pyrophosphorylase, the enzyme catalysing the formation of UMP from uracil and 5-phosphoribosyl-1-pyrophosphate. They also possess the ability to form UDP when ATP is added to the incubation mixture. Addition of pyruvate kinase and phosphopyruvate leads to UTP formation. Thus, depending on the conditions used, it is possible, starting from labelled uracil to obtain labelled UMP, UDP or UTP. Some of the properties of these reactions were studied.

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TWORZENIE NUKLEOTYDÓW URACYLOWYCH W *THERMOBACTERIUM ACIDOPHILUM*

Streszczenie

Ekstrakty z *Th. acidophilum* wykazują aktywność UMP pyrofosforylazy, enzymu katalizującego tworzenie UMP z uracylu i 5-fosforybozylo-1-pyrofosforanu. Ekstrakty te posiadają także zdolność tworzenia UDP, gdy do mieszaniny inkubacyjnej dodane jest ATP. Dalsze dodanie fosfoenolpirogronianu i kinazy pirogronianu prowadzi do powstania UTP. Tak więc używając znakowanego uracylu można, zależnie od warunków inkubacji, otrzymać znakowany UMP, UDP lub UTP. Zbadano niektóre właściwości tych reakcji.

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ADAPTATION OF YEAST TO 3-AMINO-1,2,4-TRIAZOLE*

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The cell populations exposed to the action of growth inhibitors are often able to become resistant to these agents. In such cases two major mechanisms may operate: selection of preexisting mutants or induction of resistance resulting from enzymic adjustment in response to the action of inhibitor. It is now well established that the rates of enzyme induction and repression are controlled by specific genes [8]. Therefore, cell adaptation to inhibitors constitutes another approach in the study of the relations between genome and enzyme formation.

3-Amino-1,2,4-triazole (3-AT) has been shown by Weyter & Broquist [14] and Hilton [7] to inhibit the growth of some yeasts and bacteria. In yeasts its effects can be abolished completely only by histidine [7, 14, 9]. In the presence of 3-AT the yeast accumulates imidazoleglycerolphosphate [9] which is an intermediate in histidine biosynthesis [2]. All this evidence indicates that 3-AT affects the yeast by inhibiting the biosynthesis of histidine.

In the present report the induction of resistance to 3-AT in *Saccharomyces cerevisiae* has been described. The induced resistance was not absolute; it was, however, maintained during 25 generations which suggests that it may be controlled by a reproducible factor.

METHODS

Organisms. Both yeast strains used in the present work, Yeast Foam (YF) and LM 2, were kindly supplied by Dr. M. Luzzati from Laboratoire de Génétique Physiologique in Gif-sur-Yvette, France. The strain YF is a wild *S. cerevisiae* diploid. The strain LM 2, also diploid, is a prototrophic revertant arisen by gene conversion from the strain LM 27 which is a heteroallelic $ad_{3/3}/ad_{3/6}$ mutant, requiring in consequence both histidine and a purine for growth [13, 3]. LM 2 apart from $ad_{3/3}$, is also recessive for the genes ad_2, ad_6, ur_1, hi_1, me_1 and tr_1.

The nature of the gene ad_3 is not known. Cell-free extracts of ad_3 mutants are able to synthesize earlier histidine precursors [10]. The

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mutants ad_3, unlike all the studied hi^+ strains, are incapable of accumulating imidazoleglycerolphosphate in the presence of 3-AT [9]. The second enzymic block resulting from the gene ad_3 mutation is located in AMP biosyntesis before 5-aminoimidazole ribotide [13].

The other mutated genes present in LM 2 control the respective enzymes of adenine, uracil, histidine, methionine and tryptophan biosynthetic pathways. When uncomplemented, they cause inactivity of the corresponding enzymes and respective nutritional requirements. The cells of ad_2 mutant are stained red by a product formed from accumulated 5-aminoimidazole ribotide [13]. Hence, the gene ad_2 seems to control the 5-aminoimidazole ribotide carboxylase. The cell-free extracts of hi_1 mutants do not exhibit any activity of phosphoribosyl-ATP pyrophosphorylase (Kłopotowski, unpublished experiments) which is the first enzyme of histidine biosynthesis [2].

Media. The minimum medium of Galzy & Slonimski [5] was used; it contained per liter: 6 g. of $NH_4H_2PO_4$, 0.5 g. of $MgSO_4 \cdot 7H_2O$, 2 g. of $(NH_4)_2SO_4$, 1 g. of KH_2PO_4, 0.1 g. of NaCl, 0.1 g. of $CaCl_2$ (anhydrous salt), 0.5 mg. of H_3BO_3, 0.04 mg. of $CuSO_4 \cdot 5H_2O$, 0.1 mg. of KI, 0.4 mg. of $MnSO_4 \cdot 4H_2O$, 0.2 mg. of $Na_2MoO_4 \cdot 2H_2O$, 0.4 mg. of $ZnSO_4 \cdot 6H_2O$, 0.2 mg. of $FeCl_3 \cdot 6H_2O$, 22 mg. of inositol, 2 mg. of calcium pantothenate, 2 mg. of thiamine hydrochloride, 2 mg. of pyridoxine, 0.5 mg. of nicotinic acid, 0.02 mg. of biotine and 20 g. of glucose.

The corresponding solid medium was prepared by adding 25 g. of agar per liter.

The solid peptone medium contained per liter: 10 g. of peptone, 10 g. of yeast extract (E. Gurr Ltd., London), 20 mg. of adenine free base, 20 g. of glucose and 25 g. of agar.

Cultures. The strains were maintained by trimonthly subcultures on solid peptone medium. For inocula, the yeast was allowed to grow aerobically in minimum medium at 27°. After 20 hr. the actively growing cells were harvested, washed twice with isotonic saline and sterilly centrifuged at room temperature.

Growth tests. To sterilized Evelyn tubes was added 9 ml. of sterile minimum medium alone, or with the addition of 3-AT or histidine. At zero time 1 ml. samples of yeast suspensions (freshly diluted with the medium to 20 µg. dry wt. of cells per ml.) were added to each tube. The tubes stoppered with sterile cottons were aerated at 27° by horizontal shaking at a rate of 200 cycles per minute. All growth tests were made in duplicate.

When the cultures reached about 70 µg. dry wt. of cells per ml. readings have been started, usually at intervals of 2 hr. The turbidity was read in Evelyn photometer at 660 mµ or, when indicated, in Beckman DU spectrophotometer at 420 mµ. Dry weight of cells was found from calibration curves.

Chemical methods. 3-AT was estimated by the method of Green & Feinstein [6]; the extinction was measured in Evelyn photometer at 515 mμ. The calibration curve was prepared from standard 3-AT solutions in medium.

Chemicals. All chemicals used were commercial products: 3-amino--1,2,4-triazole (Serlabo, Paris), biotine (Hoffmann, Switzerland), other vitamins (Nutritional Biochemicals Corp., Cleveland) and histidine hydrochloride monohydrate (Chemapol, Praha).

RESULTS

Yeast growth inhibition by 3-AT

The comparison of 3-AT effect on growth of the two yeast strains revealed a difference between their responses. As shown in Fig. 1A, in the wild strain YF there appeared a marked lag phase. Its duration depended on 3-AT concentration: at 0.1 mM it lasted for about 10 hr.

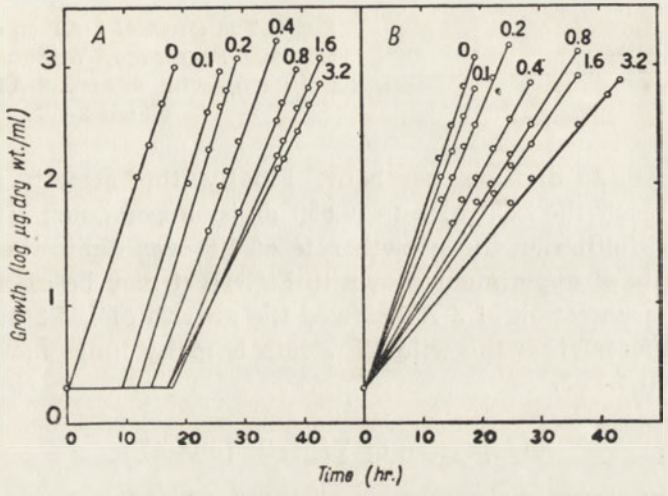

Fig. 1. Growth inhibition by 3-AT of the two *S. cerevisiae* strains, Yeast Foam (*A*) and LM 2 (*B*). The numbers represent mM concentrations of 3-AT.

and increased to about 16 hr. at 3-AT concentrations exceeding 1 mM. In cultures containing lower 3-AT concentrations the growth following the lag phase proceeded exponentially at rates virtually equal to the rate of controls. At higher concentrations, the growth rates were sensibly reduced.

The other *S. cerevisiae* strain, LM 2, grew without any lag phase in the presence of 3-AT. Its only response was the reduction in growth rate, whose extent depended on the inhibitor concentration (Fig. 1B).

To study the complex kinetics of the inhibition in YF, a special series of cultures was prepared. For the more precise observation of the early

stages of 3-AT action, inocula tenfold greater than formerly were used, and cell mass increment was followed in Beckman spectrophotometer at 420 mμ. The growth of the 3-AT containing cultures appeared to be triphasic (Fig. 2). For example, at 0.1 mM 3-AT concentration the growth proceeded initially, more or less for the time of one division, at the same rate as in controls. Then it became slower reflecting probably the increase in inhibitor content in the cells, and proceeded for about 4 hr.

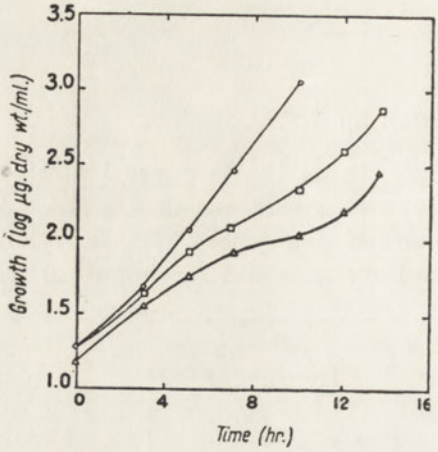

Fig. 2. The effect of 3-AT on early growth of Yeast Foam. (○), Without 3-AT (control); (□), with 0.05 mM-3-AT; (△), with 0.1 mM-3-AT.

at a rate of 0.125 division per hour. Finally the growth accelerated tending to reach the control rate (0.650 division per hour). Thus during the phase of inhibition the growth rate of YF was diminished by 80%. From the data of experiment shown in Fig. 1B it may be calculated that the same concentration of 3-AT slowed the growth of LM 2 only by 9%. It appears thereof that the wild YF strain is many times more sensitive to 3-AT than LM 2.

Adaptation of YF cells to 3-AT

The spontaneous disappearance of the 3-AT-induced growth inhibition might result either from decomposition of inhibitor or from decrease of cell sensitivity. The determinations of 3-AT content in culture media did not demonstrate any reduction of its concentration during the growth of YF cells both when it was partially inhibited and when it proceeded freely owing to presence of histidine (Table 1).

To test the alternative possibility, the cells grown in experiment shown in Fig. 1A at six 3-AT concentrations ranging from 0.1 to 3.2 mM were sterilly harvested, washed with isotonic saline and used for inoculation of minimum media. After eight generations they were harvested and washed. Then the cells from each of the six subcultures were used to inoculate separate series of media; in each the concentrations of 3-AT ranged from 0 to 3.2 mM. This experiment, shown in Fig. 3, revealed that

Table 1

Stability of 3-AT concentration during yeast growth

The minimum media were inoculated at zero time with YF cells to the density of 10 μg. dry wt. per ml. After the indicated period of growth, the cells were centrifuged and 3-AT determined in supernatants by the method of Green & Feinstein [6].

Minimum medium with 1 mM-3-AT Addition	Culture duration (hr.)	Growth (μg. dry wt./ml.)	3-AT found (mM)
None	0	0	1.0
	27	470	1.0
	27	420	1.02
L-Histidine (0.25 mM)	0	0	1.0
	17	2250	1.1
	17	2050	1.06

yeast cells grown previously in the presence of 3-AT were really resistant to the inhibitor. They were able to grow in its presence virtually without any lag phase. Some short lags (1 - 4 hr.) occurred only when media with highest 3-AT concentrations applied, were inoculated with cells derived from cultures with lowest inhibitor concentrations. However, the resistant population showed repeatedly a residual sensitivity to 3-AT, the growth rate decrease being the less the higher was the inhibitor concentration formerly used for resistance induction: when tested in medium containing 3.2 mM-3-AT the growth rate of 0.1 mM-3-AT-resistant population was inhibited by 30% and that of 3.2 mM-3-AT-resistant one, by 15%. It should be remembered that 0.1 mM-3-AT inhibited the growth of the sensitive YF strain by 80%.

It has been previously shown [7, 14, 9] that histidine is able to abolish completely the effects of 3-AT in yeast. Therefore the information concerning the effect of histidine on the development of resistance to 3-AT was thought to be of importance for elucidation of the mechanism of this process. All attempts, however, to obtain a resistant population by allowing the YF cells to grow in the presence of both 3-AT and histidine at concentrations enabling normal growth, were without success. It can be concluded, therefore, that to induce the resistance not just the presence but the inhibitory action of 3-AT is required.

In other experiments, solid minimum medium was inoculated with resistant population; subsequently single colonies were transferred to liquid medium and resistance of the obtained subcultures was studied. It appeared that in all twelve cases the degree of the resistance was the same as in the parent populations.

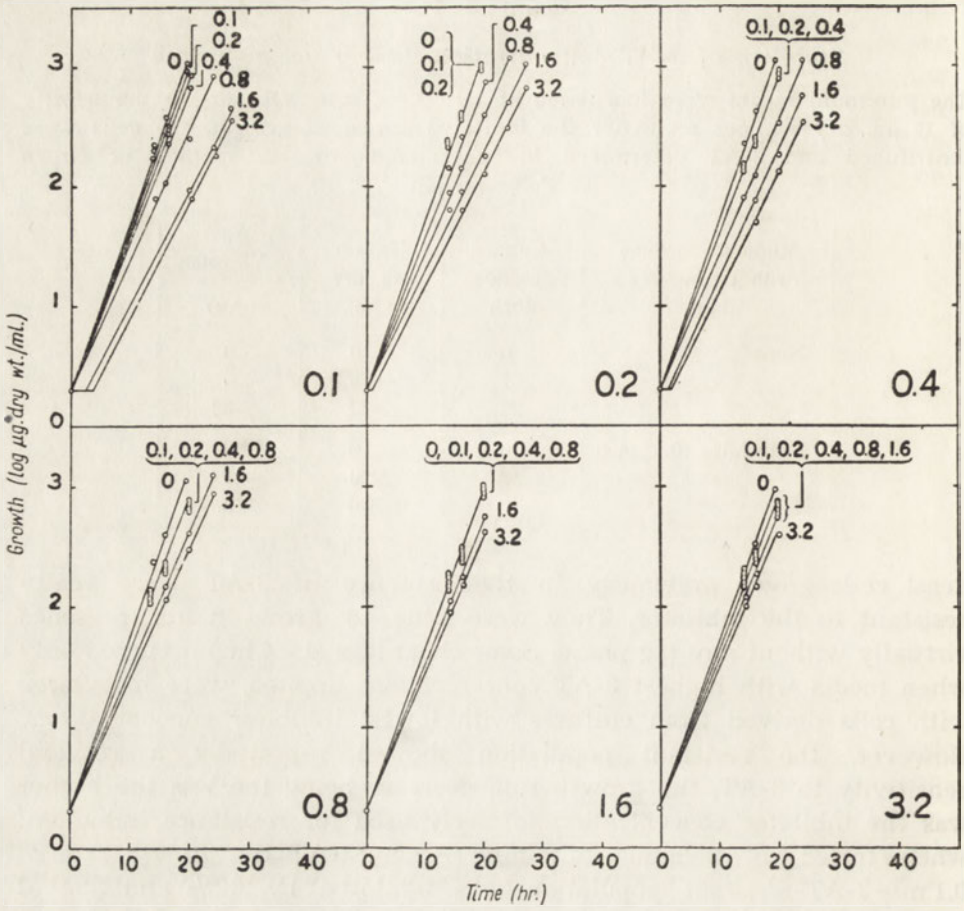

Fig. 3. Resistance of YF cells acquired at different 3-AT concentrations. The numbers facing the curves represent the mM concn. of 3-AT. The numbers in right lower corners give the mM 3-AT concn. in media in which the resistance had been acquired.

On the other hand, LM 2 which was found to be inhibited by 0.1 mM-3-AT in only about 10%, after subculture in media containing 3-AT displayed no increase in resistance.

Stability of YF resistance to 3-AT

The resistant population prepared by single culture in minimum medium with 1 mM-3-AT, was used. Subsequently, the washed resistant cells were allowed to grow in minimum medium and in another one containing 1 mM-3-AT (see Table 2, serial transfer 1). After the growth had reached ca 500 µg. dry wt. cells per ml., both yeast suspensions were harvested, washed, diluted and used for inoculating pairs of identical media (serial transfer 2). The same procedure was repeated in

serial transfers 3 and 4. Each time out of four suspensions only two were used for further transfers. In this way, the cultures shown in column A of Table 2 were inoculated, starting from the serial transfer 2, with cells grown previously in minimum medium devoid of 3-AT and those in column B were inoculated with the cells derived from 3-AT--containing media.

Table 2

Stability of acquired resistance to 3-AT

The YF yeast cell population made resistant in 1 mM-3-AT was allowed to grow for several transfers in minimum medium (A) and the medium containing 1 mM-3-AT (B). Other details are given in the text. Growth rates are expressed as cell divisions per hour. All cultures grew without any lag phase.

Serial transfer	A				B			
	Generations	Growth rate			Generations	Growth rate		
		Transfer medium (minimum)	Medium with 1 mM-3-AT	Inhibition (%)		Minimum medium	Transfer medium (with 1 mM-3-AT)	Inhibition (%)
1	8.3	0.431	0.385	11	7.5	0.431	0.385	11
2	8.5	0.486	0.433	10	8.5	0.460	0.446	3
3	8.6	0.485	0.447	8	8.2	0.485	0.456	6
4	8.6	0.478	0.423	11	8.8	0.472	0.450	5

The data of this experiment show that the lack of lag phase and ca 10% growth rate inhibition by 1 mM-3-AT, characteristic for a population which had acquired its resistance at the same 3-AT concentration, did not change after 25 generations in a medium devoid of 3-AT. On the other hand, one additional transfer of a cell population, immediately after it had been made resistant, through a medium with 3-AT (serial transfer 1 on the right) augmented somewhat the resistance, but even the two following transfers did not render the population indifferent to 3-AT.

The effect of histidine in culture medium on the maintenance of resistance to 3-AT was studied in a parallel experiment. It has been found that the presence of 0.25 mM-histidine in subculture media does not reduce the resistance.

DISCUSSION

The two *S. cerevisiae* strains used in this work, YF and LM 2, differ in their sensitivity to 3-AT. The wild strain is more sensitive than LM 2 although neither was previously exposed to 3-AT. At the same time the wild strain is easily adaptable to the inhibitor. It is possible that the

difference in adaptability is quantitative, as LM 2 has not been tested at 3-AT concentrations higher than 3.2 mM which might have provoked the appearance of a lag phase and resistance induction. It is not clear whether the low sensitivity of LM 2 strain results from the presence in its genome of mutated recessive genes involved in the control of histidine biosynthesis or depends on some other factors.

Desborough *et al.* [4] have described in *S. cerevisiae* the gene *TZ*, whose alleles determine the resistance and sensitivity to 3-AT. Analogous genes were also found by Luzzati (personal communication) in mutants derived from the strain LM 2. One of them, *AT*, is dominant and another, *at*, recessive regarding 3-AT resistance. The available information is not sufficient to decide whether the resistance studied here is related to any of these genes. Moreover, the notion of selection of preexisting mutants can hardly be accepted, as it was calculated from the data of experiments like that shown in Fig. 1A that the resistant cells would form 0.5 - 2.5% of the original sensitive population. This is definitely more than the value of 10^{-8}, generally accepted as incidence index of spontaneous mutants. Also the limited extent of the resistance seems to indicate that the difference between original and resistant populations is rather quantitative.

The existence of 3-AT resistant mutants of *S. cerevisiae* does not necessarily indicate that in every case of 3-AT resistance in this organism the specific genes are involved. Moyed [11, 12] found that in *Escherichia coli* both genetic and phenotypic resistance to 2-thiazole alanine (acting as inhibitor of phosphoribosyl-ATP pyrophosphorylase [1]) are reciprocally not related. The genetic resistance is due to mutation rendering this enzyme insensitive to the inhibitor. The kinetics of the phenotypic resistance development was similar as in the present experiments with 3-AT. The population resistant to 2-thiazole alanine was able to grow in fresh media containing the inhibitor without lag phase and at only slightly diminished rates. However, the resistance was lost when the cells were grown for 10 generations in the absence of 2-thiazole alanine. This phenotypic resistance was due to the derepression of enzymes of histidine biosynthesis. The initial reduction of histidine formation led to the diminution of its intracellular content. In consequence, the genic mechanism repressing the formation of the enzymes of histidine biosynthesis in response to histidine concentration in the cell, was shut out and the increased amount of phosphoribosyl-ATP pyrophosphorylase permitted histidine synthesis to proceed at a higher rate in spite of the inhibitor being present and the unchanged sensitivity of the enzyme. The growth of such derepressed and resistant strain in media without 2-thiazole alanine led to a relative overproduction of histidine, repression of formation of the enzymes of histidine biosynthesis and, consequently, to the loss of resistance.

Other results from our laboratory indicate [9] that 3-AT inhibits one of the enzymes of histidine biosynthesis, probably imidazoleglycerol-phosphate dehydratase [9]. Nevertheless, there is no indication at present as to the mechanism by which the resistant cells counteract the inhibitory effect of 3-AT. The derepression of the sensitive enzyme might really occur in this case also as a result of limitation of histidine synthesis. However, the resistance to 3-AT persists unchanged after 25 generations in media without 3-AT and, moreover, in media containing histidine. The resistance of *E. coli* to 2-thiazole alanine is lost during 10 generations in media without the inhibitor [11]. This difference indicates that in yeast another mechanism is operating, or that the enzyme repression has some special features. It seems probable that in yeast the inducible resistance to 3-AT is dependent on a factor ensuring the stability of this resistance and operating apparently on a higher level of cell organization. Some properties of such a factor can be deduced from the presented experiments: (1) Since 3-AT in the presence of histidine i.e. under conditions where there is no inhibition of growth, is unable to induce the resistance it follows that not the presence of 3-AT but its growth inhibiting effect is required for the appearance of the factor. (2) The degree of resistance of single colony substrains is related to 3-AT concentration during resistance development. This indicates that the factor is set on a certain level of activity or is produced in quantity depending on inhibitor concentration. (3) This level of resistance is maintained unchanged regardless of the presence of 3-AT in culture media. Its persistance through at least 25 mitotic divisions, diluting the original cell components more than million-fold, indicates that the factor is endowed with the ability to be quantitatively replicated.

Before pursuing further studies on the nature of the presumed factor, it should be first elucidated whether the selection of predisposed individuals does participate in the mechanisms rendering a yeast population resistant to 3-AT. The exclusion of such a possibility would permit to accept that a mechanism of true adaptation is operating in each cell.

SUMMARY

Sensitivity of wild yeast to 3-amino-1,2,4-triazole (3-AT) is transient, due to the fast growth of resistant cells. The degree of resistance depends on 3-AT concentration at which it has been acquired. Histidine, re--establishing the growth, prevents the development of resistance. The resistance to 3-AT is stable, lasting during at least 25 mitotic divisions in media devoid of 3-AT.

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ADAPTACJA DROŻDŻY DO 3-AMINO-1,2,4-TRIAZOLU

Streszczenie

Wrażliwość szczepu dzikiego *S. cerevisiae* na 3-amino-1,2,4-triazol (3-AT) jest przejściowa, gdyż szybko wzrastają komórki oporne. Zakres oporności zależy od stężenia 3-AT, przy którym została nabyta. Histydyna, przywracając wzrost, zapobiega powstawaniu oporności. Oporność na 3-AT jest trwała przez co najmniej 25 podziałów mitotycznych w pożywce bez 3-AT.

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IMIDAZOLEGLYCEROL ACCUMULATION BY YEAST RESULTING FROM THE INHIBITION OF HISTIDINE BIOSYNTHESIS BY 3-AMINO-1,2,4-TRIAZOLE*

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3-Amino-1,2,4-triazole (3-AT) has been shown to affect various metabolic functions in different organisms. In higher plants, it inhibits chlorophyll formation and interferes with anthocyanin metabolism [19]. In alga *Oscillatoria princeps*, 3-AT inhibits glycogen phosphorylase reaction [10], probably by spontaneous reaction with glucose-1-phosphate [9]. In another alga *Chlorella pyrenoidosa*, growth inhibitory effect of 3-AT is reversed partially by purines [24]. In animals, 3-AT inhibits haeme synthesis [6], probably by diminution of δ-aminolaevulinate dehydratase activity in tissues [22]. Besides, 3-AT inactivates irreversibly animal catalase [18], tyrosinase and fatty acid oxidase [8]. In microorganisms, 3-AT inhibits 5-aminoimidazole degradation by enzymes of *Clostridium cylindrosporum* [21]. The growth inhibition of *Escherichia coli* is partially reversed by adenine or histidine; when present together, adenine and histidine restore the growth completely [23, 7].

In yeasts, the inhibition by 3-AT of growth of *Torula cremoris* is fully reversed by histidine [23]. Hilton [15] found the same effect of histidine in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Some other amino acids and natural purines also reversed the 3-AT effect on yeast growth, but their action was distinctly weaker. Hilton concluded that in yeast, 3-AT interferes with histidine biosynthesis rather than with its utilization. The present results lend further support to this concept. The non-competitive character of histidine effect regarding 3-AT has been confirmed. In addition, it has been found that yeast inhibited by 3-AT accumulates imidazoleglycerol phosphate (IGP), an intermediate of histidine biosynthesis. This would indicate that the inhibitory effect of 3-AT in yeasts is connected with the reaction catalysed by IGP dehydratase.

MATERIALS AND METHODS

Abbreviations. 3-AT, 3-amino-1,2,4-triazole; IGP, D-erythro-imidazoleglycerol phosphate; IG, D-erythro-imidazoleglycerol.

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Organisms. All strains of *S. cerevisiae* used during this work were kindly supplied by Dr M. Luzzati from Laboratoire de Génétique Physiologique in Gif-sur-Yvette (France). Strains Yeast Foam (YF) and LM 2 are prototrophic diploids [see 16]. The haploid strains used, listed in Tables 1 and 3 are auxotrophic mutants.

Cultures. Growth experiments were made as described previously [16] using the minimum medium of Galzy & Slonimski [12]; for the auxotrophic mutants, when necessary, adenine and uracil in concn. 0.5 mM, and L-amino acids (0.25 mM) were added.

In experiments made for imidazoleglycerol (IG) accumulation the cells were allowed to grow in minimum medium, eventually supplemented as specified in Results, and dispensed sterilly into Evelyn tubes. The cotton-stoppered vessels were shaken horizontally at 27°. The initial cell density was 300 µg. dry weight per ml. Each tube contained a glass bead to prevent cell deposition on the bottom. All supplements were prepared by dissolving in the medium. Only 3-AT, forming spontaneously even at room temperature an addition product with glucose [13], was sterilized as concentrated aqueous solution and diluted before use with the medium.

Dry weight of growing yeast and cell suspensions was determined with Evelyn photometer, using 660 mµ filter.

Chemical methods. Formation of arylamine intermediates of histidine biosynthesis by cell-free extracts was estimated as described previously [17] with a slight modification since 3-AT gives a red colour with Bratton-Marshall reagents. It was observed that diazotized 3-AT, contrary to diazotized 5-aminoimidazole derivatives, is rapidly decomposed by sulphamate. The rising of sulphamate concentration to 1% and the prolongation of the interval between the addition of sulphamate and of 1-*N*-naphtyl-*p*-ethylenediamine from 2 to 15 min. permitted to avoid 3-AT interference up to at least 3 µmoles 3-AT per sample used for estimation.

Determinations of IG and IGP were done by the method of Ames [1]. In order to handle greater samples and to neutralize the acid pH of the growth medium, the method was modified without affecting the relative concentrations of reagents as follows: 0.6 ml. of medium was added with 0.05 ml. of 0.5 M-sodium phosphate buffer, pH 7.0, and 0.15 ml. of 0.33 M-sodium *m*-periodate dissolved before use in hot water. After thorough mixing, the tubes were left for 20 min. at room temperature. Then 4.2 ml. of *n*-butanol - piperidine mixture (20:1, v/v) was layered on, followed by 0.2 ml. of saturated sodium borate. The formed imidazoleformaldehyde was extracted by vigorous horizontal shaking of stoppered tubes for 20 seconds. After centrifugation, the butanol layers were pipetted off for extinction measurements at 310 mµ in Beckman DU spectrophotometer. The blank contained fresh medium treated in the same way

Another correction was introduced because when yeast was grown in the absence of 3-AT, in the medium a compound was present which in the Ames' method exhibited UV absorption. The values for this absorption, shown in Fig. 4 as curve *B*, were subtracted from actual values for media in which yeast had grown in the presence of 3-AT. The resulting values gave curve *C* resembling that for partially purified IG and IGP preparations (Fig. 5). This correction was made for all determinations except those shown in Table 3. For determinations of IG formation by auxotrophic mutants, parallel series of blank tests with water instead of *m*-periodate were made.

The IG or IGP content was calculated from net extinctions on the basis of E_{310} value 0.230 for 0.1 μmole of IGP given by Ames [1]. In this method, 3-AT does not affect the results.

Chemicals. The following chemicals were commercial products: disodium ATP, barium ribose-5-phosphate, barium phosphoglycerate and L-glutamine (Nutritional Biochemicals Corp. Cleveland); adenine hydrochloride (Feinchemie, Berlin); uracil (British Drug Houses, Poole); L-histidine dihydrochloride monohydrate (Chemapol, Praha); DL-methionine (Light, Colnbrook); L-tryptophan (Fluka, Buchs); and 3-amino-1,2,4-triazole (Serlabo, Paris).

n-Butanol (Estron, Warszawa) and piperidine (Schuchardt, Munich) were purified by redistillation. Only the fractions of *n*-butanol showing the extinction at 310 mμ lower than 0.2 were used. *n*-Butanol (K. & K., Jamaica N.Y.) having E_{310} equal to 0.068 was used without redistillation.

RESULTS

Reversal of 3-AT inhibition of yeast growth by histidine

In confirmation of earlier reports by Weyter & Broquist [23] and by Hilton [15], it has been found that L-histidine restores the yeast growth inhibited by 3-AT to normal pattern, both when YF or LM 2 strains were used in experiments. As shown in Fig. 1, histidine accelerated the initial growth rates. After a time, depending on histidine concentration, the growth became slower and entered a new exponential phase, differing from the former by its rate. The intersection points of logarithmic parts of the curves, found by extrapolation, determine cell mass yields corresponding to given amounts of histidine in media. The data presented in Table 1 show that the yields were directly proportional to histidine content, just like in the experiment with histidineless mutants. Since also the values of growth yields per mμmole of histidine were nearly the same, it can be stated that yeast response to histidine in the presence of 3-AT is identical to that of mutants with blocked histidine biosynthesis. This result suggests that in yeast the only reaction sensitive to 3-AT and simultaneously essential for growth is concerned with histidine biosynthesis.

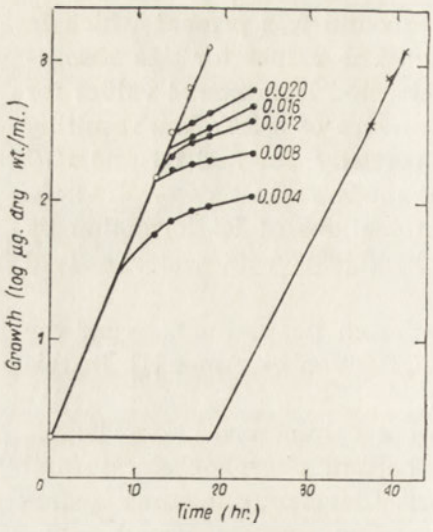

Fig. 1. Effect of L-histidine concentration on the growth of Yeast Foam cells in the presence of 1 mM-3-AT. The numbers facing the curves represent mM concentrations of L-histidine. (○), Control (without 3-AT); (×), with 1 mM-3-AT, (●), with 1 mM-3-AT and histidine added.

Alternatively, histidine would act also if 3-AT interfered with histidine utilization. However, if it were so, lowest histidine concentrations should restore growth rates only partially. As it may be seen from

Table 1

The effect of histidine on the growth of yeast YF strain inhibited by 3-AT, and of histidineless mutants

The cells were grown in minimum medium [12], supplemented for the strain 2-9 with 0.5 mM-adenine. The growth yields were calculated from the intersection points found by extrapolation of exponential parts of growth curves (Fig. 1). Temp. of incubation 27°.

Strain	Genotype	3-AT (mM)	Histidine (mM)	Growth yield	
				(µg. dry wt./ml)	(µg. dry wt./mµ-mole of histidine)
Yeast Foam	wild	1	0.004	68	17.0
		1	0.008	160	18.0
		1	0.012	215	17.9
		1	0.016	240	15.0
		1	0.020	355	17.7
				Average	17.1
2-9	hi_1, ad_2	0	0.020	440	22.0
D-230-9A	hi_4	0	0.020	430	21.5
D-256-2B	hi_6	0	0.020	430	21.5
				Average	21.7

Fig. 1, 0.004 mM-histidine reverses completely the inhibition produced by 1 mM-3-AT. The effect is temporary, of course, because the amino acid is rapidly incorporated into proteins of growing cells. The ability of histidine to counteract the 250-fold, at least, inhibitor concentration and to maintain the constant growth rate in spite of continuous disappearance at unchanged 3-AT concentration argues against the possibility of competitive interaction of 3-AT with histidine itself. Therefore it seems improbable that structural analogy between imidazole and triazole moieties of these compounds might play a role in the process of yeast growth inhibition by 3-AT.

Lack of 3-AT effect on biosynthesis of arylamine intermediates of histidine biosynthesis

To obtain more direct evidence of the inhibition of histidine biosynthesis by 3-AT, the experiments were made with cell-free extracts able to synthesize the early intermediates of this biosynthetic pathway [17]. The information concerning their exact chemical structure is rather scarce. However, it is well established that one of these compounds, named by Magasanik "Compound III" [20], yields aminoimidazolecarboxamide ribotide on mild acid hydrolysis. Therefore, the amount of non-acetylable arylamine after acid hydrolysis represents the formed "Compound III" [20, 17]. It appeared (see Table 2) that 2 mM-3-AT diminished the formation of this compound by extracts of LM 2 cells only by 20%. Since 1.6 mM-3-AT inhibits the growth rate of this strain by 51%, it follows that the effect observed *in vitro* is of only secondary, if any, significance.

Table 2

Formation of "Compound III" in the presence of 3-AT

Incubation mixture contained: potassium ribose-5-phosphate 10 mM, ATP 2 mM, potassium phosphoglycerate 15 mM, potassium phosphate buffer, pH 7.4, 50 mM, L-glutamine 7.5 mM, magnesium chloride 10 mM, and 3.0 mg. protein of dialysed cell-free extract of LM 2. Incubation temperature was 37° [17]. The non-acetylable arylamines were determined after 20 min. hydrolysis in 1 N-HCl by Bratton-Marshall method as described in Methods. "Compound III" formation was calculated from the total aminoimidazolecarboxamide formed. The results of one typical experiment are given.

Incubation time (min.)	„Compound III" (mμmoles/ml.)		Inhibition (%)
	Control	with 3-AT (2mM)	
30	97.5	80.7	17
60	168.3	134.7	20

Accumulation of IG by 3-AT inhibited yeast cells

When yeast, inhibited by 3-AT, was growing slowly, an accumulation of IG in the medium was observed. Fig. 2 shows that IG accumulation by LM 2 cultures is directly proportional to the logarithm of 3-AT concentration. With the wild strain YF the increase of the inhibitor concentration did not influence the IG accumulation. It was verified by spectrum analysis that the horizontal line drawn from the net values

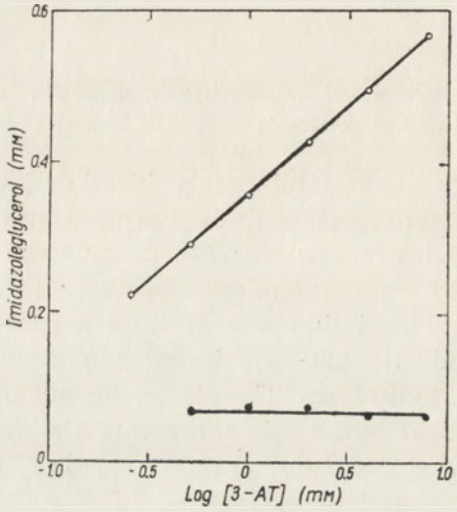

Fig. 2

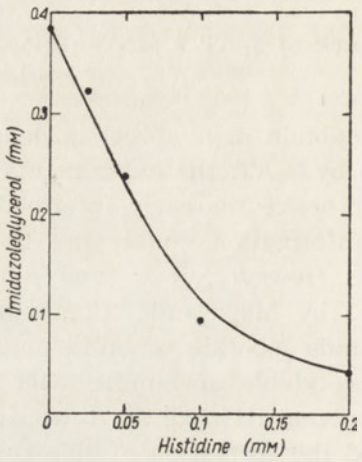

Fig. 3

Fig. 2. Accumulation of imidazoleglycerol by strains (●), Yeast Foam and (○), LM 2 inhibited by 3-AT.

Fig. 3. Effect of L-histidine on accumulation of imidazoleglycerol by LM 2 cells inhibited by 2 mM-3-AT, after 20 hr. culture.

(corrected for non-specific ultraviolet light absorption of *m*-periodate treated sample of control, without 3-AT, culture medium) really represents the accumulated IG. From growth measurements it was inferred that the lack of effect of increasing 3-AT concentration on IG accumulation is only apparent and results from decreasing final growth yields of YF. So, the higher was 3-AT concentration, the more elevated was really the formation of IG per cell and time unit. The shape of the resulting curve of IG accumulation versus 3-AT concentration suggests that a close relation exists in YF strain between growth limitation and IG accumulation. On the other hand, in LM 2 the 3-AT effect on IG production was more pronounced than that on the growth rate. This relative overproduction of IG and by inference of IGP might constitute a basis for a tentative explanation of low sensitivity of this strain to 3-AT as compared with YF, observed in other experiments [16].

Identification of the accumulated substance

To make sure that the accumulated substance is really related to histidine biosynthesis, experiments were made on mutant strains blocked in histidine or adenine biosynthesis. The results presented in Table 3 indicate that hi_1 mutants, blocked in the reaction forming phosphoribosyl-ATP (T. Kłopotowski, unpublished experiments) are unable to accumulate any compound forming by oxidation with *m*-periodate a derivative absorbing in ultraviolet and extractable by alkaline *n*-butanol. Furthermore, the adenineless mutants did not accumulate such a compound unless provided with adenine.

Also histidine, known as a feedback inhibitor of phosphoribosyl-ATP pyrophosphorylase [20, 2, 17] prevented such an accumulation. Fig. 3 shows the effect of increasing histidine concentrations on IG accumulation in the medium. It must be noted that histidine enhances yeast growth in the presence of 3-AT. Hence, the IG formation calculated per cell and time unit plotted against histidine concentration would be represented by a curve declining even more steeply.

Table 3

Inability of hi_1 *mutants to accumulate imidazoleglycerol, and adenine requirement for this accumulation by adenineless mutants in 3-AT containing media*

The symbols of biosynthetic blocks are the names of genes whose mutation resulted in respective growth requirements of adenine (*ad*), histidine (*hi*), methionine (*me*), tryptophan (*tr*) and uracil (*ur*). The sign *x* is used when the gene was not tested for allelism, and classified.

The suspensions of mutant cells were added to minimum medium supplemented with 1 mM-3-AT, and respective growth factors to the concentrations given in Methods, except for adenine added as specified in the Table and histidine which was omitted. The growth proceeded for two divisions till the exhaustion of internal histidine pool, derived from previous media. After 20 hr. the cultures were centrifuged and IG determined in supernatants.

In this experiment, the correction for non-specific ultraviolet absorption appearing in media without 3-AT after *m*-periodate treatment was not made.

Mutant	Block in the biosynthesis of			IG accumulation (mμmoles/ml.)	
	adenine	histidine	other compounds	3-AT media without adenine	3-AT media with 0.1mM-adenine
2-9	ad_2	hi_1	—	8	0
1-35	ad_6	hi_1	tr_1, ur_x	2	9
4237 B-1	ad_2	—	tr_1	19	45
4062 C	ad_4	—	me_x, ur_x	19	109
2779 A	ad_5	—	tr_x, ur_x	10	68
1-5	ad_6	—	me_x, tr_x	23	107
5178 B	ad_7	—	tr_x, ur_x	0	24
18-35	—	—	tr_x	88	85

The whole of this evidence suggests that the accumulated compound is an intermediate of histidine biosynthesis and that it is not a 3-AT derivative. However, this evidence does not suffice to state that IG is the only intermediate of histidine biosynthesis accumulated as a result of 3-AT inhibition. It is especially important to know whether the products of histidine precursors posterior to IGP appear in the medium. To clarify this point an experiment was done, in which the amount of accumulated imidazoles was established. The yeast of LM 2 strain grown

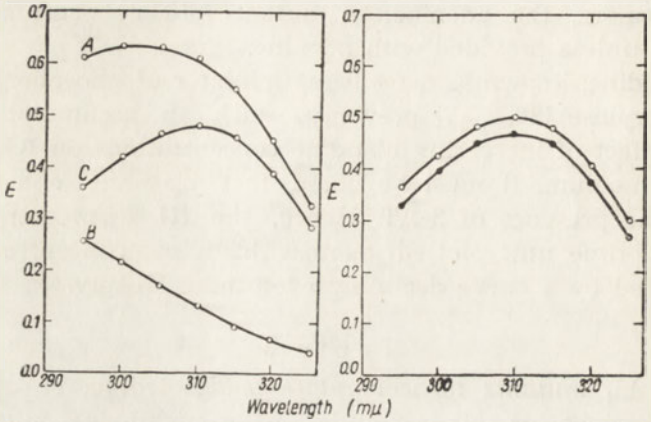

Fig. 4 Fig. 5

Fig. 4. UV absorption spectra of *m*-periodate treated culture media. Curve *A*, the medium after the growth of LM 2 in the presence of 2 mM-3-AT; curve *B*, the medium after the growth of LM 2 in the absence of 3-AT; curve *C*, resulting from subtraction of the curve *B* from *A*.

Fig. 5. UV absorption spectra of *m*-periodate treated preparations from (○), the medium, and (●), the cells of LM 2 grown in the presence of 0.5 mM-3-AT. The samples contained 0.22 μmole IG and 0.20 μmole IGP, respectively.

in 10 l. of minimum medium containing 0.5 mM-3-AT and 0.2 mM-adenine was harvested and washed with distilled water by centrifugation. The cells (26 g. of dry weight) were extracted four times with boiling water. The imidazole compounds were isolated from this extract and from cell-free medium by the procedure of Ames *et al.* [5]. After discarding sulphate and phosphate ions with barium acetate, the imidazoles were precipitated from clear supernatants by treatment with ethanolic solution of mercuric chloride. The mercuric complexes of imidazoles were extracted by repeated treatment with 0.5 N-hydrochloric acid. The mercuric ions were precipitated from the hot solution with hydrogen sulphide. The clear supernatants devoid of mercuric chloride and hydrogen sulphide were freed from 3-AT by adsorption on acid-washed active charcoal. The adsorption was repeated many times

up to the disappearance of traces of colour with Bratton-Marshall reagents for arylamines. Finally, the solutions were neutralized with potassium hydroxide and tested for imidazole by Ames' procedure [1] (Fig. 4 and 5).

The ultraviolet spectra of both preparations gave single peaks with a maximum at 310 mμ (Fig. 5). The maximum agrees with that given by Ames. The total yields of imidazoleglycerols from medium and cells were 79 and 30 μmoles, respectively.

The preparations were tested for imidazoles by ascending chromatography on Whatman no. 1 paper in the solvent system of chloroform--methanol - formic acid (3:3:1, by vol.) according to Ames & Mitchell [4]. The dried chromatograms were tested for imidazoles by Pauly reaction [3]. It appeared that the material isolated from the culture medium gave only one spot of R_F 0.30, and that from the cells also one spot, but of a lower R_F value. The number of spots and their relative positions on chromatograms seem to show that the only accumulated imidazole compound in the cells is IGP, and in the medium its dephosphorylated derivative, IG.

DISCUSSION

The present results demonstrating the non-competitive reversal of 3-AT toxicity by histidine and the identity of the response to histidine of 3-AT-inhibited yeast and of mutants blocked in histidine biosynthesis, support Hilton's assumption that 3-AT inhibits rather the process of histidine biosynthesis than that of its utilization.

Our finding that yeast inhibited by 3-AT accumulates IGP and excretes its derivative, IG, to the medium, gives a more precise indication as to the 3-AT sensitive reaction in yeast. However, it has been found by Hartman *et al.* [14] in *Salmonella typhimurium* that not only B mutants (blocked in gene B controlling IGP dehydratase and histidinolphosphate phosphatase), but also several C, and even D mutants (blocked in further reactions of histidine biosynthesis, imidazoleacetol phosphate transaminase and histidinol dehydrogenase, respectively) accumulate IG in the medium. Hence, the possibility would exist that 3-AT causes IG accumulation not necessarily by inhibiting IGP dehydratase, but by inhibiting one of the four enzymes acting between IGP and histidine. However, the fact that preparations obtained from medium and cells by Ames' procedure [5], devised to isolate all imidazole compounds, both contain only one imidazole compound (IG or IGP) makes the supposition that 3-AT inhibits IGP dehydration the most probable one.

SUMMARY

Inhibition of growth of *S. cerevisiae* produced by aminotriazole (3-AT) is non-competitively reversed by L-histidine.

The 3-AT-inhibited yeast cells accumulate imidazoleglycerol phosphate and excrete imidazoleglycerol to the medium. This suggests that 3-AT inhibits imidazoleglycerol phosphate dehydratase.

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AKUMULACJA IMIDAZOLOGLICEROLU W WYNIKU ZAHAMOWANIA BIOSYNTEZY HISTYDYNY U DROŻDŻY PRZEZ 3-AMINO-1,2,4-TRIAZOL

Streszczenie

Zahamowanie wzrostu *S. cerevisiae* przez aminotriazol (3-AT) jest niekompetytywnie znoszone przez L-histydynę.

Zahamowane przez 3-AT komórki drożdży akumulują fosforan imidazologlicerolu i wydalają do pożywki imidazologlicerol. Pozwala to przypuszczać, że 3-AT jest inhibitorem dehydratazy fosforanu imidazologlicerolu.

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PREPARATION OF POLY-5-FLUOROURIDYLIC ACID AND THE PROPERTIES OF HALOGENATED POLY-URIDYLIC ACIDS AND THEIR COMPLEXES WITH POLY-ADENYLIC ACID

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It has been previously shown that, when a methyl group is substituted on the 5-position of the uracil rings in poly-U, the resulting poly-5-methyluridylic acid (or poly-rT) [1] exhibits properties surprisingly different from those of poly-U [32, 35]. A comparison of the temperature profiles for the two polymers under identical conditions, in 0.01 M-$MgCl_2$ at neutral pH, showed that the melting point T_m (or mid-point of the temperature profile) for poly-rT was 36° as compared to 8.5° for poly-U. The former consequently exhibits a high degree of secondary structure at room temperature where poly-U is in the form of a random coil. Furthermore the twin-stranded complex of poly-rT with poly-A proved to be considerably more stable thermally than that of poly-U with poly-A, the T_m values derived from the temperature profiles at neutral pH in SSC being, respectively, 79° and 58.5°. An important consequence of this quantitative difference in behaviour between poly-U and poly-rT is that the base pair A-T is not, as has been commonly assumed [7, 29], equivalent to the base pair A-U.

The remarkable difference in stability between the base pairs A-T and A-U is, however, difficult to account for in terms of the strength of the Watson-Crick base pair hydrogen bonds, since these should be almost equivalent for uracil and thymine. It consequently follows that the stability of a polynucleotide helix is by no means dependent only on the base pair hydrogen bonds, but must involve factors other than, and additional to, hydrogen bonding. Additional evidence, both of a theoretical [5, 6] and experimental [12, 13] nature, has been accumulating and will be referred to in the Discussion, below.

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[1] The following abbreviations are used in this text: A, adenylic acid residue; U, uridylic acid residue; rT, 5-methyluridylic or ribotymidylic acid residue; FU, 5-fluorouridylic acid residue; BrU, 5-bromouridylic acid residue; ClU, 5-chlorouridylic acid residue; the prefix "d" indicates a deoxyribo analogue of any of the foregoing; the prefix "poly-" to one of the foregoing refers to the polymer resulting from the action of polynucleotide phosphorylase on the nucleoside-5′-pyrophosphate, e.g. poly-FU is poly-5-fluorouridylic acid; SSC refers to a standard saline citrate solution (0.15 M-sodium chloride and 0.015 M-sodium citrate).

In view of the foregoing, it appeared of interest to examine the properties of analogues of poly-U containing a substituent in the 5-position which would be expected to markedly modify the properties of the uracil ring. A halogeno substituent was considered to fulfil, at least partially, this requirement inasmuch as it appreciably affects the dissociation of the uracil H—N(3)—C(4)=O grouping [2] involved in hydrogen bonding to adenine residues in the poly-U + poly-A twin-stranded complex, although it likewise modifies both the magnitude and direction of the permanent dipole moment of the uracil ring. It was consequently decided to prepare poly-FU and to compare its properties with those of poly-U and poly-rT. The Br-, Cl- and I-substituted analogues of poly-U have already been reported [22] and presumably some data on their physicochemical properties will shortly be made available; but the obvious utility of these polymers in relation to the present study prompted us to make a few provisional measurements on samples of poly-BrU and poly-ClU kindly supplied by Dr. A. M. Michelson.

It remains to add that other experimental approaches will undoubtedly be required in order to arrive at an adequate understanding of the nature of the forces responsible for the stability of polynucleotide helices. A rather promising one involves the application of non-aqueous solvents, which has been extensively applied to investigations on the stability of natural nucleic acids [9, 13]. Concurrently with the above, we have initiated such studies on synthetic polyribonucleotides which, it is hoped, will provide simpler models for the interpretation of experimental findings.

Synthetic procedures

The starting material for synthetic work was 5-fluorouridine, for which we are indebted to Hoffman-LaRoche Inc. and Dr. A. R. Duschinsky. This compound was converted quantitatively to 2′,3′-*O*-isopropylidene-5-fluorouridine by the method of Levene & Tipson [7]. The latter derivative was phosphorylated by means of β-cyanoethylphosphate [36]; paper chromatography showed that phosphorylation by this procedure was fully quantitative and the only losses incurred were those resulting from the technical manipulations involved in the isolation of 5-fluorouridine-5′-phosphate (F-UMP) from the reaction mixture.

Prior to phosphorylation the stability of the fluoro substituent in the 5-position of the uracil ring was examined under the acid and alkaline conditions required for removal of the β-cyanoethyl and isopropylidene groups during the isolation of F-UMP. Paper chromatography and spectral measurements demonstrated that 5-fluorouridine was fully resistant to the action of N-HCl at 100° for 60 min. and to 0.5 N-NaOH at 100° for 40 min.

5-Fluorouridine-5'-pyrophosphate (F-UDP) was then obtained by treatment of F-UMP with dibenzylphosphochloridate [20], in 62% yield. Here again it was necessary initially to check the stability of the 5-fluoro substituent under the hydrogenation conditions normally required for the removal of benzyl groups, employing a catalyst in slightly acid (pH 4.5 - 5.0) medium. The importance of this is testified to by the fact that hydrogenation on a catalyst consisting of 10% Pd on active carbon, in alkaline medium, leads to the hydrogenation of the fluorine with the formation of uridine [8]. However, under the experimental conditions herein employed, i.e. pH 4.5 - 5.0, the fluoropyrimidine ring was found to be fully stable, as might have been anticipated.

The pyrophosphate derivative proved to be a substrate of polynucleotide phosphorylase. In initial experiments, employing standard conditions [11], the yield of polymer was rather low. This was circumvented by an increase in the concentration of Mg^{2+} ions in the incubation medium, which resulted in reproducible yields of polymer of 28 - 31%.

Properties of poly-FU

Poly-FU was completely hydrolysed by pancreatic ribonuclease and by snake venom (*Crotalus adamanteus*) with the formation of mononucleotides and nucleosides, respectively. Hydrolysis was accompanied by an increase in extinction at 269 mμ of 7.3%, measured at pH 6 where the uracil residues exist in the non-dissociated form [2].

Hydrolysis of the polymer in 1 N-HCl at 37° for 20 hr. led to an increase in extinction of about 8.5%, the measurements of absorption being made at room temperature. The foregoing value for the hyperchromicity is slightly lower than that for poly-U [34] and very much less than that for poly-rT [10, 35]. It may be inferred from this that, like poly-U, poly-FU at room temperature is in the form of a random coil.

In view of the fact that poly-U exhibits an appreciable increase in hypochromicity at low temperatures [18, 34], testifying to the formation of secondary structure under these conditions, the extinction of poly-FU was initially examined over a temperature range of 5 - 75° in 0.01 M-$MgCl_2$ at neutral pH, measurements being made at 269 mμ. No change in extinction could be detected. Only below 5°, and down to 2.5°, which was the lowest temperature attainable by installation of the spectrophotometer in the cold room, was a temperature profile placed in evidence, with a decrease in extinction of 7.5% which was reversible on raising the temperature. The fact that the T_m is well below that for poly-U, and the magnitude of the temperature hypochromicity so low, implies that even at low temperatures the degree of secondary structure of poly-FU is considerably less than that possessed by poly-U.

The pK for dissociation of the 4-carbonyl in fluorouridine and fluorouridylic acid is 7.75 [2]. In poly-FU, on the other hand, the pK is shifted to 8.3 (see Fig. 4, below).

Some question might be raised with regard to the foregoing results, in the absence of sedimentation constant measurements, on the grounds that the molecular weight of our poly-FU preparations may have been unduly low. Against this, it should be pointed out that the properties of the poly-FU samples resulting from successive polymerizations were quantitatively reproducible. Furthermore the ability of poly-FU to form a twin-stranded complex with poly-A (see below), the temperature profile of which was as sharp as that normally encountered with twin-stranded helices, suggests that our poly-FU preparations were at least as highly polymerized as those of poly-U.[2]

Complexes with poly-A

Like poly-U, poly-FU readily reacted with poly-A under appropriate conditions to form twin or triple-stranded complexes. The reactions were followed in the usual way [38] by the addition or "titration", of one polymer solution to the other and noting the decrease in extinction relative to that expected for the arithmetic sum of the two polymers. In Fig. 1 is shown the decrease in extinction at 263 mμ consequent on the formation of a twin-stranded complex between poly-A and poly-FU at room temperature in SSC at pH 6 - 7. The maximum decrease in extinction occurs, as expected, for a molar ratio of the two components of 1:1, the hypochromicity (i.e. the decrease in absorption of the complex relative to the arithmetic sum for poly-A + poly-U) being 34%.

In 0.1 M-$MgCl_2$ at pH 6 or 7 a complex was formed with a maximum hypochromicity of 41.5% at 263 mμ at a ratio of poly-A to poly-FU of 1:2.

Fig. 2 exhibits the variation in extinction at 263 mμ as a function of temperature for the 1:1 complex of poly-A + poly-FU in standard SSC. In view of the low pK of F-UMP, about 7.8 [2], the curve was also run in the same solvent buffered to pH 6, where F-UMP exists only in the non-dissociated form, with identical results. The temperature profile for the complex is seen to be fairly sharp and the dissociation of the helix is accompanied by an increase in extinction, or hyperchromicity, of 50%; while the mid-point of the transition, T_m, occurs at a temperature of 55°. These values are very similar to those for the corresponding 1:1 complex of poly-A + poly-U (hyperchromicity 53%; T_m 59°) under the same conditions.

[2] Following completion of this work, we received a sample of poly-FU prepared in the laboratory of Prof. S. Ochoa. This sample was reported to have a sedimentation coefficient, $S_{20,w}$, of 9.4 [24] similar to that normally prevailing for good poly-U preparations. The behaviour of this sample of poly-FU was practically identical with that of ours.

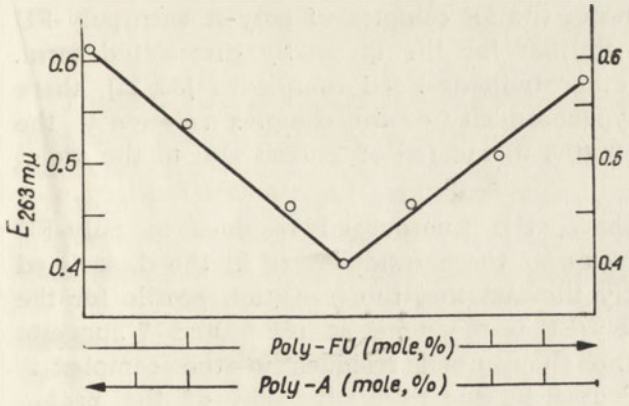

Fig. 1

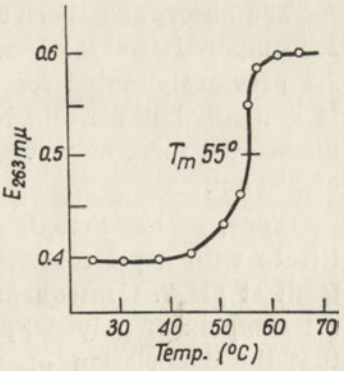

Fig. 2

Fig. 1. Complex formation between poly-FU and poly-A in SSC at 25° and pH 6 - 7. Increasing concentrations of one polymer are added to a solution of the other and the decrease in extinction from the expected arithmetic sum noted. Maximum decrease in extinction (263 mμ, $\lambda_{max.}$ for complex) occurs at ratio of poly-FU: poly-A of 1 : 1.

Fig. 2. Variation with temperature of extinction at 263 mμ of 1:1 complex of poly-FU+poly-A in SSC at pH 6 or pH 7. Mid-point of transition of temperature profile, T_m, is at 55°

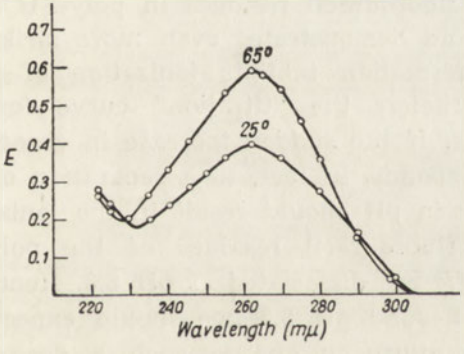

Fig. 3

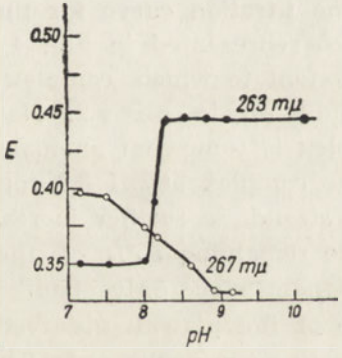

Fig. 4

Fig. 3. Absorption spectrum in SSC, pH 6 or 7, of twin-stranded complex of poly-FU+poly-A at 25° and at 65° where the helix is fully dissociated. Note hypochromicity of complex, with respect to dissociated form, at wavelengths to red of 290 mμ and to violet of 230 mμ

Fig. 4. Titration curve at 25° in 0.1 M-borate buffer+0.01 M-sodium citrate of poly-FU (○) and the 1:1 complex of poly-FU+poly-A (●). The apparent pK of poly-FU from the curve is 8.3. Note that complex dissociates very abruptly at pH 8.2, but that beyond this pH there is no evidence for further ionization of fluorouracil residue of poly-FU component, as would be expected if the twin-strands underwent complete separation at and above pH 8.2

The absorption spectrum of the 1:1 complex of poly-A with poly-FU is compared in Fig. 3 with that for the thermally dissociated form. As previously noted for other twin-stranded complexes [30, 35], there is a small, but definite hypochromicity of the complex relative to the dissociated form at wavelengths to the red of 290 mμ and to the violet of 230 mμ.

Since, as mentioned above, the fluorouracil residues in poly-FU titrate with a pK of 8.3, some of these residues are in the dissociated form at pH 7. Consequently the fact that the transition profile for the 1:1 complex of poly-A+poly-FU is the same at pH 6 and 7 suggests that the dissociation of the fluorouracil residues in the complex is inhibited. This must, of course, be due to involvement of the proton on $N_{(3)}$ of the fluorouracil residues in hydrogen bonding with the adenine residues in poly-A. The extent to which the fluorouracil residues are prevented from dissociating in the twin-stranded complex is demonstrated in Fig. 4, which exhibits the "titration" curve for a 1:1 complex of poly-A+poly-FU. It will be observed from the figure that the twin-stranded helix is fully stable at room temperature up to a pH of 8.15, where a sudden break occurs followed by the rapid dissociation of the helical structure over a very narrow pH range of 8.15 - 8.30. The "titration" curve is completely reversible, the complex reforming once more as the pH is reduced.

The titration curve for the fluorouracil residues in poly-FU alone is also represented in Fig. 4, and demonstrates even more strikingly the extent to which complex formation inhibits ionization of these residues up to pH 8.2. Nonetheless the "titration" curve for the complex is somewhat anomalous. If the sudden increase in absorption of the complex at pH 8.2 corresponds, in fact, to a separation of the twin strands, a further increase in pH should result in the ionization of the remaining 50% of the fluorouracil residues of the poly-FU component, since only about 50% are dissociated at pH 8.3. Hence, in place of the plateau observed beyond pH 8.3, we should expect the curve to slope downwards. The failure to observe such a downward slope, even to a pH as high as 11, suggests that the twin strands may not have undergone complete separation. An exactly analogous behaviour is exhibited by the 1:1 complex of poly-A+poly-U [cf. ref. 33], which dissociates at pH $\sim$ 10.

The behaviour of the poly-A+poly-FU twin-strand is quantitatively similar to that for other twin-stranded polynucleotide helices [33, 28]. Quantitatively, on the other hand, the pH for complex dissociation is considerably lower than that for poly-A+poly-U, in agreement with the lower pK of the fluorouracil residues in poly-FU (pK 8.3) as compared to that of the uracil residues in poly-U (pK 9.6).

Comparison with other halogenated poly-uridylic acids

The findings for poly-FU are of some interest in relation to those of Michelson *et al.* [22] for poly-BrU and poly-ClU. The latter were observed to form triple-stranded (2:1) complexes with poly-A in 0.01 M-ammonium acetate + 0.001 M-$MgCl_2$ and some preliminary measurements indicated that these possessed a higher temperature stability than the corresponding 1:2 complex of poly-A + poly-U.

The foregoing prompted us to make some provisional comparative measurements on samples of poly-BrU and poly-ClU kindly supplied by Dr. A. M. Michelson. In 0.01 M-$MgCl_2$, both of these polymers exhibit very broad temperature profiles extending over room temperature and indicative of the presence of a moderate degree of secondary structure up to about 35°.[3]

We have found that, in SSC at neutral pH, each of these polymers forms only a three-stranded complex with poly-A, with fairly sharp temperature profiles, the respective T_m values being 82° for poly-ClU + + poly-A (2:1 complex) and 87° for poly-BrU + poly-A (2:1 complex). These are indeed relatively high values as compared to what might be expected for poly-U + poly-A (2:1 complex).

To provide a more quantitative comparison, an examination was made of the temperature profiles for the 1:1 complexes with poly-A of poly-U, poly-FU and poly-BrU. All the foregoing readily form 1:1 complexes with poly-A in ten-fold diluted SSC at neutral pH, and the T_m values were found to be, respectively, 43°, 40° and 69°. For poly-rT, the T_m value of its 1:1 complex with poly-A, which is 79° in SSC, may be calculated to be 63° in ten-fold diluted SSC.

DISCUSSION

Bearing in mind the provisional findings reported above for poly-ClU and poly-BrU, it seems rather difficult to interpret in terms of hydrogen bonding the fact that poly-FU possesses a lower degree of secondary structure than poly-U and that its 1:1 complex with poly-A is somewhat less stable than that of poly-U with poly-A.

Twin-stranded poly-dA-dBrU (alternating sequence) has been found to be more stable, in low ionic strength medium, than the corresponding poly-dA-dT (alternating sequence), the T_m for the former being 9° higher [14]. This is in reasonably good agreement with our own findings

[3] In estimating the residual hyperchromicity of these homopolymers, Michelson *et al.* [22] reported that considerable variations were encountered in the measured values. Such variations would be expected in the absence of temperature control during absorption measurements since, in view of the existence of a temperature profile in the neighbourhood of room temperature, the extinction of the polymer prior to hydrolysis is a function of temperature (cf. analogous situation for poly-rT [10, 32, 35]).

for poly-A+poly-BrU and poly-A+poly-rT at low ionic strength where 1:1 complexes are formed, the T_m for the former under these conditions being 6° higher. Inman & Baldwin [14] have proposed that the greater stability of the brominated polymers is due to the influence of the electronegative Br substituent on the proton donor and acceptor abilities of the $N_{(3)}$ and $O_{(4)}$ positions, respectively, of the uracil rings; this explanation is, however, open to some doubt. While a similar argument formulated by the same authors [14] to account for the increased stability of poly-dG+poly-BrC as compared to poly-dG+poly-dC [26] appears to be more convincing, this line of reasoning is difficult to reconcile with the behaviour of poly-FU, where the influence of the electronegative F substituent is much more pronounced and in the reverse sense. If, in addition, we recall the remarkable increase in stability of poly-rT+ +poly-A, as compared to poly-U+poly-A (T_m of former 20° higher than latter), for which no interpretation based on such a line of reasoning may be invoked, it becomes clear that hydrogen bonding between base pairs is inadequate to account for the stability of polynucleotide helices. Mention should also be made of the fact that the difference in T_m values between poly-dA-dBrU and poly-dA-dT disappears at high ionic strength, thus further invalidating any explanation for this difference in terms of hydrogen bonding properties alone.

Following formulation of the Watson-Crick [4] structure for DNA as a twin-stranded helix with base pairing *via* hydrogen bonds, it was perhaps not surprising that the implicit assumption arose that the hydrogen bonds between base pairs provided the forces necessary for maintenance of the helix structure. This conception was in particular reinforced by the observation of Marmur & Doty [19] that the thermal stability of a DNA helix was a function of its G-C (or A-T) base pair content. Since, as pointed out by Pauling & Corey [25], the G-C base pair can form three hydrogen bonds as against two for the A-T base pair, it appeared logical to conclude that a higher G-C content should impart greater stability, as was found to be the case by Marmur & Doty [19] for both natural and synthetic twin-stranded polynucleotide helices. The view also prevailed for some time that the hypochromicity of polynucleotides was due, in large part, to the effects of hydrogen bonding on the absorption of the individual bases [1]. This latter postulate was soon shown to be incorrect [16, 21, 34] and, to a considerable extent, studies on the origin of hypochromicity in polynucleotides [15, 37, 27] provided the stimulus for a theoretical investigation of the factors responsible for the degree of stability of such polynucleotide helices. The results of these theoretical studies [5, 6], although as yet insufficiently refined, lead to the conclusion that hydrogen bonding contributes relatively little to stability, and that other factors must be involved. These are extensively enumerated by DeVoe & Tinoco [5, 6], the principal ones

being considered to be electrostatic and London forces between the regularly "stacked" bases in the helix. This should not, of course, be interpreted as mitigating the importance of the twin-strand hydrogen bonds which undoubtedly are a necessary prerequisite for the specifity required to line up the base pairs in the formation of a helix. In any event the theoretical predictions [6] do not show very good agreement with experiment, e.g. poly-dA-dBrU is expected to be more stable than the non-brominated polymer because of the greater polarizability of BrU [6], but, as indicated above, this is true only at low ionic strength [14]. It is clear that further theoretical studies and additional experimental data will be required in order to fully elucidate the sources of helix stability; and it is perhaps not out of place to draw attention to two additional points.

If the stability of a polynucleotide helix were due largely to hydrogen bonding, one might expect an increase in stability of a helix in heavy water since a hydrogen bond to deuterium is intrinsically stronger. However, neither poly-A (unpublished work from this laboratory), natural DNA [3], nor the twin-stranded complex of poly-A+poly-U [23] exhibit any essential differences either in their temperature profiles or T_m values in light and heavy water. The temperature of inactivation of transforming DNA is altered only slightly, if at all, in heavy water (unpublished experiments). While theoretical considerations [31] indicate that it is not feasible as yet to predict quantitatively the effects of deuteration on the helix-coil transition in polypeptides, the foregoing results argue, albeit indirectly, against hydrogen bonding as a major factor in the stability of polynucleotide helices.

Other promising experimental methods of approach have been based on the use of organic solvents [9, 13] and various electrolytes [12] for studying the nature of the helix-coil transition in natural nucleic acids under conditions in which the transition may be reversible or irreversible. The results of such investigations also provide strong evidence for participation of forces additional to hydrogen bonding in maintenance of helix stability; in addition they emphasize the significant role of the solvent itself. The application of these procedures to synthetic polymers, such as those employed in the foregoing investigation, might be expected to facilitate an interpretation of the results. Such studies are under way in this laboratory.

EXPERIMENTAL

A Hilger Uvispek and a Unicam SP-500 were used for spectral measurements. Temperature profiles for the various polymers were run on the Hilger instrument, equipped with a specially constructed three-cuvette compartment block, the temperature of which was varied at

will by means of a current of water or water-glycerine mixtures from a Hoepler ultrathermostat. Temperature measurements were standardized against those of the thermostat by means of copper-constantan thermocouples. Chromatographic data were obtained as indicated in Table 1.

Table 1

R_F values of various uridine derivatives

Ascending chromatography, Whatman paper no. 1. Solvents: (A), Ethanol - 1 M-ammonium acetate (5:2, v/v); (B), propan-2-ol - 1% ammonium sulphate (3:2, v/v); (C), propan-2-ol - NH_4OH (d-0.88) - water (7:1:2, by vol.); (D), butanol saturated with 0.2 N-HCl.

Compound	R_F in solvent system			
	A	*B*	*C*	*D*
Uridine	0.72	—	0.50	0.23
5-Fluorouridine	0.73	—	0.50	0.47
2′ : 3′-*O*-Isopropylidene-5-fluorouridine	0.95	—	0.87	—
5-Fluorouridine-5′-phosphate	0,27	0.52	0.19	0.22
Uridine-5′-phosphate	0.25	0.50	0.18	0.19
Uridine-5′-pyrophosphate	0.10	0.38	—	—
5-Fluorouridine-5′-pyrophosphate	0.12	0.42	—	—

2′:3′-Isopropylidene-5-fluorouridine. This was prepared from 5-fluorouridine as described by Levene & Tipson [17] for the analogous derivative of uridine. The yield was essentially quantitative and, following recrystallization from ethyl acetate by addition of petroleum ether (40 - 60°), the compound melted at 177 - 178°. For $C_{12}H_{15}O_6N_2F$: calculated N, 9.27%, determined N, 9.12%.

5-Fluorouridine-5′-phosphate. The foregoing compound was phosphorylated with β-cyanoethylphosphate as described by Tener [36], the reaction being essentially quantitative. Following removal of the cyanoethyl group in 0.5 N-NaOH at 100° for 40 min., the isopropylidene group was removed by hydrolysis in 1 N-HCl at 100° for 30 min. The fluorouridine-5′-phosphate was isolated as the calcium salt [20] and purified by three precipitations with ethanol from aqueous solution. It was chromatographically homogeneous in solvents *A* and *B* and gave a positive periodate reaction and, under the influence of snake venom, was quantitatively transformed to fluorouridine.

5-Fluorouridine-5′-pyrophosphate. 95 mg. of the calcium salt of F-UMP (0.25 mM) was dissolved in water and the calcium removed by means of Dowex-50-H^+. The solution was concentrated to small volume and the remaining water removed by azeotropic distillation with water and benzene. The residue was then transformed to the mono-(tri-*n*-octylamine) salt and phosphorylated with an excess of dibenzylphosphochloridate [20, 34]. The F-UDP was then isolated as the calcium salt,

freed from traces of F-UMP and inorganic phosphate by two precipitations with ethanol from acid (pH 2.5 - 3.0) aqueous medium, and transformed to the sodium salt by means of Dowex-50-Na^+. The yield was 85 mg. by weight and, spectrally (λ_{max} 269 mμ and ε_{max} 9.2×10^3 as for fluorouridine at pH 5 [2]) 60 mg., which corresponds to the formula $C_9H_{10}O_{12}N_2P_2FNa_3 \cdot 2H_2O$, the overall yield thus being 62%. The product was chromatographically homogeneous in solvents *A* and *B*, gave a positive periodate reaction and was quantitatively hydrolysed by snake venom to fluorouridine. It was active as a substrate of polynucleotide phosphorylase.

Poly-FU. The enzymic synthesis of poly-FU was carried out as described by Grunberg-Manago *et al.* [11, cf. 35], but it was found necessary to increase the $MgCl_2$ concentration from 2.5 mM to 10 mM in order to obtain a reasonable yield of polymer (28 - 32%), which was twice precipitated with ethanol, dialysed for 24 hr. at 3° against 0.005 M-NaCl and stored in aqueous medium in the deep-freeze.

The polymer was quite stable in aqueous medium, and did not undergo degradation such as frequently encountered with preparations of poly-rT [35] and poly-C [39].

Secondary structure. This and complexing ability with poly-A, etc. were examined by standard methods as previously described [38, 28, 34].

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SUMMARY

Poly-5-fluorouridylic (poly-FU) acid has been prepared, and found to exist as a random coil at room temperature. In 0.01 M-magnesium chloride it exhibits some secondary structure below 5°. Poly-FU forms a twin-stranded complex with poly-A in 0.15 M-NaCl + 0.015 M-sodium citrate the resulting helix being only slightly less stable than that formed between poly-U and poly-A. In 0.1 M-borate buffer the poly-FU + poly-A helix dissociates reversibly at pH 8.2 as compared to pH 9.5 for the corresponding poly-U + poly-A helix, in agreement with the lower pK for dissociation of fluorouracil as compared to uracil residues.

Both poly-ClU and poly-BrU exhibit secondary structure at room temperature and, in 0.15 M-NaCl, form only triple-stranded complexes

with poly-A. In 0.015 M-NaCl, they form twin-stranded complexes with poly-A; and the poly-BrU+poly-A twin-strand is more stable than those formed by poly-A with either poly-U, poly-FU or poly-rT.

The results are discussed in relation to the factors conferring stability on twin-stranded helical poly-nucleotides.

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OTRZYMYWANIE KWASU POLI-5-FLUOROURYDYLOWEGO, WŁAŚCIWOŚCI 5-CHLOROWCOPOCHODNYCH KWASU POLI-URYDYLOWEGO I ICH KOMPLEKSÓW Z KWASEM POLI-ADENYLOWYM

Streszczenie

Przeprowadzono syntezę kwasu poli-5-fluorourydylowego (poli-FU) i stwierdzono, że w pokojowej temperaturze w 0.01 M-$MgCl_2$ nie posiada on struktury drugorzędowej, a w temperaturze poniżej 5° wykazuje pewien stopień uporządkowania łańcucha. Poli-FU w 0.15 M-NaCl+0.015 M-cytrynianie sodowym tworzy dwułańcuchowy heliks z kwasem poli-adenylowym, nieznacznie mniej trwały od powstającego pomiędzy poli-U i poli-A. W 0.1 M buforze boranowym kompleks poli-FU+ +poli-A dysocjuje odwracalnie w pH 8.2. Analogiczna wartość dla kompleksu poli-A+poli-U wynosi 9.5; różnica ta jest zgodna z niższą wartością pK 5-fluorouracylu w porównaniu z uracylem.

Kwasy poli-5-chlorourydylowy (poli-ClU) i poli-5-bromourydylowy (poli-BrU) wykazują obecność struktury drugorzędowej w 0.01 M-$MgCl_2$ w pobliżu temperatury pokojowej, a w 0.15 M-NaCl tworzą z poli-A kompleksy trójłańcuchowe. W 0.015 M-NaCl tworzą kompleksy dwułańcuchowe z poli-A. Stwierdzono, że podwójny łańcuch poli-BrU+poli-A jest trwalszy niż odpowiednie kompleksy zawierające kwasy poli-urydylowy, poli-5-fluorourydylowy i poli-rybotymidylowy.

Wyniki przedyskutowano z punktu widzenia czynników stabilizujących dwułańcuchową strukturę polinukleotydów.

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THE TRANSFER OF THE PHOSPHORIC ESTER OF *N,N*-DIETHYLAMINOETHANOL FROM ITS CYTIDYLYL DERIVATIVE INTO PHOSPHOLIPIDS OF RAT AND CHICKEN TISSUE HOMOGENATES

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The *in vivo* formation of lecithin analogues containing "unnatural" bases other than choline has been reported. When arsenocholine [19] and triethylaminoethanol [16] were administrated orally to rats these "unnatural" bases were found in the phospholipid fraction. The *in vivo* incorporation of 2-amino-2-methyl-propan-1-ol [14] and of trimethylaminopropan-2-ol [3] into phospholipids has also been described. This could be the result of an exchange reaction similar to the mechanism of incorporation of free choline into lecithin described by Dils & Hübscher [7], or the incorporation of an "unnatural" base could be explained by low specificity of the enzymes catalysing the reactions of the cytidine-dependent pathway [11] of phospholipid formation. The description given by Kennedy [11] indicated that two enzymes, ethanolaminephosphate cytidylyltransferase (EC 2.7.7.14) and cholinephosphate cytidylyltransferase (EC 2.7.7.15), catalyse the formation of cytidine diphosphate ethanolamine and cytidine diphosphate choline[1]. This implicates their specificity for phosphorylethanolamine and for phosphorylcholine, respectively. However, Ansell & Chojnacki [1] found that the next step in the reaction chain, i.e. the transfer of the phosphorylated bases onto diglycerides catalysed by ethanolaminephosphotransferase (EC 2.7.8.1) and cholinephosphotransferase (EC 2.7.8.2), was not so specific. When namely the mono- or dimethylaminoethanol phosphates were coupled with CMP they were used for phospholipid biosynthesis at the same rates as CMP-PC or cytidine diphosphate ethanolamine [1]. This may be considered as due to a lack of specificity of the respective enzymes. However, the presence of *N*-monomethylated and *N,N*-dimethylated analogues of phosphatidylethanolamine in a mutant of *Neurospora crassa* and in liver tissue, described by Wolf & Nyc [20] and Bremer & Greenberg [4], could suggest another possibility, viz. of the presence of enzymes

[1] Abbreviations used: CMP, cytidine-5′-monophosphoric acid; CMP-PC, cytidine diphosphate choline; CMP-PDEAE, cytidine diphosphate *N,N*-diethylaminoethanol; DEAE, *N,N*-diethylaminoethanol; PC, phosphorylcholine; PDEAE, *N,N*-diethylaminoethanol phosphate.

specifically transferring *N*-monomethylaminoethanol phosphate and *N*,*N*-dimethylaminoethanol phosphate from their CMP derivatives onto diglycerides.

In the present study an attempt was made to replace the natural bases of phospholipids by *N*,*N*-diethylaminoethanol (DEAE) which as far as we are aware has not been reported to be a natural constituent of phospholipids. The incorporation of this "unnatural" base was achieved by incubating tissue homogenates with the chemically synthetised ^{32}P-labelled cytidine diphosphate *N*,*N*-diethylaminoethanol (CMP-^{32}PDEAE). The procedures of isolating the "unnatural" DEAE-containing phospholipid are also described.

MATERIALS AND METHODS

Animals. Albino rats 6 weeks old and chickens 3 - 4 months old were used. The 20% tissue homogenates were prepared in a Potter-Elvehjem homogenizer in a medium consisting of 10 parts of 0.154 M-KCl and 3 parts 0.154 M-NaF. The grey matter of rat brain was obtained by cutting off the surface parts of the haemispheres.

Special reagents. ^{32}P-Labelled phosphoric acid of French origin was distributed by Nuclear Research Institute, Warszawa; CMP, Sigma, USA; dicyclohexylcarbodiimide, BDH, London; *N*,*N*-diethylaminoethanol, BDH, redistilled (b.p. 161 - 162°); aluminum oxide, Brockman activity 2, was Savory & Moore (Great Britain) product.

Assay of phosphorus and ^{32}*P.* Phosphorus was assayed according to Strickland *et al.* [17], and the amount of ^{32}P with a VA-Z 410 VEB Vacutronic liquid counter.

Chemical syntheses of ^{32}*P-labelled compounds*

CMP-^{32}*PDEAE.* This synthesis involved two steps: the synthesis of ^{32}P-labelled phosphoric ester of DEAE and the coupling with CMP in the presence of dicyclohexylcarbodiimide by the method of Kennedy [12]. The [^{32}P]*N*,*N*-diethylaminoethanol phosphate (^{32}PDEAE) was obtained by heating equimolar amounts of DEAE and $H_3{}^{32}PO_4$ in the presence of P_2O_5 at 130° under reduced pressure for 6 hr. Then the reaction mixture was dissolved in water, heated for 15 min. at 100°, neutralized to pH 8, and the ester adsorbed on Dowex 1 formate column. Then the column was washed with water and ^{32}PDEAE was eluted with 0.05 M-HCOOH. The yield of the synthesis was about 5%. As revealed by paper chromatography in different solvents the obtained ^{32}PDEAE was identical with the standard compound synthetised in this laboratory from DEAE and $POCl_3$.

To 15 μmoles of ^{32}PDEAE and an equimolar amount of CMP, 0.1 ml. of water, 0.7 ml. of pyridine, and 150 mg. of dicyclohexylcarbodiimide

were added, and the mixture left for three days at 37°. The semisolid reaction mixture was then mixed with 10 ml. of water, washed with ethyl ether, the lower aqueous phase after filtration was again washed twice with ether, and the trace of ether removed *in vacuo*. The solution was neutralized to pH 8, diluted to 100 ml. and passed through the Dowex 1 formate column (10 × 1.2 cm.). Elution was made by concentration gradient of HCOOH (300 ml. of water in the mixing chamber, 300 ml. of 0.04 M-HCOOH in the reservoir). Fractions of 10 ml. were collected and their ^{32}P contents and extinctions at 280 mμ were estimated. The first and the third peaks (Fig. 1) contained the unreacted ^{32}PDEAE

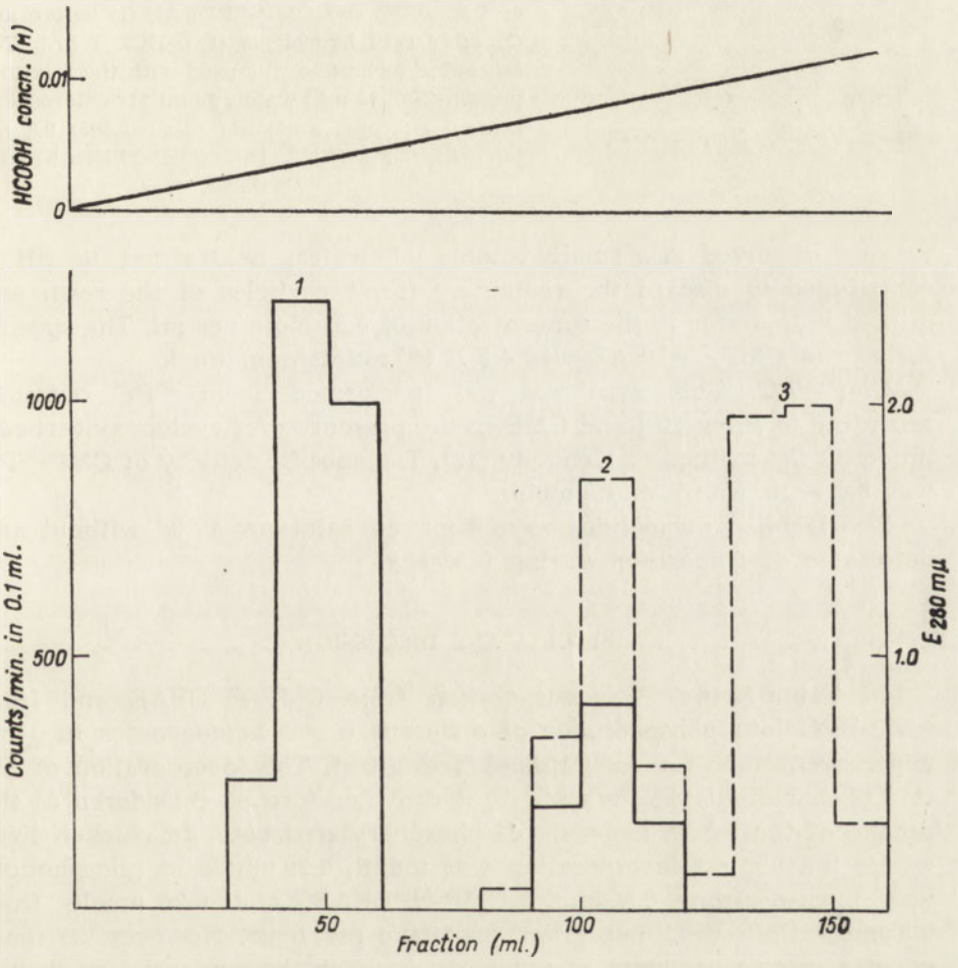

Fig. 1. Column chromatography of the products of the reaction of CMP with [^{32}P]*N*,*N*-diethylaminoethanol phosphate in the presence of dicyclohexylcarbodiimide. The elution was made by concentration gradient of formic acid. (————), Counts/min., (— — —), E_{280}. Peak 1, [^{32}P]*N*,*N*-diethylaminoethanol phosphate; peak 2, cytidine diphosphate *N*,*N*-diethylaminoethanol; peak 3, CMP

and CMP, respectively. The second peak containing CMP-^{32}PDEAE eluted between 90 - 120 ml. of effluent showed the ratio of cytidine to phosphorus 1 : 1.89 (calculated 1 : 2), and the ratio of its specific activity (counts/min./μmole P) to that of free ^{32}PDEAE was 0.53 (calculated 0.5). On acid hydrolysis (1 hr., 100°, 1 N-HCl) it was decomposed similarly to the synthetic (see below) CMP-^{32}PC with the formation of CMP and ^{32}PDEAE (Fig. 2). The fraction from the column containing CMP-^{32}PDEAE was evaporated to dryness at room temperature, the

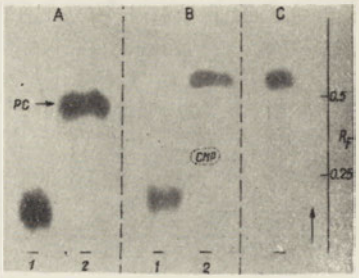

Fig. 2. Autoradiogram of paper chromatogram of CMP-^{32}PC and CMP-^{32}PDEAE (1) before and (2) after acid hydrolysis (1 N-HCl, 1 hr.). The ascending technique was used with the mixture: propan-2-ol, 75 ml.; water, 25 ml.; trichloroacetic acid, 5 g.; conc. ammonia ($d_{20} = 0.895$) 0.3 ml. [8]. (A), CMP-^{32}PC; (B), CMP-^{32}PDEAE; (C), ^{32}PDEAE

residue dissolved in a small volume of water, neutralized to pH 7, centrifuged to discard the remaining finest particles of the resin and diluted with water to the concentration of 0.5 μmole per ml. The specific activity of CMP-^{32}PDEAE was 4.5×10^4 counts/min./μmole.

CMP-^{32}PC. This synthesis was performed from ^{32}PC obtained according to Riley [18] and CMP in the presence of dicyclohexylcarbodiimide by the method of Kennedy [12]. The specific activity of CMP-^{32}PC was 8.8×10^5 counts/min./μmole.

The labelled nucleotides were kept as solutions at 0° without any detectable decomposition during 6 weeks.

RESULTS AND DISCUSSION

In Table 1 the ^{32}P incorporation from CMP-^{32}PDEAE and from CMP-^{32}PC into phospholipids of different tissue homogenates in 1-hr. experiments (one for each tissue) are given. The incorporation of ^{32}P into phospholipids was very high, it can therefore be considered as the uptake of the whole molecule of phosphorylated base. In chicken liver where the highest incorporation was found, 0.29 μmole of phospholipid was formed from 0.5 μmole CMP-^{32}PDEAE, and 0.26 μmole from 0.5 μmole CMP-^{32}PC, per gram of tissue per hour. However, in these experiments no net increase of phospholipid phosphorus could be shown, since a large amount of lipid phosphorus was already present in tissue homogenate (1 g. of chicken liver contained about 30 μmoles of lipid phosphorus). In rat tissues the incorporation of ^{32}P into phospholipids from CMP-^{32}PDEAE was about 30 - 40% lower than that from CMP-^{32}PC.

To evaluate the effect of a possible difference in the decomposition rate of CMP-^{32}PDEAE and CMP-^{32}PC in rat tissues on the incorporation of ^{32}P into phospholipids, the amount of the remaining, non-incorporated substrate was estimated at the end of 1-hr. incubation. The trichloroacetic acid filtrate was treated with charcoal, and the radioactivity of the adsorbed nucleotide was estimated. In rat brain 58% of the radioactivity present in trichloroacetic acid filtrate was in the nucleotide form in the case of CMP-^{32}PDEAE. The corresponding value for CMP-^{32}PC was 64%. Thus the effect of different decomposition rates was negligible. In rat liver both compounds were decomposed more rapidly, and after one hour only traces of radioactivity were adsorbed on charcoal.

Table 1

The incorporation of ^{32}P from CMP-^{32}PDEAE and from CMP-^{32}PC into phospholipids of tissue homogenates

Each incubation sample contained 100 mg. of homogenized tissue in a medium containing: 0.0316 M-KCl; 0.0095 M-NaF; 0.02 M-$MgCl_2$; 0.004 M-Na_2HPO_4; 0.0266 M-tris - HCl buffer, pH 7.4; CMP-^{32}PDEAE, 0.05 μmole, or CMP-^{32}PC, 0.05 μmole, were added as indicated. The final volume of incubate was 1.5 ml. Incubation period 1 hr. at 37°. The phospholipids were extracted according to Folch [9] and the radioactivity was measured as described in Methods.

Tissue homogenate	CMP-^{32}PDEAE added		CMP-^{32}PC added	
	^{32}P incorporated (%)	Total lipid P extracted (μg.)	^{32}P incorporated (%)	Total lipid P extracted (μg.)
Rat brain (grey matter)	16.1	105	23.8	90
Rat liver	23.6	88	40.0	90
Chicken liver	58.5	86	53.5	86

To obtain an amount of the expected "unnatural" phospholipid sufficient for analytical purposes, the radioactive phospholipids formed from CMP-^{32}PDEAE obtained by the incubation of homogenate from 0.5 g. of rat liver, were chromatographed on a column of aluminum oxide using chloroform - methanol (1 : 1, v/v) mixture with increasing concentrations of water [13] (Fig. 3). In the first peak eluted with the sole chloroform - methanol (1 : 1, v/v) mixture, which showed only slight radioactivity, choline-containing lipids were also present. The second peak, which was the main radioactive peak, was eluted with chloroform - - methanol mixture (1 : 1, v/v) containing 1 - 3% of water. This was similar to the elution of phosphatidyl *N,N*-dimethylaminoethanol [2]. On paper chromatography according to Marinetti [15] the radioactive materials of these two peaks moved as single spots free from any other

material stained with rhodamine (Fig. 4). The radioactive phospholipids formed in rat liver homogenate from CMP-^{32}PDEAE were converted to the water-soluble derivatives on mild alkaline hydrolysis [5]. The labelled water-soluble material was then separated from other phosphorus compounds of the hydrolysate by two-dimensional chromatography according to Dawson [6]. It moved as a single spot, and had identical R_F as glycerylphosphorylcholine in the phenol - ammonia sol-

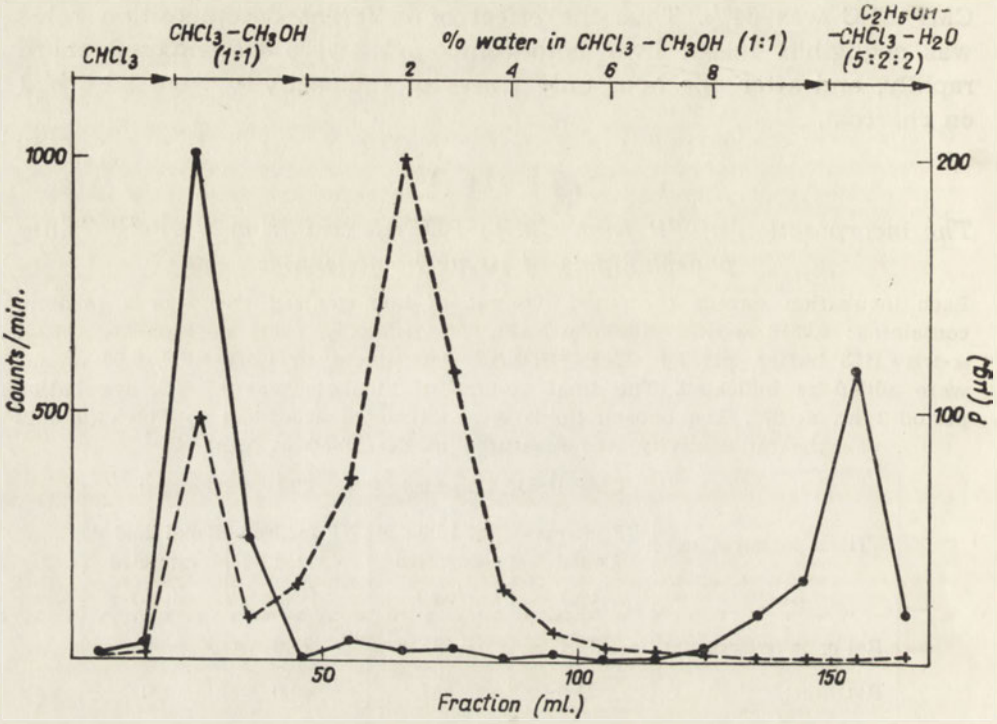

Fig. 3. Chromatography on aluminum oxide of the lipids obtained after the incubation of rat liver homogenate with CMP-^{32}PDEAE. An amount of lipid corresponding to 0.5 g. of fresh tissue was applied to a 5 g. column (0.8 × 12 cm.) and the elution carried out in a step-wise fashion. (+), Counts/min.; (●), µg. P

vent, but had a higher R_F in the second solvent composed of tert. butanol - water - trichloroacetic acid (Fig. 5). Since the chromatography of intact phospholipids on the aluminum oxide column has shown the presence of two radioactive peaks, we assume that the second, i.e. the main one is due to the phosphatidyl *N,N*-diethylaminoethanol, and the first one, smaller, could be due to the product formed by the methylation of phosphatidyl *N,N*-diethylaminoethanol according to the mechanism proposed by Bremer & Greenberg [4]. The water-soluble derivatives of phosphatidyl *N,N*-diethylaminoethanol and of its methylated form could not be separated by the method of Dawson [5, 6].

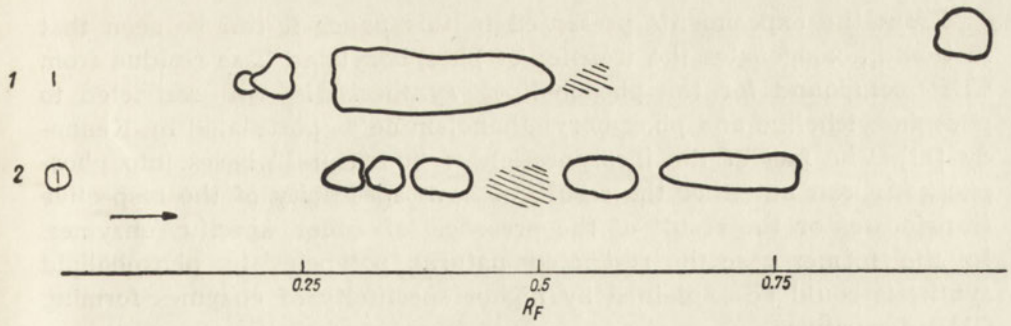

Fig. 4. Autoradiogram (scheme) of radioactive phospholipids formed from CMP-^{32}PDEAE in rat liver homogenate. Chromatography on silicic acid paper was performed with the mixture of diisobutylketone - acetic acid - water (40:20:3, by vol.) [15]. The hatched areas represent the position of radioactive material, the rhodamine-positive spots are marked with pencil. (1), Phospholipids eluted from aluminum oxide column with the mixture of chloroform - methanol (1:1); (2), phospholipids eluted from aluminum oxide column with the mixture of chloroform - methanol (1:1), containing 1 - 3% of water (Fig. 3)

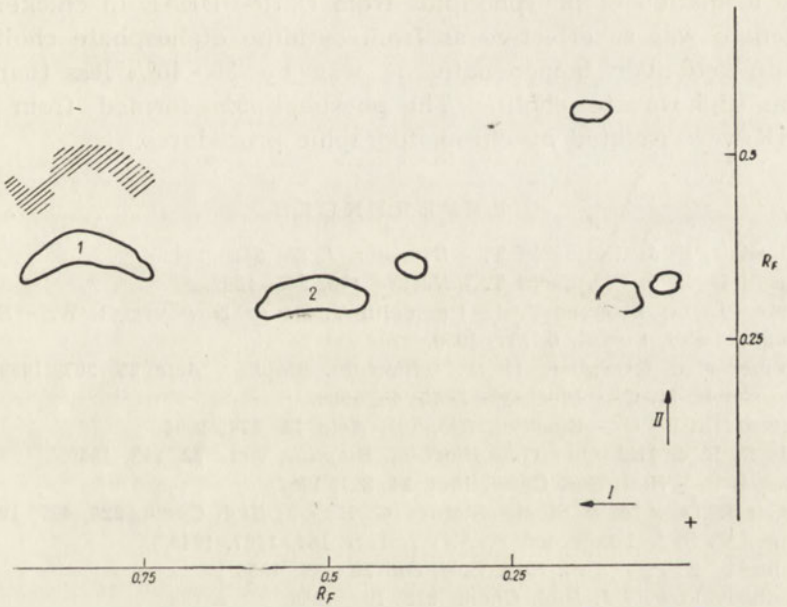

Fig 5. Autoradiogram (scheme) of a two-dimensional chromatogram of an alkaline hydrolysate [5] of the lipids of rat liver homogenate incubated with CMP-^{32}PDEAE. Details of the incubation as in Table 1. The following solvent systems were employed: *I*, phenol - water - conc. ammonia, $d_{20} = 0.895$ (80:20:0.3, w/v/v); *II*, trichloroacetic acid (10%, w/v) in 2-methylpropan-2-ol - water (62:38, v/v) [6]. The hatched area represents the radioactive material. The phosphorus-containing spots, (1), glycerylphosphorylcholine, (2), glycerylphosphorylethanolamine, and others were localized with acid molybdate spray [10] and marked with pencil

From the experiments presented in this paper it can be seen that in tissue homogenates the transfer of phosphorylated base residue from CDP compound for the phospholipid synthesis is not restricted to phosphorylcholine and phosphorylethanolamine as postulated by Kennedy [11]. The fact of the incorporation of "unnatural" bases into phospholipids can be either the result of low specificity of the respective transferases or the result of the presence of other specific enzymes. In the former case the restricted natural potencies in phospholipid synthesis could be explained by higher specificity of enzymes forming CMP derivatives.

The use of a phosphorylated base coupled with CMP may provide a very efficient method for the *in vitro* biosynthesis of various phospholipids, which could be also applied for preparing phospholipid analogues on a larger scale.

SUMMARY

The chemical synthesis of cytidine diphosphate *N,N*-diethylaminoethanol (CMP-PDEAE) is described.

The formation of phospholipids from CMP-PDEAE in chicken liver homogenate was as effective as from cytidine diphosphate choline; in rat brain and liver homogenates it was by 30 - 40% less than from cytidine diphosphate choline. The phospholipids formed from CMP--PDEAE were isolated by chromatographic procedures.

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PRZENOSZENIE ESTRU FOSFOROWEGO *N,N*-DWUETYLOAMINOETANOLU Z POCHODNEJ CYTYDYLOWEJ DO FOSFOLIPIDÓW W HOMOGENATACH TKANEK SZCZURA I KURCZĘCIA

Streszczenie

Przeprowadzono syntezę chemiczną cytydynodwufosfo-*N,N*-dwuetyloaminoetanolu (CMP-PDEAE). Związek ten okazał się równie efektywnym jak cytydynodwufosfocholina dla biosyntezy fosfolipidów w homogenacie wątroby kurczęcia. W homogenatach wątroby i mózgu szczura powstawanie fosfolipidów z CMP-PDEAE było o 30 - 40% mniejsze niż z cytydynodwufosfocholiny. Fosfolipidy powstające w homogenatach tkanek z CMP-PDEAE wydzielono metodami chromatograficznymi.

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