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Formation of Uridine Phosphates from Uracil in Extracts of Ehrlich Ascites Tumor

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The enzymic formation of uridine monophosphate from uracil in extracts from Ehrlich ascites tumor has been demonstrated to proceed according to two different mechanisms. The first involved the formation of uridine from uracil + ribose-1-phosphate by the action of nucleoside phosphorylase and the subsequent phosphorylation of uridine with ATP. This mechanism was by far the predominant one in the acetone powder extracts.

The second mechanism involved the direct condensation of uracil with phosphoribosyl pyrophosphate. In this case only very small amounts of UMP synthesis were observed.

The work of Plentl and Schoenheimer [1] originally demonstrated that uracil-^{15}N when injected into rats was not utilized for the biosynthesis of polynucleotide pyrimidines. Even though later work from different laboratories with uracil-^{14}C showed that there was a slight incorporation of the pyrimidine especially into fast growing organs [2,3], comparison of the incorporation data with results obtained with labeled nucleosides or orotic acid [4] demonstrated that the utilization of uracil was very much smaller than that of the other compounds. In the mouse, however, the incorporation of uracil and orotic acid was of the same order of magnitude [5].

The present investigation was started with the aim to investigate the pathway of uracil incorporation in the mouse. *In vivo* experiments demonstrated that labeled uridine phosphates appeared in the acid soluble fraction of mouse liver after injection of uracil-^{14}C in a similar manner as after injection [6] of orotic acid-^{14}C. It seemed justified to assume that the incorporation of uracil into polynucleotide pyrimidines proceeded *via* uridine phosphates. Our investigation was therefore confined to a study of the enzymic formation of uridine monophosphate (UMP)* from uracil.

* The following abbreviations are used throughout this paper: UMP = uridine-5′-phosphate, ATP = adenosine triphosphate, PGA = 3-phosphoglyceric acid, PCA = perchloric acid, PRPP = phosphoribosyl pyrophosphate.

EXPERIMENTAL

Uracil-^{14}C (80 000 ct/min/μmole) was synthesized according to Johnson and Flint [7]. Ribose-5-phosphate, ATP and PGA were commercial products (Schwarz Laboratories, Inc. and Sigma Chemical Company). *Ribose-1-phosphate* was a gift from Dr. H. Klenow, Copenhagen, for which we are very much indebted. *Phosphoribosyl pyrophosphate* was prepared enzymically as described earlier [8]. The preparation was 62 % pure judged from ribose analysis and had a ratio of ribose: acid labile phosphate of 1:1.96. It contained no ATP.

Preparation of enzyme extract. The ascites tumor was originally obtained through the courtesy of Dr. G. Klein, Institute of Cell Research, Karolinska Institutet. Routinely, it was kept by transfer of 0.1 ml of ascitic fluid 8—10 days after the previous inoculation. For the preparation of the acetone powder the ascitic fluid from 20—50 mice on the tenth day after inoculation was pooled and centrifuged cold at 1 000 *g* for 10 min. The sediment was washed twice with physiological sodium chloride and finally suspended in enough sodium chloride solution to give the original ascites volume. The solution was mixed in a Waring blendor with 10 volumes of acetone (−15°), filtered by suction and washed with cold acetone. The residue was dried in a desiccator and kept dehydrated at −15°. *Ca.* 4 g of acetone powder was obtained per 100 ml of ascites.

Immediately before use the powder was extracted with ice cold 0.05 M potassium phosphate buffer, pH 7.4 (10—50 ml per g powder) in a Potter-Elvehjem homogenizer. Three treatments were carried out at 10 min intervals. After centrifugation (15 000 *g* for 15 min) at 0° a clear red extract was obtained which was directly used for the experiments.

Assay of enzyme activity. The enzyme reaction was stopped by addition of 0.1 volume of 4.4 M PCA to the cold solution. The protein precipitate was centrifuged and washed once with 1 ml of 0.6 M PCA.

Two different methods were used after that. In *method 1* the combined supernatants were neutralized with 4 M KOH (phenol red) and the perchlorate was allowed to precipitate overnight in the refrigerator. The supernatant was then chromatographed on a Dowex-2 column according to Hurlbert *et al.*[9] Fractions within the different radioactive peaks were combined and the total radioactivity of each compound was determined on infinitely thin samples. This method gave the amount of the different radioactive uridine phosphates formed from uracil-^{14}C. The identity of the uridine phosphates was based on their localization on the chromatogram.

In *method 2* pyrophosphate linkages were hydrolyzed with acid so that all uridine phosphates were converted to UMP. This was achieved by heating the PCA supernatant at 100° for 1 h. Neutralization with KOH was then carried out as described above. The neutralized solution was then adsorbed on a Dowex-2-formate column (diameter 0.9 cm, length 5 cm). The column was washed with 50 ml of water which removed all uracil and uridine. Thereafter UMP was removed from the column with 60 ml of 6 N formic acid. The total radioactivity of the UMP fraction was determined and from this value and the specific activity of the uracil-^{14}C the amount of UMP formation could be calculated.

A blank which contained no enzyme was carried through the whole procedure and a small correction for "UMP formation" in the blank was applied.

Uracil and uridine could be separated and their specific activities determined by the following procedure: The first fraction from the Dowex-2 column was passed through a Dowex-50-H^+ column (0.9 cm diameter, 5 cm length). After washing with 30 ml of water the eluent was evaporated to dryness *in vacuo*. The dry residue was dissolved in butanol-water and chromatographed on a starch column (2 cm diameter, 20 cm length). The amount of the separated uracil and uridine was determined by light absorption and their specific activities were calculated after determination of radioactivities on infinitely thin samples.

Experiments with whole cells. (*cf.*[5]). In each of two 200 ml Erlenmeyer flasks 6 ml of ascites was mixed with 10 ml of Krebs-Henseleit substrate, from which $CaCl_2$ had been omitted. 50 μmoles of fructose diphosphate was added to each flask, and 25 μmoles of orotic acid-^{15}N to one vessel. The flasks were filled with an atmosphere of oxygen:carbon dioxide (95:5) and kept in an ice bath for 2 h in order to permit orotic acid to penetrate into the cells.[5] After that 25 μmoles of uracil-^{14}C was added and incubation carried out at 37° for another 2 h period.

The reaction was stopped by addition of 1.6 ml of 4.4 M PCA and the solution was mixed in a Waring blendor. The cell mass was filtered, washed with cold 0.2 M PCA and dried with alcohol and ether.

The PCA filtrate was neutralized with KOH and the acid soluble uridine phosphates were prepared from it as described above.

The polynucleotides from the ascites cells were prepared according to Hammarsten [10]. The pyrimidine ribosides were obtained by starch chromatography as described earlier [11]. The different compounds were analyzed for ^{15}N in the mass spectrometer and for ^{14}C in a Tracerlab SC-18 windowless flow counter (infinitely thin samples).

RESULTS

Extracts from acetone powder of Ehrlich ascites tumor showed a small but readily demonstrable capacity to form UMP from uracil in the presence of ribose-5-phosphate, ATP, Mg^{++} and an ATP regenerating system. The *same experimental conditions*, however, did not allow UMP synthesis in extracts from mouse liver acetone powder. All our subsequent experiments were carried out with the ascites tumor.

When starting our investigation we visualized three different pathways which might explain UMP formation: (1) Uracil might be carboxylated to orotic acid and orotic acid might subsequently by known enzymic pathways [12] be converted to UMP. (2) Uracil might replace orotic acid in the condensation with Kornberg's phosphoribosylpyrophosphate [12] and UMP be formed directly. (3) Uracil might react with ribose-1-phosphate [13] (formed from the added ribose-5-phosphate[14]) and form uridine. After that uridine might be phosphorylated by ATP with the formation of UMP, a reaction which we had earlier observed in rat liver extracts.

Formation of orotic acid

The influence of the presence of orotic acid-^{15}N on the formation of acid soluble uridine phosphates and polynucleotide pyrimidines from uracil^{14}-C is demonstrated in Table 1. Freshly obtained tumor cells were incubated with

Table 1. Non-involvement of orotic acid in UMP formation from uracil.

	Uracil + orotic acid			Uracil	
	μmoles formed	ct/min/μmole	atom per cent excess ^{15}N	μmoles formed	ct/min/μmole
Acid soluble fraction:					
UMP	0.12			0.15	
Uridine pyrophosphates	0.02			0.02	
Pentose nucleic acid:					
Uridine		1 290	0.252		1 330
Cytidine		410	0.063		570
Deoxypentose nucleic acid:					
Thymine		210	0.078		170
Cytosine		120	—		150

Ascites cells were incubated with uracil-^{14}C (80 000 ct/min/μmole) and orotic acid-^{15}N (16 atom per cent excess). For further explanation see text.

Krebs-Henseleit solution together with orotic acid. After 2 h at 0° uracil was added and incubation continued for two more hours. In the control vessel preincubation was carried out without orotic acid. The acid soluble nucleotides and the pyrimidines from polynucleotides were prepared in both experiments and the amount of ^{14}C in the different fractions was measured. Clearly the presence of orotic acid very little influenced the incorporation of uracil. The ^{15}N-data show that orotic acid did penetrate into the cells.

A similar experiment was carried out with an acetone powder extract from the tumor in a bicarbonate buffer. Again the presence of orotic acid did not influence the formation of radioactive uridine phosphates from uracil-^{14}C.

Reaction of uracil with phosphoribosyl pyrophosphate

The formation of UMP was greatly stimulated by the inclusion of ribose-5-phosphate and ATP in the medium (Table 2). Our experiments had to be carried out with an undialyzed extract since dialysis almost completely destroyed the ability of the system to synthesize UMP. It was conceivable that the requirement for ATP and ribose-5-phosphate indicated the intermediate formation of PRPP from these compounds. In Table 2 are also recorded two experiments, in which PRPP had been substituted for ATP and ribose-5-phosphate. More than six times the amount of "background synthesis" was observed. Inclusion of fluoride (to inhibit enzymic breakdown of PRPP) did not increase UMP synthesis.

Table 2. Involvement of PRPP.

Additions	μmoles UMP formed
Ribose-5-phosphate	0.007
ATP, PGA	0.007
Ribose-5-phosphate, ATP, PGA	0.092
PRPP	0.047
PRPP, NaF	0.037

Each vessel contained 75 μmoles of $MgCl_2$, 10 μmoles of uracil-^{14}C, 150 μmoles of phosphate buffer, pH 7.4 and enzyme (corresponding to 190 mg of acetone powder). Incubation 40 minutes at 37°. Final volume 4.5 ml.

Where indicated ribose-5-phosphate (40 μmoles), ATP (20 μmoles) PGA (60 μmoles), PRPP (17 μmoles) and NaF (200 μmoles) were added.

In these and the following experiments method 2 was used for enzyme assay. In order to ascertain the identity of the analyzed compound with UMP the radioactive fractions obtained by the acid washing from the PRPP experiments were combined and rechromatographed according to method 1. The main part of the radioactivity was localized in the UMP region of the chromatogram.

Intermediate formation of uridine

The possible participation of uridine during the reaction was tested in one experiment in which nonlabeled uridine was included during the incubation of the tumor extract with uracil-^{14}C, ribose-5-phosphate and ATP. With

method 2 it was found that a considerable increase of radioactivity appeared in the UMP fractions as compared to the control. The specific activity and the total amount of the formed UMP were determined after rechromatography. Uracil and uridine were purified by starch chromatography and the total amounts and specific activities of the two compounds were determined. The results (Table 3) show that a large amount of UMP had been formed, while most of the added uridine had disappeared. The specific activity of uracil had been lowered to about half of its original value. Uridine had received isotope during the incubation and its specific activity was between that of uracil and UMP.

Table 3. Uridine as intermediate in UMP formation.

Isolated after incubation	Addition		
	Uridine		None
	μmoles	ct/min/μmoles	μmoles
Uracil	9.0	49 000	*ca.* 7.5
Uridine	0.5	29 000	—
UMP	5.9	10 700	0.07

Each vessel contained 75 μmoles of $MgCl_2$, 20 μmoles of ATP, 60 μmoles of PGA, 40 μmoles of ribose-5-phosphate, 10 μmoles of uracil-^{14}C (80 000 ct/min/μmole) 150 μmoles of phosphate buffer, pH 7.4, and enzyme (corresponding to 190 mg acetone powder). Incubation 40 min at 37°. Volume = 4.5 ml.
Where indicated 10 μmoles of uridine was added.

This experiment gave strong support to the idea that uridine indeed was an intermediate in UMP formation. The fact that the *total amount* of ^{14}C in UMP was very much increased indicated that uridine was not only an intermediate in the formation of UMP from uracil but also had supplied the substrate for the conversion of uracil-^{14}C to uridine-^{14}C. In order to test this possibility inosine was substituted for ribose-5-phosphate in one experiment (Table 4). Good synthesis of UMP was obtained, but only in the presence of ATP.

Table 4 also includes results obtained with ribose-1-phosphate. Good synthesis of UMP was observed in the presence of ATP and Mg^{++}. When uracil and uridine were purified from the incubation mixture it was found that uridine was formed in the absence of ATP and Mg^{++}, while there was little nucleoside in the presence of these substances.

The localization of the phosphate group on the 5′-position of ribose was demonstrated by enzymic dephosphorylation of the formed UMP with snake venom (*Crotalus adamanteus*) 5′-nucleotidase. Complete liberation of the phosphate group was observed under conditions which allowed no dephosphorylation of the 2′- or 3′-phosphates of uridine.

Table 4. Formation of UMP and uridine from ribose-1-phosphate.

Additions	μmoles UMP formed from ^{14}C	μmoles uridine formed		μmoles uracil left	
		from light absorpt.	from ^{14}C	from light absorpt.	from ^{14}C
Inosine, ATP, PGA, Mg^{++}, phosphate	0.37				
Inosine, Mg^{++}, phosphate	0				
Ribose-1-phosphate, ATP, PGA, Mg^{++}	1.32	0		7.1	6.9
Ribose-1-phosphate, ATP, versene	0.05	2.6	2.4	5.0	4.8
Ribose-1-phosphate, versene	0	2.5	2.5	5.8	5.6

Each experiment contained enzyme (corresp. to 60 mg acetone powder), 10 μmoles of uracil-^{14}C and 150 μmoles of tris buffer, pH 7.4. Volume = 4.5 ml, incubation 40 min at 37°.

Where indicated ATP (20 μmoles), PGA (30 μmoles), inosine (10 μmoles), $MgSO_4$ (75 μmoles), versene (10 μmoles), ribose-1-phosphate (7 μmoles), and phosphate, (100 μmoles, pH 7,4) were added.

DISCUSSION

The results obtained by Hurlbert *et al.* [6] from *in vivo* experiments with orotic acid-^{14}C have made it very probable that uridine phosphates from the acid soluble fraction of cells are intermediates in the biosynthesis of polynucleotide pyrimidines. An investigation of the mechanism of uracil incorporation into polynucleotides in the Ehrlich ascites tumor should consequently start with the elucidation of uridine phosphate formation from uracil.

Three possibilities were from the beginning envisaged for the formation of uridine phosphate from uracil. Of these, transformation of uracil to orotic acid is clearly ruled out from our experiments, both with whole tumor cells and with extracts of acetone powder.

The results with acetone powder extracts demonstrated that UMP formation from uracil could take place in the presence of ATP and ribose-5-phosphate. The latter two compounds could be replaced by phosphoribosyl pyrophosphate, a compound which is an intermediate in UMP formation from orotic acid[12,8]. This resulted, however, in a lower synthesis of UMP than in the presence of ATP + ribose-5-phosphate. It seemed therefore unlikely that the function of these compounds was merely to form PRPP. We interpret the results to indicate that a quite low formation of UMP through direct condensation of uracil + PRPP did take place, though the reaction did only account for a fraction of the UMP formed. It is not probable that PRPP broke down to ribose-1-phosphate and that UMP was formed from this compound, since no ATP was added in these experiments and since UMP was not formed from ribose-1-phosphate in the absence of added ATP.

It is evident, however, that the main pathway of the reaction involved the intermediate formation of uridine. Uridine was formed either from uracil+ribose-1-phosphate or from uracil+inosine+phosphate. The enzyme for

uridine formation, pyrimidine nucleoside phosphorylase [13], is well known. In the presence of ATP, uridine was then phosphorylated and formed UMP. This second reaction required Mg^{++}. The reaction sequence is represented below:

$$\text{Uracil} + \text{ribose-1-phosphate} \xrightarrow[\text{phosphorylase}]{\text{nucleoside}} \text{uridine} + \text{phosphate}$$

$$\text{Uridine} + \text{ATP} \xrightarrow[\text{kinase, } Mg^{++}]{\text{nucleoside}} \text{uridine monophosphate} + (\text{ADP})$$

It is not quite clear why uracil is very poorly utilized for polynucleotide and UMP synthesis in many tissues, *e.g.* rat liver, while it is well utilized by the mouse. Nucleoside phosphorylase is a wide spread enzyme in rat tissues and also the kinase has been found by us in rat liver. Canellakis [15] has suggested that the enzymic reduction of uracil to dihydrouracil in rat liver proceeds at such a fast rate that very little uracil is available for synthetic reactions, unless large amounts are administered. Other explanations might also be considered, such as the availability of ribose-1-phosphate in the different tissues or the concentration of the nucleoside kinase.

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