OPERATION OF OROTIC ACID PATHWAY AS THE MAJOR SOURCE OF PYRIMIDINES IN THE HEMATOPOIETIC MOUSE SPLEEN[1/]

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Most of animal tissues other than the liver seemed to lack the enzyme activity to synthesize carbamyl phosphate (Cohen and Brown, 1960; Jones et al., 1961), one of the initial substrates of the orotate pathway for pyrimidine biosynthesis. These findings have posed a problem as to the mechanism of pyrimidine supply to those tissues. It must be determined whether an alternative pathway operates in those tissues, or the tissues depend on preformed pyrimidines or some precursors supplied from the liver, or carbamyl-P synthetase is actually present though its detection has not been possible for some technical difficulty. Ehrlich ascites tumor cells have been studied regarding this problem. Although carbamyl-P synthetase has not been detected (Jones et al., 1961; Kusama and Roberts, 1963), evidence so far presented is consistent with operation of the complete orotate pathway in these cells (Lagerkvist et al., 1955; Kusama and Roberts, 1963; Hager and Jones, 1965). However, the quantitative significance of the pathway in vivo is yet to be elucidated.

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In this paper, evidence is presented that the hematopoietic mouse spleen, a rapidly-growing normal tissue, is provided with the complete orotate pathway starting with bicarbonate in spite of its apparent lack of carbamyl-P synthetase and that this pathway is the major source of uracil in this tissue. The transport of preformed pyrimidines or precursors from the liver seems to play a minor role, if any.

Young adult mice of dd strain were given daily injections of acetylphenylhydrazine, 1 mg per mouse for 4 days. As a result of the hemolytic anemia thus induced, hyperplasia of the spleen took place with concomitant increase in its hematopoietic activity. A synthetic diet containing no nucleic acid (Pitot et al., 1961) was given for the last 2 days before the animal was sacrificed. The in vivo synthesis of uracil in acid-soluble nucleotides in the spleen and the liver was followed after a subcutaneous injection of $NaH^{14}CO_3$. Uracil was isolated as 5-UMP for counting, since the radioactivity of the sugar moiety of UMP was practically none. As shown in Fig. 1, the labeling of uracil in the spleen was rapid and took place without any noticeable lag, reaching a maximum after 30 min following the tracer administration. In addition, the specific radioactivity of the spleen UMP was higher than that of the liver UMP for the time period from 0 to 4 hours. Both of these findings suggest direct entry of $^{14}CO_2$ into the spleen uracil, and it seems unlikely that the hepatic uracil plays the role as main source of pyrimidines in nonhepatic tissues. The liver is known to contain a high activity of carbamyl-P synthetase and is the only organ in higher animals where the operation of complete orotate pathway has been so far established.

Further evidence against the possibility that the spleen uracil may have the hepatic origin was provided by comparing distribution of the ^{14}C label in uracil molecules isolated from both

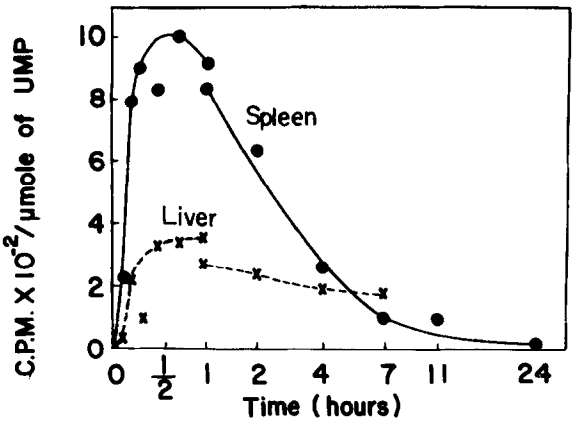

Fig. 1. Incorporation in vivo of $^{14}CO_2$ into nucleotide uracil of the spleen and the liver. Each mouse was given a subcutaneous injection of $NaH^{14}CO_3$ (1 X 10^7 cpm) contained in 0.1 ml of 0.15 M NaCl and killed at the time indicated by exsanguination from the femoral artery. Acid-soluble nucleotides were extracted from the tissues with trichloroacetic acid and subjected to acid hydrolysis in 0.5 N HCl at 100° for 30 min. UMP in the hydrolysate was fractionated on a Dowex 1 formate column (Hurlbert, et al., 1954) followed by rechromatography on paper (Wyatt, 1951) and on a Dowex 1 chloride column. UMP thus isolated was determined spectrophotometrically, then applied on a stainless steel planchet and counted in a gas flow counter. Each point in the figure represents the result from a single animal.

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the tissues (Table I). As shown, the highly preferential labeling of C-2 makes a feature of the spleen uracil, which can be distinguished from the liver uracil in this respect. It can be concluded that the greater part of uracil in the spleen has an origin different from that of the liver uracil.

The above conclusion drawn for uracil could be extended and applied to carbamylaspartate or the other pyrimidine precursors of the orotate pathway, because the distribution of ^{14}C in the pyrimidine ring is determined at an initial step of its biosynthesis, i.e., at the step of carbamylaspartate formation. Thus, the results of uracil decomposition experiments also exclude the possibility that these pyrimidine precursors of hepatic origin may serve as the main source of pyrimidines in the spleen, being transfered from the

Table I.

Distribution of ^{14}C in the uracil molecules

Source	Time after $NaH^{14}CO_3$ injection (min)	Total activity of sample (cpm)	C-2 (cpm)	C-4 (cpm)	C-5,6 (cpm)	C-2 / C-2+C-4 +C-5,6 (%)	Recovery of ^{14}C (%)
Spleen	15	248	233	14	5	92	98
"	120	192	187	6	5	95	103
Liver	15	1248	1049	192	81	79	106
"	120	339	237	38	46	73	95
Uracil -2-^{14}C		1977	1937	4	5	99	98

Labeled UMP was hydrolyzed by prostatic acid phosphatase and bacterial nucleoside hydrolase (unpublished). Uracil in the hydrolysate was isolated by chromatography on paper with n-butanol saturated with 3 N NH_4OH as developing solvent and subsequently on a Dowex 1 chloride column. To the uracil isolated was added 5 µmoles of non-radioactive uracil and the mixture was subjected to decomposition according to procedures of Kusama and Roberts (1963). Evolved CO_2 was absorbed in 1 ml of hyamine and counted by a Tri-Carb liquid scintillation spectrometer with the aid of a toluene counting fluid. Uracil-2-^{14}C was purchased from New England Corp., Boston, Mass.

liver through blood stream. Further implication of the preferential labeling of C-2 of the spleen uracil is the operation of complete orotate pathway within the tissue *in situ*, since C-2 should be derived from HCO_3^- if this pathway operates. This interpretation is supported by preliminary results of experiments designed to detect the accumulation of label in some intermediates of this pathway following $^{14}CO_2$ administration.

Although attempts were not successful to detect the enzyme activity for synthesis of carbamyl-P or some other carbamyl group donor in cell-free preparations of the spleen, its presence was definitely shown by slice experiments as described below. Anemic mice were given 10 µmoles of 6-azauridine for each by intraperito-

neal injection and killed after 1 hour. Slices of the hematopoietic spleen (0.5 g) were incubated for 1 hour at 37° in 5 ml of Krebs improved Ringer phosphate solution II (Krebs, 1950) with $H^{14}CO_3^-$ (1 X 10^7 cpm) in the presence of excess amounts of DL-carbamylaspartate (3 mM) and orotate (0.5 mM). 6-Azauridine (azaUR), a potent inhibitor of the orotate pathway (Pasternak and Handschumacher, 1959; Handschumacher, 1960), was also included to a concentration of 0.5 mM. The medium was fortified with dialyzed rabbit serum as well as with amino acids (Eagle, 1955). The other flask contained the slices from anemic mice, not treated with azaUR, and the medium was devoid of this analogue. After the incubation, carbamylaspartate and orotate were isolated and their specific activities were determined. The total radioactivities that these compounds possessed at the end of incubation were calculated thereof and shown in Table II. As seen, there was a considerable accumulation of the label in both of these compounds for the slices prepared from azaUR-treated animals. Pretreatment of the animal with azaUR and/or its inclusion in media seems to be necessary to obtain a significant label in the pyrimidine precursors under the conditions employed.

It is pertinent here to attempt a quantitative evaluation of the activity shown by the slices in comparison with the demand of the spleen *in situ* for uracil supply. The net amount of CO_2 that the sliced tissue fixed in the pyrimidine precursors was obtained from the data in Table II, though an accurate assessment is difficult because of dilution of the label by endogenous non-radioactive CO_2. An approximate estimation gave a value of 1 μmole of CO_2 fixation for 1 hour by 1 g of the slices. The detailed process of the calculation will be published elsewhere. On the other hand, the demand of the tissue for uracil supply was obtained from the data in Fig. 1. If some assumptions, including a steady state of

Table II.

Synthesis of intermediates of orotic acid pathway by spleen slices

Treatment of animal with azaUR	AzaUR in medium	Radioactivity in Carbamylaspartate (cpm)	Radioactivity in Orotate (cpm)
-	-	260	300
+	+	28500	45000

Carbamylaspartate and orotate were fractionated on a Dowex 1 formate column by stepwise elution with formic acid of varying concentrations and further purified by repeated recrystallization on addition of carriers and subsequent rechromatography on Dowex 1 formate columns. Amounts of both compounds were assayed at each step of the purification. Carbamylaspartate was determined colorimetrically (Koritz and Cohen, 1954) and orotate spectrophotometrically by the absorption at 280 mμ. Radioactivity was measured by a gas flow counter.

uracil metabolism, are permitted, the disappearance of label from uracil could represent the demand of the tissue for uracil supply. As illustrated by the figure, half life of the spleen uracil was about 4 hours and the total content of uridine nucleotides in the tissue was about 0.6 μmole per g of wet weight (Tatibana, unpublished). A calculation based on these values gave an estimate of about 0.1 μmole for the uracil demand of 1 g of the tissue for 1 hour. Thus, the activity of the initial steps for pyrimidine biosynthesis of this tissue is sufficient enough to cover its need for uracil supply.

The findings described in this paper emphasize the importance of the detection and characterization of the enzyme which is responsible for the synthesis of carbamyl-P or some other carbamyl group donor in nonhepatic tissues. Studies along this line are currently in progress in our laboratory.

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