CARBAMYL PHOSPHATE SYNTHETASE OF THE HEMATOPOIETIC MOUSE SPLEEN AND THE CONTROL OF PYRIMIDINE BIOSYNTHESIS[1/]

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All the attempts of previous investigators to detect carbamyl-P synthetase in animal tissues other than liver and intestine have been consistently unsuccessful (Cohen and Brown, 1960; Jones *et al.*, 1961; Kusama and Roberts, 1963). This led to varying speculations concerning the source of pyrimidines in these other tissues, since carbamyl-P is considered the initial intermediate in the orotate pathway of pyrimidine biosynthesis. It was reported, however, in a previous communication (Ito and Tatibana, 1966) that the hematopoietic mouse spleen is provided with the complete orotate pathway starting from bicarbonate in spite of its apparent lack of carbamyl-P synthetase. Hager and Jones (1965) also demonstrated glutamine-dependent operation of the orotate pathway in the intact cells of Ehrlich ascites tumor and proposed the possible presence of glutamine-dependent carbamyl-P synthetase in these cells. This type of enzyme activity was found initially in mushroom (Levenberg, 1962) and later in *E. coli* (Piérard and Wiame, 1964), but not thus far in

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animal tissues.

The present report deals with the detection of carbamyl-P synthetase in the hematopoietic mouse spleen and some of its properties. The partially purified enzyme does not require the presence of N-acetyl-L-glutamate for full activity and seems to utilize L-glutamine as well as ammonia as a substrate. Available evidence indicated a key role of this enzyme in the regulation of pyrimidine biosynthesis.

Male young mice were treated with acetylphenylhydrazine as described (Ito and Tatibana, 1966) and the hyperplastic spleen with increased hematopoietic activity was used as the enzyme source.

Carbamyl-P formation was followed by its conversion to L-citrulline in the presence of ornithine and purified ornithine transcarbamylase from beef liver (Marshall and Cohen, Personal Communication). The standard enzyme assay system contained the following constituents in a final volume of 0.6 ml: potassium N-2-hydroxyethylpiperazine-N-ethanesulfonate (Calbiochem), pH 7.5, 18 µmoles; NH_4HCO_3, 15 µmoles; ATP, 6 µmoles; $MgCl_2$, 9 µmoles; 2-mercaptoethanol, 1.8 µmoles; L-ornithine, 0.6 µmole; ornithine transcarbamylase, 5 µg (at least 10 units); enzyme to be assayed; $NaH^{14}CO_3$, 3.8 X 10^6 c.p.m.; glycerol, 60 mg. Use of a high concentration of glycerol was found to be necessary to stabilize the enzyme through its purification steps as well as during assay. The enzyme was inactivated almost instantaneously in a buffer solution without glycerol. Sucrose could substitute for glycerol to some extent. The reaction was allowed to proceed for 15 minutes at 37° and was terminated by the addition of trichloacetic acid. The radioactivity, fixed in an acid-stable form, was counted in a dioxane scintillator fluid (Bray, 1960) in a Tri-Carb liquid scintillation spectrometer. The radioactivity in L-citrulline accounted for more than 97% of the $^{14}CO_2$

fixed by the reaction with purified enzyme. In assays of crude enzyme, the product was partially purified before counting, by adsorption on a small column of Dowex 50 (H^+) followed by elution with aqueous pyridine. Under the conditions employed, citrulline formation was linear with time and proportional to enzyme concentration within the range of 5 mμmoles per tube in 15 minutes.

Fresh hematopoietic spleen was homogenized with 9 volumes of ice-cold 0.25 M sucrose containing 0.05 M potassium phosphate, pH 6.8, 1 mM ATP, 2 mM $MgCl_2$ and 3 mM 2-mercaptoethanol. ATP and Mg^{++} seem to be effective in stabilizing the enzyme, although precise evaluation of the effect remains to be investigated. The homogenate was centrifuged for 20 minutes at 20,000 g and the supernatant was subjected to ammonium sulfate fractionation (0 - 38% saturation). Subsequent steps included removal of insoluble material by centrifugation at 104,000 g, treatment with Sephadex G-25 and chromatography on a hydroxylapatite column. The procedures were carried out at 0 to 4° and all buffers contained 12.5% to 30% glycerol. About 18 fold purification was achieved by these procedures with a yield of 22% over the supernatant from the sucrose homogenate. The final preparation could catalyze the formation of about 25 mμmoles of carbamyl-P per mg of protein in 15 minutes under the standard assay conditions.

As shown in Table I, when either NH_4^+, ATP or Mg^{++} was omitted from the complete reaction mixture, $^{14}CO_2$ fixation was decreased to zero or to a very low level. Ornithine or ornithine transcarbamylase was also necessary. L-Glutamine could replace, and was equally as active as NH_4^+. The activities were not additive. Since the enzyme preparation was practically free from glutaminase activity as tested with L-glutamine-^{14}C as substrate, it is most likely that L-glutamine serves directly as amino group donor. Approximate

Table I

Requirements of Reaction

System	$^{14}CO_2$ Fixation
	c.p.m. per tube
Complete	254
- Enzyme	0
- NH_4^+	32
- ATP	0
- Mg^{++}	0
- Ornithine	34
- Ornithine transcarbamylase	34
- NH_4^+, plus glutamine (6 mM)	280
+ Glutamine (6 mM)	258
+ N-Acetyl-L-glutamate (5 mM)	261
+ N-Carbamyl-L-glutamate (5 mM)	250

The complete reaction mixture (0.6 ml) consisted of the following: potassium phosphate (pH 7.5), 25 µmoles; ammonium acetate, 15 µmoles; $KHCO_3$, 15 µmoles; ATP, 3 µmoles; $MgSO_4$, 6 µmoles; L-ornithine, 0.3 µmole; ornithine transcarbamylase, 5 µg; 2-mercaptoethanol, 1.8 µmoles; $NaH^{14}CO_3$, 3.8 X 10^6 c.p.m.; enzyme of hydroxylapatite fraction, 80 µg; glycerol, 60 mg. Incubation, 10 minutes at 37°.

values of K_m for L-glutamine and NH_4^+ were found to be 0.3 mM and 1.8 mM, respectively. L-Asparagine did not serve as substrate at the concentration of 1 mM.

While the liver carbamyl-P synthetase requires the presence of N-acetylglutamate or a related derivative for full activity (Metzenberg et al., 1959), the spleen enzyme apparently does not, as shown in Table I. No significant increase in catalytic activity was observed by the addition of acetylglutamate or carbamylglutamate to the assay system.

The presence of enzyme-bound activator was possible, but only a negative result was obtained when a hot water extract of crude enzyme preparation was tested (less than 0.1 µµmole as N-acetyl-L-

glutamate per mg original protein) by assay with frog liver carbamyl-P synthetase (Marshall et al., 1961). The assayed preparation was previously treated with Dowex 1 (Cl^-) and was fully active in the absence of N-acetylglutamate. Thus it is also suggested that spleen tissue is devoid of the liver type enzyme whose activity is dependent on the presence of N-acetyl-L-glutamate.

The radioactive product of the reaction was identified as L-citrulline by its behavior on paper electrophoresis (Rothman and Higa, 1962) and by its constant specific activity through the steps of repeated recrystallization with non-radioactive L-citrulline. When the recovered L-citrulline was submitted to arsenolysis (Reichard, 1957) by incubation with arsenate in the presence of ornithine transcarbamylase, most of the radioactivity was released from the amino acid and recovered as $^{14}CO_2$, indicating that the carbamyl carbon of L-citrulline was the site of its labeling.

The feedback inhibition of aspartate transcarbamylase (ATC) of E. coli by UTP and CTP (Gerhart and Pardee, 1962) is a classical example of metabolic control of enzyme activity. A general conception seems to have developed that ATC plays the key role in the regulation of pyrimidine biosynthesis in most biological systems. The situation is quite different, however, in the hematopoietic mouse spleen. The ATC of spleen may not be the limiting step of the orotate pathway, in view of its relatively high activity, i.e., formation of 100 μmoles of carbamylaspartate per hour per gm tissue. The value is 100 times as great as the maximum synthesis of carbamylaspartate so far observed in tissue slices (Ito and Tatibana, 1966). Moreover, it has not been possible to obtain any indication that the spleen enzyme is subject to significant inhibition by nucleosides or nucleotides at physiological concentrations (Inagaki and Tatibana, unpublished observations).

On the other hand, the newly detected carbamyl-P synthetase of spleen seems to be intimately involved in the regulation of pyrimidine biosynthesis in this tissue. The hypothesis is supported by the following findings. Firstly, enzyme activity is inhibited by the presence of UTP, as is shown in Table II along with the effects of some other nucleosides and nucleotides. The inhibition is highly specific and would be a typical case of control of the activity

Table II

Effect of Nucleosides and Nucleotides on the Spleen Carbanyl-P Synthetase Activity

Compounds added	Activity (%)
None	100
Uridine	102
UMP	114
UTP	27
Cytidine	94
CMP	103
CTP	102
Thymidine	82
TMP	85
TTP	143
Orotidine	128
IMP	107
ITP	100
GMP	130
GTP	88

Standard assay conditions were employed with the following exceptions: use of a lower concentration of ATP (3 mM) and MgCl (12 mM); replacement of NH_4HCO_3 by L-glutamine (2 mM) and $KHCO_3$ (25 mM); addition of nucleoside or nucleotide as indicated (2 mM). $^{14}CO_2$ fixation in the tube without the special additions was 232 c.p.m. and this value is expressed as 100%.

of an initial enzyme through feedback inhibition by the final product of the metabolic pathway. The physiological significance of the inhibition as well as its kinetic nature is currently under in-

vestigation. A recent report by Anderson and Meister (1966) showed that carbamyl-P synthetase of E. coli is also subject to inhibition by uridine nucleotides and to stimulation by IMP. Secondly, the enzyme activity in the spleen is so low as to make a limiting step in the pyrimidine biosynthesis. Enzyme from 1 gm of the tissue could synthesize only 1 μmole of carbamyl-P in an hour and the value approximately corresponds to the maximum flow of the orotate pathway of this tissue.

This type of regulation of pyrimidine biosynthesis seems to be common to many non-hepatic animal tissues including some experimental cancer cells. The presence of carbamyl-P synthetase in these tissues have been proved through the use of glycerol as stabilizing agent. More detailed results will be published elsewhere.

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