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CONTROL OF PYRIMIDINE BIOSYNTHESIS IN MAMMALIAN TISSUES

III. MULTIPLE FORMS OF ASPARTATE TRANSCARBAMOYLASE OF MOUSE SPLEEN

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SUMMARY

1. Aspartate transcarbamoylase (carbamoyl phosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) in hematopoietic mouse spleen can be fractionated into multiple forms. The activity in the soluble fraction can be separated into two fractions by chromatography on hydroxylapatite and are designated as Form I and II according to the order of elution from the column. Some activity sediments with the microsomal fraction (Form III). All these forms catalyze the formation of carbamoylaspartate from carbamoyl-*P* and aspartate.

2. When Form I was stored at 0° for 1 week and then rechromatographed on hydroxylapatite, some portion of activity behaved as Form II. The "conversion" is stimulated in the presence of dithiothreitol or mercaptoethanol. The physiological significance and the precise nature of the "conversion" are not yet known.

3. Upon centrifugation in a sucrose density gradient, Form I sediments at a S value of about 19, while Form II shows a broad distribution of S values, ranging from 22 to 45, with a peak at 35 S.

4. The three forms are not distinguished from each other by kinetic properties. All three forms have the same apparent K_m values for aspartate (5.6 mM), and are inhibited by higher concentrations of aspartate, 20 mM or more. The Lineweaver-Burk plots for varying concentrations of carbamoyl-*P* are not linear, but concave downward, with all three forms. The apparent K_m values obtained at lower concentrations of carbamoyl-*P* are smaller than those obtained at higher concentrations.

5. Inhibition by pyrimidine derivatives was examined at pH 9.2 and 7.6 for each of the three forms. Of eleven pyrimidine derivatives so far tested, only deoxythymidine and deoxyuridine at 4 mM inhibit the enzymic activity by about 15–30% at pH 9.2. Of the three forms, Form II is inhibited to a slightly greater extent than the others. At pH 7.6, the inhibition is more marked, and in addition to deoxy-

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thymidine and deoxyuridine, TTP and CTP at 4 mM inhibit the enzyme activity by about 20–30%. In any case, the extent of inhibition is lower than 40%.

6. The function of aspartate transcarbamoylase regarding regulation of pyrimidine biosynthesis in mouse spleen as well as in other tissues is discussed.

INTRODUCTION

It has been a problem as to how pyrimidine biosynthesis is regulated in biological systems. Aspartate transcarbamoylase of *Escherichia coli* is shown to be subject to feedback inhibition to a significant degree by a low concentration of CTP and is considered to be a regulatory enzyme of pyrimidine biosynthesis[1]. This fact, however, does not lead to a generalization that aspartate transcarbamoylase plays the key role in regulation of pyrimidine biosynthesis in other biological systems. Aspartate transcarbamoylases of *Streptococcus faecalis* and *Bacillus subtilis* are shown to be insensitive to pyrimidine nucleotides[2]. In mammalian tissues, rat liver aspartate transcarbamoylase was shown to be subject to feedback inhibition by deoxynucleosides such as deoxythymidine[3]. However, the high concentrations of the effectors required for a significant inhibition make one wonder if such a mechanism does play a major role in control of pyrimidine biosynthesis *in vivo*[4].

Recently, the existence of glutamine-utilizing carbamoyl-*P* synthetase was demonstrated in hematopoietic mouse spleen[5,6] as well as in other mammalian tissues[7–9], and it was suggested that the enzyme plays a primary role in regulation of pyrimidine biosynthesis in these tissues[5]. These observations prompted us to evaluate more precisely the role that aspartate transcarbamoylase plays in the metabolic regulation. During the course of these studies we found that aspartate transcarbamoylase in hematopoietic mouse spleen was fractionated into at least three forms. This paper deals with some of the properties of each form, with special emphasis on the effects of nucleosides and nucleotides on the activity of the fractionated enzymes.

While the present paper was in preparation, OLIVER *et al.*[10] reported that aspartate transcarbamoylase of rat liver is separated into two forms by sucrose density gradient centrifugation, and BETHELL AND JONES[11] found that the bacterial enzymes are divided into three classes according to differences in molecular size.

MATERIALS AND METHODS

Animals

Young adult mice of dd strain were made anemic by treatment with acetylphenylhydrazine as described previously[9] and the hyperplastic spleen was used as the source of enzyme.

Chemicals

Dilithium carbamoyl-*P* was synthesized according to the method of JONES *et al.*[12] and further purified by the method of GERHART AND PARDEE[1]. [^{14}C]Carbamoyl-*P* was prepared by the method of LOWENSTEIN AND COHEN[13]. The final product had a specific activity of 35 000 counts/min per μmole. Hydroxylapatite was prepared by

the method of TISELIUS *et al.*[14]. Crystalline protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa*[15] was kindly provided by Drs. H. Fujisawa and O. Hayaishi in this laboratory. Yeast alcohol dehydrogenase was purchased from Sigma Chemical Co. (St. Louis). All other chemicals were obtained commercially and were of highest purity available.

Determinations

The activity of aspartate transcarbamoylase was assayed by following the production of carbamoylaspartate in a system containing the following: dilithium carbamoyl-*P* (dissolved immediately before use), 10 μmoles; L-aspartic acid (brought to pH 9), 3.75 μmoles; enzyme; Tris HCl buffer (pH 9.2), 150 μmoles; in a volume of 0.75 ml. The mixture was incubated at 37° for 20 min. The reaction was stopped by the addition of 0.25 ml of 4 M $HClO_4$, and the denatured protein was removed by centrifugation. Carbamoylaspartate was determined with a 0.5-ml aliquot of the supernatant by a modification[1] of the method of KORITZ AND COHEN[16]. Suitable blanks were run in parallel. One unit of aspartate transcarbamoylase is defined as the amount that produces 1 μmole of carbamoylaspartate in 20 min at 37°. under the conditions. Protocatechuate 3,4-dioxygenase was assayed spectrophotometrically according to the method of STANIER AND INGRAHAM[17]. Sucrose density gradient centrifugation was performed according to the method of MARTIN AND AMES[18]. Yeast alcohol dehydrogenase ($s_{20,w}$ — 7.6 S) (ref. 19) and protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa* ($s_{20,w}$ 19.4 S) (ref. 15) were used as standards. Protein determinations and radioactivity measurements were carried out as described previously[9].

RESULTS

Separation of three forms of aspartate transcarbamoylase of hematopoietic mouse spleen

Fresh hematopoietic mouse spleens were homogenized for 2 min with 9 vol. of ice-cold 0.25 M sucrose containing 0.01 M potassium phosphate buffer (pH 7.4) and 2 mM mercaptoethanol in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 13 000 × *g* for 15 min. The supernatant was centrifuged at 105 000 × *g* for 60 min. The precipitate was suspended in a volume, equal to 2 times the original tissue weight, of 0.25 M sucrose containing 0.01 M potassium phosphate buffer (pH 7.4) and 2 mM mercaptoethanol, and followed by centrifugation at 105 000 × *g* for 60 min. The precipitate was suspended in 0.01 M potassium phosphate buffer (pH 7.4) containing 2 mM mercaptoethanol and designated as the microsomal fraction (Form III).

To the 105 000 × *g* supernatant was added solid $(NH_4)_2SO_4$ to 40% saturation (243 g/l). The precipitate formed was collected by centrifugation, dissolved in 0.01 M potassium phosphate buffer (pH 7.4) containing 2 mM mercaptoethanol and desalted with a Sephadex G-25 column equilibrated with the same buffer. This eluate, which was designated as the 0–40% $(NH_4)_2SO_4$ fraction, was then placed on a hydroxylapatite column; 1 ml of hydroxylapatite was used for 3–5 mg of protein. The column was washed with 2 column bed vol. of 0.01 M potassium phosphate buffer (pH 7.4) containing 2 mM mercaptoethanol and then successively eluted with 5 column vol. of 0.1 and then of 0.3 M potassium phosphate buffer (pH 7.4) containing 2 mM

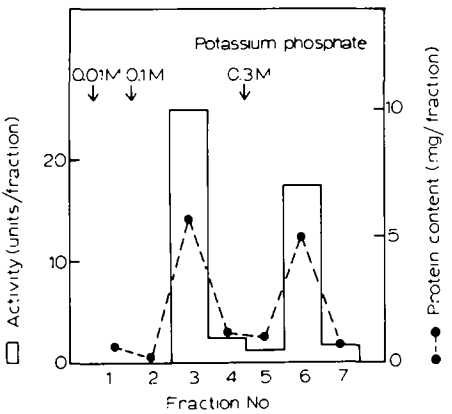


Fig. 1. Column chromatography on hydroxylapatite of aspartate transcarbamoylase in the soluble fraction. 14.4 mg of 0.40% $(NH_4)_2SO_4$ fraction was applied on a column of hydroxylapatite (bed volume, 4 ml), washed with 7 ml of 0.01 M potassium phosphate buffer (pH 7.4) containing 2 mM mercaptoethanol and then successively eluted with 18 ml of 0.1 M and 18 ml of 0.3 M potassium phosphate buffer (pH 7.4) containing 2 mM mercaptoethanol, as indicated by the arrows. The volume of each fraction collected was 4.8 ml (Fraction 1, 7 ml; Fractions 2 and 5, 4 ml; Fractions 3 and 6, 6 ml; Fractions 4 and 7, 8 ml). White bar, enzyme activity; ● ·· ●, protein content.

mercaptoethanol. Activities were eluted in both of the fractions, as shown in Fig. 1, and designated as Form I and Form II in the order of elution, although it remains to be seen whether each form represents a single molecular species of enzyme. A typical result of the fractionation of the enzyme is shown in Table I. The separation of the two forms could also be achieved by elution with a linear gradient from 0.01 to 0.60 M potassium phosphate buffer (pH 7.4) containing 2 mM mercaptoethanol, instead of the stepwise elution described above.

TABLE I

PURIFICATION AND SEPARATION OF THREE FORMS OF ASPARTATE TRANSCARBAMOYLASE FROM HEMATOPOIETIC MOUSE SPLEEN

Details of procedure of purification and separation are described in the text. The starting material was 8.1 g, wet weight, of mice spleen.

	Total activity (units)	*Total protein (mg)*	*Specific activity (units/mg protein)*
Homogenate (10%)	316	1300	0.24
13 000 × *g* supernatant	221	570	0.39
105 000 × *g* supernatant	128	400	0.32
0–40% $(NH_4)_2SO_4$ fraction	116	56	2.04
Hydroxylapatite fractions			
Form I	87	14	5.66
Form II	25	11	2.00
Microsomal fraction (Form III)	26	40	0.60

Identification of reaction product

The reaction was conducted with the use of [^{14}C]carbamoyl-*P* in place of nonradioactive carbamoyl-*P*. The radioactive reaction product was chromatographed on Dowex 1 on addition of nonradioactive carbamoylaspartate. There appeared a peak of radioactivity coincident with the authentic carbamoylaspartate. The specific radioactivities of carbamoylaspartate in the fractions of the column eluates were constant within limits of experimental error. Essentially the same results were obtained with any one of the three forms and the data for Form I were illustrated in Fig. 2. When

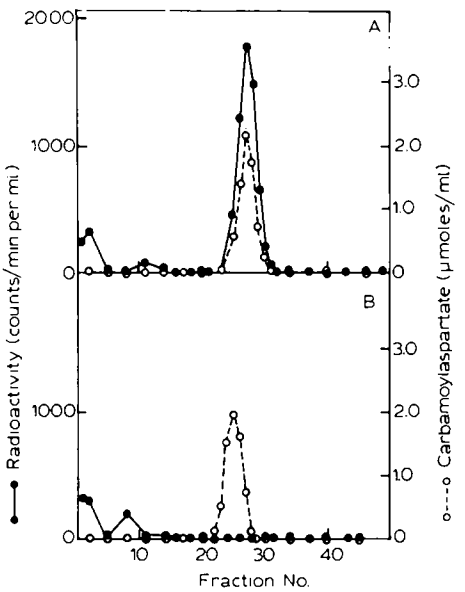


Fig. 2. Formation of carbamoylaspartate by Form I. A. 0.12 mg of Form I was incubated in the reaction mixture containing 3.0 μmoles of [^{14}C]carbamoyl-*P*, 3.75 μmoles of aspartate and 150 μmoles of Tris–HCl buffer (pH 9.2) in a volume of 0.75 ml for 20 min at 37°. The reaction was stopped by adding trichloroacetic acid and the mixture was centrifuged. The supernatant was extracted with ether to remove trichloroacetic acid. The solution was adjusted to pH 3, kept at 100° for 90 sec and then neutralized, followed by chromatography on a Dowex 1 column (3 ml) upon addition of 10 μmoles of DL-carbamoylaspartate. Elution was carried out with 60 ml of a linear gradient of ammonium formate from 0 to 1.6 M (pH 4.3), and fractions of 1.2 ml were collected. B. A control tube with water in place of aspartate was treated in the same way as above. ●—●, radioactivity; ○ – – ○, carbamoylaspartate determined colorimetrically.

aspartate was omitted from the reaction mixture, there was no peak of radioactivity coincident with the authentic carbamoylaspartate as shown in Fig. 2. About 85% of the total radioactivity fixed in an acid-stable form was recovered in fractions of carbamoylaspartate.

Activity in microsomal fraction

About 10% of activity in the 13 000 × *g* supernatant sedimented with the microsomal fraction upon centrifugation at 105 000 × *g*. The activity in this fraction could not be "solubilized" by 0.04 or 0.12% Triton X-100, 0.05 or 0.5% deoxycholate,

TABLE II

COMPARISON OF ACTIVITY IN MICROSOMAL FRACTIONS OF SPLEEN FROM ACETYLPHENYLHYDRAZINE-TREATED AND NONTREATED MICE

Treatments of mice with acetylphenylhydrazine were performed as described in the text.

Acetyl phenyl-hydrazine treatment	*Microsomal fraction*		*105 000 × g supernatant*	
	Activity (units/g wet wt. of spleen)	*Specific activity (units/mg protein)*	*Activity (units/g wet wt. of spleen)*	*Specific activity (units/mg protein)*
−	3.2	0.6	15.8	0.32
+	0.7	0.1	15.0	0.34

or 0.01 M imidazole–HCl buffer containing 2 mM mercaptoethanol. It was "solubilized" only by 1% digitonin, so far as examined.

The activity in the microsomal fraction of hematopoietic spleen from acetylphenylhydrazine-treated mice was higher than the activity in the corresponding fraction of spleen from untreated mice. As shown in Table II, the activity of this form, when expressed in terms of either the tissue weight or the protein, was several times higher in the treated mice than in the untreated. On the other hand, there was no such difference in the activity of the soluble fraction.

Partial "conversion" of Form I to Form II

When Form I, obtained by chromatography on hydroxylapatite, was stored for several days at 0° and then rechromatographed, a portion of the activity behaved

TABLE III

PARTIAL "CONVERSION" OF FORM I TO FORM II

A portion of freshly prepared Form I was passed through a Sephadex G-25 column that had been equilibrated with 0.01 M potassium phosphate buffer (pH 7.4). The eluate containing 17.6 units of enzyme and 3.0 mg of protein was immediately rechromatographed on a hydroxylapatite column (1 ml). To the major part of the freshly prepared Form I was added solid $(NH_4)_2SO_4$ to 60% saturation (390 mg/ml). The precipitate formed was collected by centrifugation, dissolved in 0.01 M potassium phosphate buffer (pH 7.4) and briefly dialyzed against the same buffer (the period of dialysis was only 20 min to minimize enzyme inactivation). The preparation was diluted with the same buffer to make the protein concentration 1 mg/ml and divided into three portions (3.0 ml each). These were stored for 7 days at 0° under the conditions specified and then rechromatographed as described for Fig. 1.

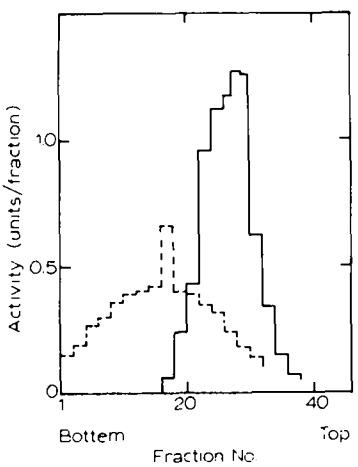
Storage conditions	*Enzyme prior to fractionation*		*Enzyme fractionated as*			
			Form I		*Form II*	
	Activity (units)	*Protein (mg)*	*Activity (units)*	*Protein (mg)*	*Activity (units)*	*Protein (mg)*
0 day	17.0	3.0	14.9	2.4	0.8	0.2
7 days without sulfhydryl compound	8.6	3.0	4.9	1.4	1.7	0.2
With 2 mM dithiothreitol	13.2	3.0	0.7	1.0	4.7	0.2
With 2 mM mercaptoethanol	12.0	3.0	1.4	1.2	3.4	0.3

as Form II. The "conversion" was marked for the enzyme stored in the presence of a sulfhydryl compound, dithiothreitol or mercaptoethanol (Table III). When Form I was stored at 25° for 86 h in the presence of 2 mM mercaptoethanol, similar "conversion" was observed. However, when the freshly prepared Form I was immediately rechromatographed on hydroxylapatite, there was practically no shift in the position of the elution.

As noted in Table III, the recovery of enzyme activity and of protein upon rechromatography was sometimes as low as 40%. It is possible that a portion of Form I was converted to some forms other than Form I and II, which were not recovered from hydroxylapatite by the procedures used. The "conversion" of Form I to Form II is not a reflection of changes in properties of proteins in general, since the appearance of Form II was not accompanied by a significant change in the elution pattern of protein (Table III).

Attempts were made to convert Form II to Form I by the following procedures: storage for 7 days at 0° in the solution containing various concentrations of GSSG or iodoacetate, or in the solution saturated with O_2. In neither case was observed formation of Form I.

The observations presented here do not permit any definite conclusion as to the nature and the physiological significance of the "conversion". The elucidation of the problem awaits further investigation.



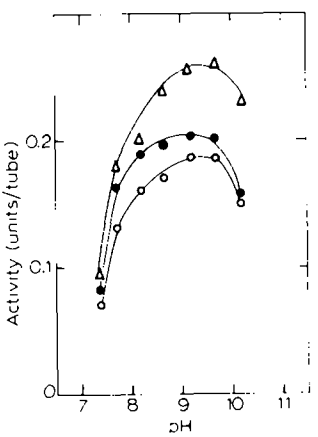


Fig. 3. Sedimentation in sucrose of Form I and Form II. Enzyme solutions (0.2 ml) were placed on a linear gradient between 5 and 20% sucrose in 0.01 M potassium phosphate buffer (pH 7.4) containing 2 mM mercaptoethanol. The amounts of enzyme protein used were 1.2 mg of Form I or of Form II, 0.2 mg of yeast alcohol dehydrogenase, and 1.4 mg of protocatechuate 3,4-dioxygenase. Centrifugation was performed in a Spinco Model L ultracentrifuge for 150 min at 37 500 rev./min at 4° in a SW-39 rotor. The results of experiments for Form I and Form II are presented together. The peaks of activity of yeast alcohol dehydrogenase and protocatechuate 3,4-dioxygenase were observed in Fractions 36 and 28, respectively, in both experiments. — · — ·, Form I; - - - -, Form II.

Fig. 4. Effect of pH on aspartate transcarbamoylase. The reaction mixture (0.75 ml) contained 10 μmoles of dilithium carbamoyl-*P*, 3.75 μmoles of L-aspartic acid, 70 μg of Form I or of Form II, or 380 μg of microsomal fraction, and 0.2 M buffer (Tris-HCl, pH 7.4–9.7; and diethanolamine-HCl, pH 9.7–10.2). The activities in the two different buffers at pH 9.7 were essentially the same. ○—○, Form I; ●—●, Form II; △—△, microsomal fraction (Form III).

Sedimentation velocities of Form I and Form II

Molecular weight estimations were performed by the sucrose density gradient method[18]. Yeast alcohol dehydrogenase, $s_{20,w}$ = 7.6 S (ref. 19) and protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa*, $s_{20,w}$ = 19.4 S (ref. 15), were used as standards. As shown in Fig. 3, Form I sedimented at an S value of about 19, while Form II showed a broad distribution of S values with a peak at 35 S but ranging from 22 to 45 S. The S values of 19 and 35 were tentatively calculated to correspond to 600 000 and 1 500 000 of molecular weights, respectively.

pH optimum

As shown in Fig. 4, the effects of pH on the activity of the three aspartate transcarbamoylase forms were similar, with pH optima between 9.2 and 9.7.

Kinetics

As shown in Fig. 5, the apparent K_m values for aspartate were almost the same (5.6 mM) for the three forms of the enzyme. All the forms are also subject to inhibition by higher concentrations of aspartate, 20 mM or more.

It should be noted that the Lineweaver Burk plots for carbamoyl-*P* were not linear but concave downward, as shown in Fig. 6. The possibility that this was a result of gross changes in concentrations of carbamoyl-*P* during incubation may be excluded, since the degradation of carbamoyl-*P* for 7 min at 37° in the presence or absence of enzyme (Form I) was less than 10%, either at a high (13 mM) or low

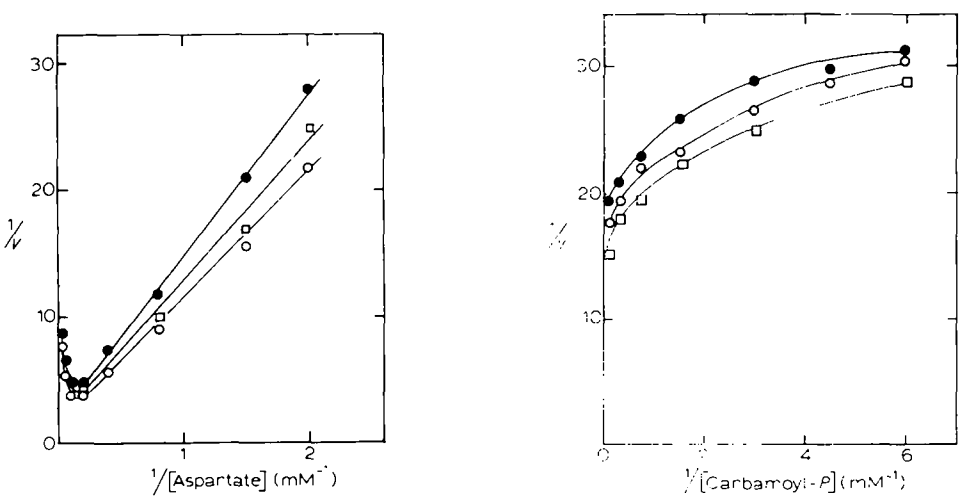


Fig. 5. Plots of reciprocal initial velocity *vs.* reciprocal aspartate concentration. The reaction mixture (0.75 ml) contained 150 μmoles of Tris–HCl buffer (pH 9.2), 10 μmoles of dilithium carbamoyl-*P*, 70 μg of Form I or of Form II, or 380 μg of microsomal fraction and the indicated amounts of L-aspartic acid. ● ●, Form I; ○ ○, Form II; □ □, microsomal fraction (Form III).

Fig. 6. Plots of reciprocal initial velocity *vs.* reciprocal carbamoyl-*P* concentration. The reaction mixture (0.75 ml) contained 150 μmoles of Tris–HCl buffer (pH 9.2), 3.75 μmoles of L-aspartic acid, 70 μg of Form I or of Form II, or 380 μg of microsomal fraction and the indicated amounts of dilithium carbamoyl-*P*. ● ●, Form I; ○ ○, Form II; □ □, microsomal fraction (Form III).

(0.23 mM) concentration of carbamoyl-*P*. Aspartate was omitted from the incubation mixture in these experiments.

The implications of the nonlinear kinetics observed are discussed later.

Stability properties

When each form was allowed to stand at 25° for 46 h in 0.01 M potassium phosphate buffer (pH 7.4) containing 2 mM mercaptoethanol, losses in activity during this period were 42, 4 and 10% for Form I, Form II, and Form III, respectively. The protein concentrations were 1.4, 1.1 and 7.6 mg/ml, respectively.

Effects of pyrimidine derivatives

It was of particular interest to know if any of the three forms might be intimately involved in the regulation of pyrimidine biosynthesis through "feedback inhibition". To minimize possible changes in the regulatory properties of the enzyme

TABLE IV

INHIBITION OF ASPARTATE TRANSCARBAMOYLASE BY PYRIMIDINE DERIVATIVES

The reaction mixture contained $4 \cdot 10^{-4}$ M carbamoyl-*P*, $2 \cdot 10^{-3}$ M aspartate, $2 \cdot 10^{-3}$ or $4 \cdot 10^{-3}$ M nucleoside or nucleotide (neutralized), 0.2 M Tris HCl (pH 9.2) or 0.05 M potassium phosphate (pH 7.6) and enzyme (Form I, 100 and 350 μg; Form II, 110 and 380 μg; microsomal fraction, 660 μg and 1.1 mg; for the experiments at pH 9.2 and 7.6, respectively).

Additions	*Concn. (mM)*	*Inhibition (%)*					
		Form I		*Form II*		*Microsomal fraction*	
		pH 9.2	*pH 7.6*	*pH 9.2*	*pH 7.6*	*pH 9.2*	*pH 7.6*
Uridine	2	2		0		0	
Deoxyuridine	2	3	21	24	23	5	9
Deoxyuridine	4	15	37	30	39	16	21
UMP	2	0		0		0	
UTP	2	4	3	0	6	0	0
Deoxythymidine	2	6	21	21	23	10	15
Deoxythymidine	4	18	40	27	37	31	24
TMP	2	8		6		6	
TTP	2	6	24	12	20	9	9
TTP	4		31		28		21
Cytidine	2	4		0		3	
Deoxycytidine	2	8		7		0	
CMP	2	0		0		0	
CTP	2	4	10	0	19	9	9
CTP	4		21		22		18

as far as possible, all three forms were prepared at a low temperature and assayed within 12 h after mice were killed, and a high dilution of the enzyme solutions was avoided during the purification process. The colorimetric assay of carbamoylaspartate was not interfered by the presence of pyrimidine derivatives at the concentrations used.

As shown in Table IV, at pH 9.2, most of the pyrimidine derivatives including CTP did not inhibit the enzymic activity. Only deoxythymidine and deoxyuridine at 4 mM showed about 15–30% inhibition of the activity, and of the three forms,

Form II was most sensitive to the inhibitors. At pH 7.6, inhibition by deoxythymidine or deoxyuridine was more marked than at pH 9.2, and furthermore, TTP and CTP at 4 mM also inhibited the enzymic activity by about 20–30%. Lower concentrations of aspartate and carbamoyl-*P*, each close to the respective apparent K_m value, were used in these experiments.

DISCUSSION

Aspartate transcarbamoylase in hematopoietic mouse spleen was demonstrated to be present in at least three forms; one is present in the microsomal fraction and the others are in the soluble fraction. Previous observations on the subcellular distribution of mammalian aspartate transcarbamoylase were not in accord with each other. BRESNICK[3] reported that all the activity of the homogenate of rat liver was recovered in the supernatant after centrifugation at 105 000 × *g* for 30 min. On the other hand, BOTTOMLEY AND LOVIG[20] recovered a major part of aspartate transcarbamoylase activity in the microsomal fraction of rat tissues. According to their report, however, there was a great difference in sedimentation behaviors between aspartate transcarbamoylase and glucose-6-phosphatase that is known to be bound to microsomes; the former required about 3 h for complete sedimentation, whereas the latter only 30 min at 105 000 × *g*. In view of our results it is likely that the major part of aspartate transcarbamoylase in mammalian tissues is not bound to microsomes but only behaved similarly because of large molecular size.

It was found that the activity of microsomal fraction of mouse spleen is higher when the hematopoietic activity of the tissue is supposed to be enhanced. Possibilities may be that this form serves as a precursor of the enzyme in the supernatant or that this form carries some special functions related in some manner to the rapid cellular growth. The elucidation of the problems awaits further investigations.

While the present paper was in preparation, OLIVER *et al.*[10] reported the presence of two forms of aspartate transcarbamoylase in the soluble fraction of rat liver, based on analysis by sucrose density gradient centrifugation. The estimated molecular weights of the two forms are 600 000 and 900 000, respectively. The large molecular weights and the relative amounts of the two forms in the soluble fraction agree fairly well with our results. Detailed properties of the two forms are not reported. Two forms of aspartate transcarbamoylase are also shown to be present in the extracts from *Citrobacter freundii* and *Proteus vulgaris*[11].

Both Form I and Form II were detected, even when the enzyme purification was promptly carried out, and this may support the natural occurrence of the two forms in the tissue. However, a possibility still remains that Form II is a kind of artifact formed from Form I during the enzyme purification. It is conceivable that Form I is converted to Form II by formation of intermolecular disulfide bridges in the presence of low concentrations of sulfhydryl compounds and O_2. The broad range of S values of Form II suggests aggregates of various sizes.

As shown in Fig. 6, all the forms showed nonlinear Lineweaver-Burk plots for carbamoyl-*P*. This kind of kinetics could be explained by assuming that each form has two or more separable carbamoyl-*P* sites with different binding constants or that each form is a mixture of two or more polymorphic forms of enzymes. An alternative possibility is the negative cooperativity between subunits of enzymes in the binding

of substrates, as emphasized by LEVITZKI AND KOSHLAND[21]. Examples are seen in CTP synthetase[21], phosphoenolpyruvate carboxylase[22] and glyceraldehyde-3-phosphate dehydrogenase[23]. It may be interesting to see if such cooperativity occurs for aspartate transcarbamoylase of mouse spleen.

As reported, there were no remarkable distinctions between the three forms regarding effects of pH on the activities, kinetic properties, and sensitivities to "feedback inhibition" by pyrimidine derivatives. It is to be noted that relatively high concentrations of pyrimidine derivatives are required to inhibit the enzymic activity to measurable extents. According to GERHART AND PARDEE[1], aspartate transcarbamoylase of *E. coli* is inhibited by 44 and 74% with 0.05 and 0.5 mM CTP, respectively. In contrast, the spleen enzymes are inhibited by only no more than 25% by 2 mM deoxythymidine, the most effective inhibitor so far found. In hematopoietic mouse spleen, the activity of aspartate transcarbamoylase is about 100 times greater than that of carbamoyl-*P* synthetase, the first enzyme of pyrimidine biosynthesis. This may indicate that in the spleen the rate-limiting enzyme is carbamoyl-*P* synthetase rather than aspartate transcarbamoylase, and that the observed inhibition of the latter enzyme by pyrimidine derivatives does not play a significant role in control of pyrimidine biosynthesis.

The difference in the properties of aspartate transcarbamoylase of *E. coli* and of hematopoietic mouse spleen with respect to the "feedback inhibition" may be related to the difference in the metabolic pathways of carbamoyl-*P* in the two biological systems. In *E. coli*, carbamoyl-*P* is utilized for the synthesis of both pyrimidine and arginine, and therefore aspartate transcarbamoylase is considered the first enzyme for pyrimidine biosynthesis. On the other hand, in nonhepatic animal tissues where the urea cycle is lacking, carbamoyl-*P* may be utilized principally for pyrimidine biosynthesis, and thus carbamoyl-*P* synthetase is the first enzyme for the pyrimidine pathway. In fact, carbamoyl-*P* synthetase (glutamine-utilizing) from hematopoietic mouse spleen was demonstrated to be subject to "feedback inhibition" by UTP (about 70% inhibition at 2 mM)[2]. In nonhepatic animal tissues and possibly also in the hepatic tissue (see ref. 24), aspartate transcarbamoylase may coordinate with carbamoyl-*P* synthetase and catalyze the quantitative and rapid conversion of the labile carbamoyl-*P* into carbamoylaspartate. The kinetic analysis of the enzyme (Fig. 6) showed that when the concentration of carbamoyl-*P* is lower, the apparent K_m for this is also smaller. On the assumption that similar kinetics is valid at physiological levels of aspartate, the property could be regarded as an advantage for the enzyme to carry out the expected function. The increase in the intracellular level of the enzyme in rapidly growing tissues, observed by previous investigators[25-29], can be explained from the same teleological point of view.

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