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INHIBITION BY PYRIMIDINES OF ASPARTATE TRANSCARBAMYLASE PARTIALLY PURIFIED FROM RAT LIVER

EDWARD BRESNICK

Department of Biochemistry, Baylor University College of Medicine, Houston, Texas (U.S.A.)

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SUMMARY

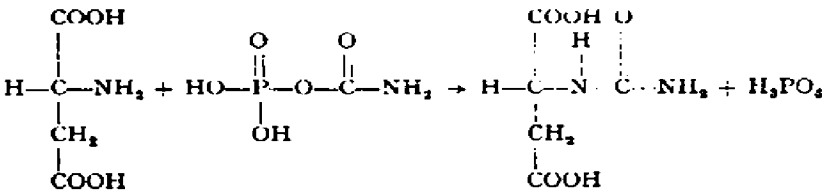
Aspartate transcarbamylase has been partially purified by 56-fold from rat liver employing 30% saturation with ammonium sulfate, absorption and elution from calcium phosphate gel, and fractionation upon DEAE-cellulose columns.

The enzyme has a pH optimum of 9.2; a K_m for aspartate of $5.3 \cdot 10^{-3}$ M and a K_m for carbamyl phosphate of $2.5 \cdot 10^{-4}$ M. Mercaptoethanol was necessary for stabilization of the enzyme. The enzymic reaction resulted in the release of equivalent concentrations of carbamylaspartate and inorganic phosphate. The enzyme was inhibited by heavy metals, *e.g.*, Ag^+, Hg^{2+}, Cu^{2+} and Cd^{2+}, and the inhibition could be reversed upon addition of EDTA to the incubation mixture. A crude supernatant preparation of rat liver was also inhibited by *p*-hydroxymercuribenzoate.

Uridine, cytidine or their nucleotides inhibited the enzymic activity. The deoxyribonucleosides or deoxyribonucleotides were more effective inhibitors of this enzyme than the ribose derivatives and of the former, thymidine was the most potent. The inhibition by thymidine and deoxycytidine was of the reversible, competitive type as determined by the methods of ACKERMANN AND POTTER and LINEWEAVER AND BURK. The inhibition constants were $4.1 \cdot 10^{-4}$ M and $7.0 \cdot 10^{-4}$ M, respectively.

INTRODUCTION

Aspartate transcarbamylase is an enzyme which catalyzes the irreversible transcarbamylation of L-aspartic acid by carbamyl phosphate to form carbamylaspartic acid (Eqn. 1).



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The enzyme has been shown to be subject to "feedback" inhibition by pyrimidine nucleotides in *E. coli*[1,2] and in Ehrlich ascites cells[3]. The pyrimidines that inhibited

this enzyme differed in Ehrlich ascites cells and in *E. coli*. In the latter, only cytidine and cytidine 5'-monophosphate appeared to exert maximal inhibition[1], whereas in the former system, uracil derivatives were equally effective[3]. The deoxyribonucleosides and deoxyribonucleotides were better inhibitors of this enzyme in a crude rat liver preparation; deoxyuridine or thymidine compounds were more active than the corresponding deoxycytidine derivatives[4].

The enzyme from *E. coli* has subsequently been purified[5] and the structural requirements for feedback inhibition have been reexamined[6]. In agreement with data obtained in liver preparations[4], GERHART AND PARDEE[6] observed that deoxyribonucleotides also inhibited the activity of the *E. coli* enzyme, although the uridine- and deoxyuridine nucleotides, as well as thymidine triphosphate, were ineffective. These results suggested the desirability of a purification and characterization of the mammalian enzyme prior to any comparison of the inhibitory efficacy of the ribo- and deoxyribonucleotides. A method for the partial purification of aspartate transcarbamylase from rat liver and the properties of this enzyme are presented in this report. In addition, the inhibition of this partially purified enzyme by pyrimidine ribo- and deoxyribonucleosides, and ribo- and deoxyribonucleotides has been investigated and the results are reported herein.

EXPERIMENTAL PROCEDURE

Chemicals: All chemicals were obtained from commercial sources. Commercial dilithium carbamyl phosphate (90% pure) was further purified by the method suggested by GERHART AND PARDEE[6], and stored over calcium chloride in a desiccator at −5°.

Enzyme assay: Aspartate transcarbamylase was assayed by the production of carbamylaspartate in a system containing the following: carbamyl phosphate, 20 μmoles; L-aspartic acid, 7.5 μmoles; enzyme; 0.2 M Tris buffer, pH 9.2, to a volume of 1.5 ml. The system was incubated at 37° for 30 min. At the end of this time the reaction mixture was deproteinized with 0.5 ml of 4 N perchloric acid and centrifuged to remove the denatured protein; 0.5 ml of the extract was employed in the assay of carbamylaspartate by the recent modification[6] of the method of KORITZ AND COHEN[7]. In all cases, the amount of carbamylaspartate produced non-enzymically was determined.

In some experiments, the activity of the enzyme was assayed by measuring the amount of inorganic phosphate produced[8]. In this procedure, all solutions were kept cold to minimize decomposition of the carbamyl phosphate prior to colorimetric assay.

Protein was assayed either by the colorimetric procedure of LOWRY *et al.*[9] or by the spectrophotometric method based upon the absorbance at 280 mμ[10].

RESULTS

Purification of rat liver aspartate transcarbamylase

All steps in the purification procedure were performed at 4°. Fresh rat livers obtained from male Holtzman rats weighing 125–150 g were homogenized in ice-

cold 0.25 M sucrose (1 g in 9 ml of sucrose) in a Potter-Elvehjem homogenizer with a teflon pestle. The homogenate was centrifuged at 105 000 × g for 30 min and the resultant supernatant solution was filtered through glass wool to yield a clear red solution.

Solid $(NH_4)_2SO_4$ was added to this solution to 30% saturation and the precipitat collected by centrifugation, was redissolved in 0.01 M imidazole buffer (pH 7.0) containing 0.002 M mercaptoethanol. This solution was dialyzed against the imidazole buffer for one hour in the cold room in a rotating dialysis apparatus*. Dialysis against water, 0.25 M sucrose, phosphate buffers of various concentrations at pH 7.0, or 0.01 M imidazole buffer at pH 7.0 resulted in a complete loss of activity.

The dialysate was mixed with 2 mg of calcium phosphate gel[11] per mg protein for 15 min. The gel was then collected by centrifugation and was washed with 0.1 M phosphate buffer, pH 7.4 (100 ml/1.5 g dry weight of gel). The resultant slurry was centrifuged, the supernatant solution was discarded, and the gel was extracted twice by stirring for 5 minutes with 0.3 M phosphate buffer (pH 7.4) (100 ml/1.5 g gel). The gel was removed by centrifugation and the combined eluates were dialyzed against 0.01 M imidazole–0.002 M mercaptoethanol buffer (pH 7.0) for 1 h in the rotating dialyzer.

DEAE-cellulose was activated by homogenizing the cellulose in a Waring blendor with 0.1 M potassium hydroxide. The DEAE-cellulose was then washed with distilled water until the pH of the wash was neutral, was centrifuged, was resuspended in 0.01 M imidazole–0.002 M mercaptoethanol buffer (pH 7.0) and was packed in a column under a pressure of 5 lb/in². The combined extracts from the calcium phosphate gel were added to a column of DEAE-cellulose (1.5 cm × 30 cm) that had been allowed to equilibrate at 4° in a cold room. The flow rate was 40 ml/h.

Fractionation of the dialyzed preparation on DEAE-cellulose was accomplished according to the elution scheme depicted in Fig. 1. The column was washed with

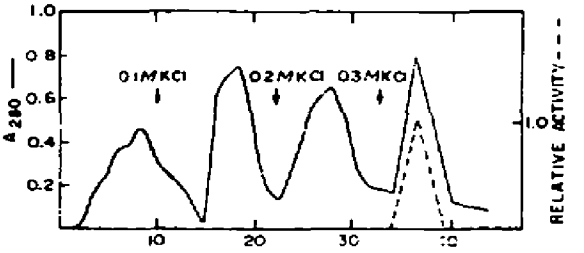

Fig. 1. Elution of the enzyme from DEAE-cellulose columns. Relative activity is defined as the enzymic activity of the particular fraction in units/mg relative to the fraction with the peak activity.

the imidazole buffer and then was eluted with 0.1 M KCl in 0.01 M imidazole–0.002 M mercaptoethanol buffer (pH 7.0). Fractions of 10 ml were obtained every 15 min. The eluant was changed to 0.2 M KCl in the imidazole buffer and 10 more fractions were collected. No enzymic activity was observed and consequently, the KCl con-

* Buchler Instruments, New York, N.Y.

centration was increased to 0.3 M in the imidazole buffer. A large increase in the absorbance at 280 mμ was observed at approximately the 6th fraction with a concomitant increase in elution of enzymatic activity. The total enzymic activity was eluted in about 10 tubes (~ 100 ml).

The purification scheme is presented in Table I starting with 150 ml of a 10% rat liver homogenate representing 334 units of activity. Saturation with ammonium

TABLE I

SUMMARY OF PURIFICATION DATA

	Total enzyme (units)*	*Total protein (mg)*	*Specific activity (units/mg)*	*Enzyme yield (%)*
Homogenate (10%)	334	4760	0.07	
105 000 × *g* extract	363	1460	0.25	109
0–30% $(NH_4)_2SO_4$ fraction	272	262	1.04	81
Gel extract	170	78	2.18	51
DEAE-cellulose fractions				
1. Purest fraction	19.5	5	3.90	22
2. Other fractions	54	20	2.70	

* unit = number of μmoles of carbamylaspartate produced in 30 min under the conditions of the assay as presented in the experimental section.

sulfate to 30% resulted in an overall purification of 15-fold. A further 2-fold purification was produced by absorption and subsequent elution from calcium phosphate gel. The overall purification to the fraction from the DEAE-cellulose column that possessed the most enzymatic activity was 56-fold with a yield of 22%.

Further attempts at purification by means of ethanol or acetone precipitation, or concentration of the enzyme by lyophilization resulted in inactivation of the enzyme. After the DEAE-cellulose step the enzyme could not be frozen without

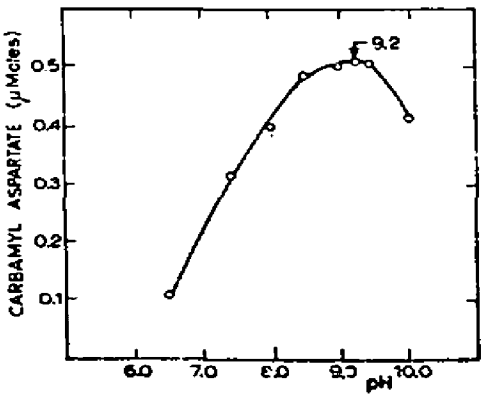

Fig. 2. The dependence of the enzymatic velocity upon pH. The reaction mixture was incubated for 30 min and contained the following: carbamyl phosphate, 20 μmoles; L-aspartic acid, 7.5 μmoles; enzyme, 90 μg protein; and 0.2 M buffers as indicated. Phosphate was used for pH 6.5 and Tris for all other pH's.

immediate loss in activity, but could be kept for 5 days at 4°. The preparation was run at 8° in a Spinco analytical ultracentrifuge at 50 740 rev./min. Photographs taken at 16-min intervals indicated the heterogeneity of the preparation (at least 3 major peaks were observed).

Properties of aspartate transcarbamylase

Dependence of velocity upon pH: The effect of pH upon the enzymic velocity is presented in Fig. 2. Maximum velocity was observed at pH 9.2. Beyond this pH, it was difficult to assess the rate of the enzymic reaction, because of the increase in the non-enzymic reaction of carbamyl phosphate with L-aspartate.

Dependence of enzymatic velocity upon incubation time and enzyme concentration: The rate of formation of carbamylaspartate was linear with respect to time for 30 min (Fig. 3) and was also proportional to the concentration of the enzyme up to 90 μg of protein (Fig. 4).

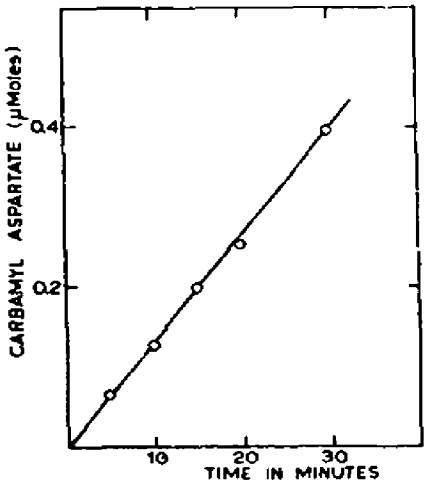

Fig. 3. The dependence of the velocity upon the time of incubation. The reaction mixture consisted of: carbamyl phosphate, 20 μmoles; L-aspartic acid, 7.5 μmoles; 0.2 M Tris buffer (pH 9.2) to 1.5 ml; enzyme, 90 μg protein. The incubation was conducted at 37° for the specified time.

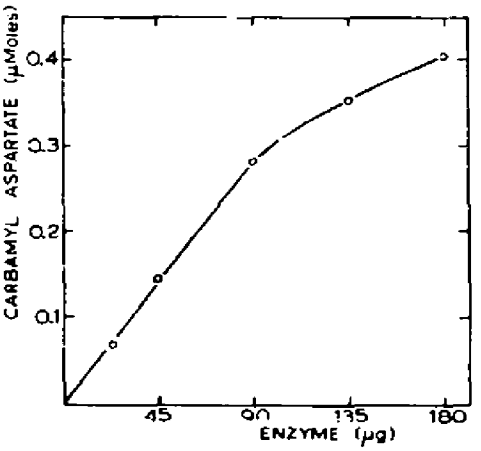

Fig. 4. The dependence of the velocity upon the enzyme concentration. The reaction mixture was similar to that described in the legend to Fig. 3, with the exception that the enzyme concentration was varied as indicated in the figure. The incubation time was 30 min.

Dependence of velocity upon substrate concentration: The velocity of aspartate transcarbamylase was proportional to the L-aspartate concentration to 30 μ moles (Fig. 5). A LINEWEAVER–BURK plot[12] is presented in the insert of Fig. 5. The K_m for aspartate is $5.3 \cdot 10^{-3}$ M. The reaction was also linear with respect to carbamyl phosphate concentration to 0.17 μmoles (Fig. 6). The Lineweaver–Burk plot (insert on Fig. 6) indicated that the K_m for carbamyl phosphate is $2.5 \cdot 10^{-4}$ M.

Stoichiometry of aspartate transcarbamylase reaction: The stoichiometry of the reaction was investigated at three enzyme concentrations (Table II). It is evident

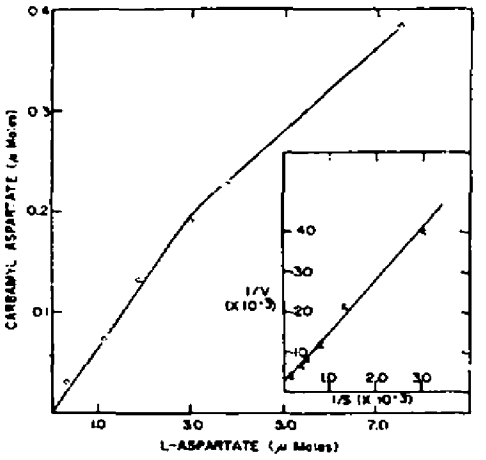

Fig. 5. The dependence of the velocity upon the concentration of L-aspartic acid. The reaction mixture was similar to that described in the legend to Fig. 3, with the exception that the L-aspartic acid concentration was varied as indicated in the figure.

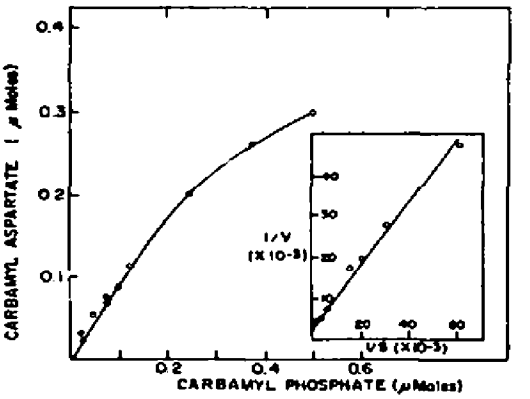

Fig. 6. The dependence of the velocity upon the concentration of carbamyl phosphate. The reaction mixture was similar to that described in Fig. 3, with the exception that the concentration of carbamyl phosphate was varied as indicated in the figure.

that for each mole of carbamylaspartate that was produced, an equivalent mole of inorganic phosphate was generated.

Inhibition of aspartate transcarbamylase by heavy metals: Aspartate transcarbamylase activity was inhibited in the presence of heavy metals (Table III). The addition of Ag^{+}, Hg^{2+}, Cd^{2+}, or Cu^{2+} at $3.35 \cdot 10^{-4}$ M resulted in an inhibition of 56–68% in the rate of the reaction. Zn^{2+} at a comparable concentration had no

TABLE II

STOICHIOMETRY OF THE ASPARTATE TRANSCARBAMYLASE REACTION

The enzyme (90–270 μg of protein) was incubated with L-aspartic acid (7.5 μmoles) and carbamyl phosphate (5 μmoles) for 30 min at 37° as indicated in the experimental section. The amount of carbamylaspartic acid and inorganic phosphate was determined by the methods cited in the text.

Enzyme (μg)	*Carbamylaspartate (μmoles)*	*Inorganic phosphate (μmoles)*
90	0.169	0.130
180	0.300	0.302
270	0.490	0.409

effect upon the enzymic activity. The addition of Fe^{3+} to the incubation mixture resulted in a non-specific interference with the color reaction, hence, no data on possible inhibition was obtained.

The inhibition by Cu^{2+} was completely reversed by EDTA (Table III) if the chelating agent was present at the time of the addition of the metal. The inhibition

TABLE III

INHIBITION OF ASPARTATE TRANSCARBAMYLASE BY HEAVY METALS

Expt. A: The enzyme (42 μg protein) was incubated with carbamylphosphate and L-aspartic acid as described in the experimental section. The inhibitors were added at zero time. Expt. B: $CuSO_4$ ($2.0 \cdot 10^{-4}$ M) was added at zero time and EDTA ($6.7 \cdot 10^{-4}$ M) was added at the indicated times.

	Additions	Amount (M)	Carbamylaspartate (μmoles)	Inhibition (%)
Expt. A	None		0.217	
	$AgNO_3$	$3.35 \cdot 10^{-4}$	0.069	68
	$Hg(NO_3)_2$	$6.7 \cdot 10^{-5}$	0.175	20
		$3.35 \cdot 10^{-4}$	0.096	56
	$CdSO_4$	$6.7 \cdot 10^{-5}$	0.100	54
		$3.35 \cdot 10^{-4}$	0.083	62
	$CuSO_4$	$6.7 \cdot 10^{-5}$	0.140	30
		$3.35 \cdot 10^{-4}$	0.076	65
Expt. B	None		0.214	
	$CuSO_4$		0.093	57
	$CuSO_4$ – EDTA at 0 time		0.210	2
	$CuSO_4$ – EDTA after 5 min		0.139	35
	$CuSO_4$ – EDTA after 15 min		0.125	42
	$CuSO_4$ – EDTA after 20 min		0.111	48

TABLE IV

INHIBITION OF ASPARTATE TRANSCARBAMYLASE BY PYRIMIDINES

The enzyme (40 μg protein) was incubated under conditions described under EXPERIMENTAL. The amount of carbamylaspartate produced in 30 min under these conditions was 0.383 μmoles. In those instances where two experiments were run, both the average value and the individual values (within the parenthesis) are indicated.

Additions	Amount (μmoles)	% Inhibition	Additions	Amount (μmoles)	% Inhibition
Uridine	20	10.1	Deoxyuridine	20	31.5 (30.6, 32.4)
Uridine	50	28.9 (23.7, 34.1)	Deoxyuridine	50	59.1 (54.7, 63.4)
UMP-5′	10	11.5	DeoxyUMP-5′	10	6.7
UMP-5′	20	20.0 (20.0, 20.0)	DeoxyUMP-5′	20	20.0 (21.0, 19.0)
Cytidine	20	17.3	Deoxycytidine	20	41.1
Cytidine	50	22.4 (22.0, 22.8)	Deoxycytidine	50	50.1
CMP-5′	10	0.7			
CMP-5′	20	8.6 (6.2, 10.4)	DeoxyCMP-5′	20	21.6 (20.8, 22.4)
CMP-5′	50	25.0 (22.0, 28.0)	DeoxyCMP-5′	50	43.0 (41.0, 45.0)
UTP-5′	20	21.4	Thymidine	10	20.3 (18.7, 22.0)
			Thymidine	20	44.3
			Thymidine	50	64.5 (63.4, 66.5)
CTP-5′	20	15.6	TMP-5′	10	11.5
			TMP-5′	20	18.3
			TMP-5′	50	42.1 (40.6, 43.6)

increased progressively when the Cu^{2+} was allowed to react with the enzyme prior to the addition of EDTA.

Since the enzyme required sulfhydryl groups for stabilization (mercaptoethanol), it was difficult to assess the effects of the compounds that react with sulfhydryl groups of the enzyme. However, employing a crude supernatant preparation, *p*-hydroxymercuribenzoate at $4 \cdot 10^{-5}$ M inhibited the enzymic activity by 50%.

The addition of pyrimidine nucleosides and nucleotides to the system resulted in inhibition of the enzymic activity (Table IV). Uridine and UMP-5' appeared to be more effective than the corresponding cytidine derivatives. The triphosphates of both uridine and cytidine were no more effective as inhibitors than the mono-

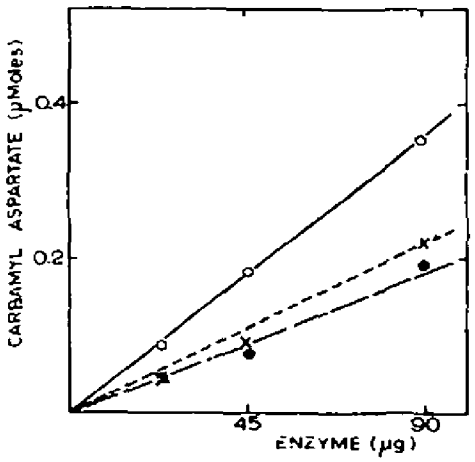

Fig. 7. ACKERMANN-POTTER plot of enzyme vs. inhibition by deoxyribonucleosides. The reaction mixture was similar to that described in the legend to Fig. 3 with the addition of either 15 μmoles of thymidine, ×- - - ×, or 20 μmoles of deoxycytidine, ●——●.

phosphates. The deoxyribonucleosides and deoxyribonucleotides were much more potent as inhibitors than the ribose derivatives. Maximal inhibition was obtained in the presence of thymidine. The deoxyribonucleotides as well as the ribonucleotides were no better as inhibitors of this enzyme than the nucleosides. In fact, in the deoxy-series, the nucleotide manifested less activity.

The inhibition produced by deoxycytidine and thymidine was determined as a function of the amount of enzyme (ACKERMANN-POTTER plot[13]) and the data (Fig. 7) would indicate competitive inhibition. Further proof as to the reversibility of the inhibition was supplied by employing the method of LINEWEAVER AND BURK[13]. The inhibition constants for thymidine and for deoxycytidine as determined by this method were $4.1 \cdot 10^{-4}$ M and $7.0 \cdot 10^{-4}$ M, respectively.

DISCUSSION

Aspartate transcarbamylase from rat liver appears to be quite different from the aspartate transcarbamylase isolated from *E. coli*[5] in terms of pH optimum, heat stability, stability at 4°, precipitability upon the addition of ammonium sulfate, and elution from DEAE-cellulose columns.

GERHART AND PARDEE[6] state that the addition of Ag^+ or Hg^{2+} to the bacterial system resulted only in a loss in feedback inhibition by the cytidine derivatives, but had no effect upon enzymic activity. Furthermore, in an earlier communication[13], they mentioned that the addition of *p*-hydroxymercuribenzoate did not inhibit, but in fact, enhanced enzymic activity. These data led them to postulate that inhibition by cytidine derivatives is a result of attachment of the pyrimidines to an adjacent site to that occupied by aspartate. This adjacent site presumably contained a sulfhydryl group which may be selectively destroyed by heating at 60° without any destruction of enzymic activity[6].

The data presented in this report indicate that Ag^+, Hg^{2+}, and *p*-hydroxymercuribenzoate inhibit mammalian aspartate transcarbamylase. No selective destruction of the site at which feedback inhibition occurs can be accomplished by heating at 60°, without a concomitant loss in activity of the enzyme. Hence, it may be inferred that a sulfhydryl group is intimately connected with enzymic activity in rat liver and may also be associated with feedback inhibition.

The inhibitions observed upon the addition of the pyrimidine nucleosides and nucleotides to the system containing the partially purified enzyme agree quite well with that observed in crude liver preparations[4]. Thymidine was the most potent inhibitor of this enzyme, while the uracil derivatives were as effective as the corresponding cytosine derivatives. These data again point out the basic difference between the mammalian and bacterial systems. Unfortunately, neither the ribonucleotide nor deoxyribonucleotide possessed greater inhibitory efficacy than the corresponding nucleoside in the former system, although the degree of inhibitory activity of the bacterial enzyme increased from nucleoside to nucleoside triphosphate.

Although the mammalian enzyme has been partially purified, still a large amount of deoxyribonucleoside (approx. $7 \cdot 10^{-3}$ M) is required for any degree of inhibition. These data would appear to cast some doubt upon the role that feedback inhibition of aspartate transcarbamylase plays in the regulation of pyrimidine biosynthesis in the mammalian cell. However, it is difficult to assess the degree of concentration of deoxyribonucleosides or deoxyribonucleotides and aspartic acid within the mammalian cell especially in conditions of rapid growth, *e.g.*, embryonic or regenerating liver. Sufficient concentration of pyrimidine derivatives may accumulate within the cell in these conditions to exert feedback inhibition. A comparison of the inhibitory efficacy of the pyrimidine deoxyribonucleosides upon this enzyme in various rapidly growing systems (embryonic or regenerating liver, hepatomas) may supply some further data on the role of feedback inhibition in the regulation of pyrimidine biosynthesis. These studies are presently in progress.

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