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GUINEA PIG ASPARAGINE SYNTHETASE

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SUMMARY

The partial purification and properties of guinea pig asparagine synthetase (L-aspartate:ammonia ligase (AMP), EC 6.3.1.1) are described. The enzyme activity is present largely in the high-speed supernatant fractions and is found in extracts from all tissues with the highest specific activities in extracts from spleen, liver, intestine and lymph nodes.

High concentrations of $(NH_4)_2SO_4$ or K_2SO_4 with glutamine are required to demonstrate this activity in crude enzyme preparations. L-Asparagine appreciably inhibits the reaction in the presence of glutamine but not in the presence of high concentrations of $(NH_4)_2SO_4$.

INTRODUCTION

Asparagine has been shown to be an essential amino acid for growth and protein synthesis in certain tumors[1–3]. Treatment of animals with L-asparaginase (L-asparagine aminohydrolase, EC 3.5.1.1) causes a marked reduction in circulating levels of asparagine and depletes tissue stores. The reduction (or depletion) of this essential amino acid produces a regression of susceptible tumors (see reviews[4,5]). The difference in sensitivity to L-asparaginase of various tumors and the host animal has led to a reexamination of the biosynthesis of asparagine. Sensitivity of tumors to this enzyme has been correlated with a low or absent level of asparagine synthetase[6–8] (L-aspartate: ammonia ligase (AMP), EC 6.3.1.1).

Unlike other common laboratory animals, the guinea pig has high circulating levels of L-asparaginase. Most likely, its tissues must synthesize asparagine to meet the cellular requirements for this amino acid, making the guinea pig a natural model for the study of asparagine synthesis in the L-asparaginase-treated animal. As expected, enzymic synthesis of asparagine has been readily demonstrated[8,9].

As a first step in analyzing the physiological regulation of asparaginase, a detailed study of guinea pig asparagine synthetase was undertaken. This paper reports the properties of a partially purified preparation of asparagine synthetase in this animal.

MATERIALS AND METHODS

Mixed breed male guinea pigs weighing 250–500 g were stunned by a blow on the neck and killed by exsanguination. The animal was perfused *in situ* through the descending aorta with ice-cold saline; the tissues were removed and homogenized in 3 vol. media A (ref. 10) in a Sorvall Omnimixer for 1 min. Cell debris was removed by brief centrifugation, and the supernatant was centrifugated at 105 000 × *g* for 60 min. The enzymatic activity of this supernatant fraction was stable for several months at −20°.

For the fractionation study the crude homogenate was centrifuged sequentially as follows: 3000 rev./min during 10 min (approx. 1000 × *g*); 10 000 rev./min during 60 min (approx. 12 000 × *g*); 17 600 rev./min during 30 min (approx. 38 000 × *g*) in a Sorvall Model RC2B; and 40 000 rev./min during 60 min (105 000 × *g*) in a Beckman Model L-2, type 40.0 rotor.

Asparagine synthetase assay

The enzyme was incubated with 1.5 mM of uniformly labeled L-[^{14}C]-aspartic acid ($3–6 \cdot 10^4$ counts/min per μmole), 10 mM neutralized disodium ATP, 10 mM $MgCl_2$, 200 mM neutralized $(NH_4)_2SO_4$ and 100 mM Tris–HCl buffer (pH 8.0) in a final volume of 0.5 ml. After 30–60 min incubation at 37° the reaction was stopped by the addition of 0.5 ml 0.8 M $HClO_4$. Protein was removed by centrifugation and each supernatant was transferred to another tube containing 0.6 ml 1.0 M potassium acetate. The $KClO_4$ formed on chilling the tubes was removed by centrifugation and 0.5 ml of the supernatant was passed through a column (4 cm × 0.4 cm) of HCl-washed alumina[11]. The column was eluted with 3.5 ml of 0.5 M acetic acid. This technique retained all the aspartic acid and eluted over 85% of the asparagine. The radioactivity in an aliquot of the asparagine-containing eluate was determined by liquid scintillation techniques. These samples were counted in Bray's solution[12] with a counting efficiency of about 50%.

The alumina eluates from incubations with crude enzyme from guinea pig liver contained asparagine *plus* other radioactive compounds[9]. Addition of *Escherichia coli* asparaginase to the reaction mixtures eliminated the asparagine radioactivity in these eluates. Therefore, duplicate incubations containing excess *E. coli* asparaginase (12 hourly units) were included in routine assays. The radioactivity in these eluates was subtracted from that of the eluates without added asparaginase. The synthesis of asparagine was calculated from this net radioactivity. With enzyme from Step 3 (see below) the radioactivity in the eluates from reactions containing added asparaginase was less than 25 counts/min over background radioactivity, and represented the small residual impurity in the L-[^{14}C]-aspartic acid. No correction was made for the incomplete recovery of asparagine from the columns (loss of 5–15%). 1 unit of the enzyme was defined as that amount which will catalyze the formation of 1 nmole asparagine per h under these conditions.

Additional methods

L-Asparaginase activity was routinely assayed by a measurement of ammonia release by a direct Nesslers reaction[13]. L-Glutaminase was measured in a similar fashion. In each case duplicate blank incubation mixtures were used to correct for nonenzymatic

hydrolysis of the amides and the ammonia in the various solutions. The amidase activities were expressed in μmoles NH_3 formed per h.

To assay asparaginase in the presence of $(NH_4)_2SO_4$, the enzyme samples were incubated 30 min at 37° with 8 mM L-[^{14}C]-asparagine ($1.3 \cdot 10^5$ counts/min) and 0–0.1 M $(NH_4)_2SO_4$ in a 100 mM Tris–HCl buffer (pH 8.0). The reaction was stopped, perchlorate precipitated, and the supernatant chromatographed as in the asparagine synthetase assay. Enzymatic activity was calculated from the loss of asparagine radioactivity in the alumina eluate.

Inorganic pyrophosphatase activity was assayed by measurement of phosphate release upon incubation of the enzyme preparation with 0.2 mM $Na_4P_2O_7$, 6 mM $MgCl_2$ and 60 mM Tris–HCl buffer (pH 7.5) for 15 min at 37°. Aspartic acid-dependent phosphate release was assayed by incubation of the enzyme preparation with 10 mM neutralized disodium ATP, 10 mM $MgCl_2$, 200 mM neutralized $(NH_4)_2SO_4$, 100 mM Tris–HCl buffer (pH 8.0) and 5 mM L-aspartic acid. Duplicate incubations contained no aspartic acid. After 60 min incubation at 37°, the protein was precipitated by trichloroacetic acid, and the supernatant was assayed for phosphate. Phosphate was assayed by a modification of the Fiske and SubbaRow method[14].

Protein concentrations were determined by a biuret method and by absorption at 280 and 260 nm, with bovine serum albumin as a standard[15].

Purification of asparagine synthetase

Step 1. The 105 000 × g supernatant fractions from liver, small intestine and occasionally spleen were pooled. 0.2 vol. of a fresh solution of protamine sulfate (10 mg/ml water) was added slowly. After 10 min at 0° the precipitate was removed and discarded. The protamine sulfate step produced little purification but removed material absorbing at 260 nm without decreasing the enzyme activity.

Step 2. $(NH_4)_2SO_4$ fractionation was performed at 5° using solid $(NH_4)_2SO_4$. The protein precipitating between 40 and 55% saturation was dissolved in 0.05 M Tris–HCl buffer (pH 7.5).

Step 3: Alumina Cγ absorption. The $(NH_4)_2SO_4$ fraction was diluted with distilled water to a conductivity less than that of 0.2 M $(NH_4)_2SO_4$. Alumina Cγ gel (0.35 mg dry wt. per mg protein) was added. After 30 min at 0°, the suspension was centrifuged and the supernatant discarded. The gel was then washed sequentially 4 times each with 0.2 M $(NH_4)_2SO_4$ and 0.51 M $(NH_4)_2SO_4$. These solutions were made from a saturated solution of $(NH_4)_2SO_4$ containing 0.2 mM EDTA and adjusted to pH 7. The volumes of the washes were 1/2 the original volume of gel added.

The 0.51 M $(NH_4)_2SO_4$ eluates lost about 50% of their activity in 1 week at −20°. Therefore, the combined eluates were immediately fractionated by addition of solid $(NH_4)_2SO_4$. The proteins precipitating between 13–43, 43–55 and 55–60% saturation were each dissolved in small volumes of 0.05 M Tris–HCl buffer (pH 7.5). These concentrated solutions were stable for several weeks at −20°.

Reagents and enzymes

Special reagents were obtained as follows: *E. coli* L-asparaginase from Worthington (Freehold) and Sigma (St. Louis); uniformly ^{14}C-labeled L-aspartic acid from Nuclear Chicago (Des Plaines) and New England Nuclear (Boston); uniformly ^{14}C-labeled L-asparagine from Nuclear Chicago; Tris base and disodium ATP from Sigma;

protamine sulfate from CalBiochem (Los Angeles); and enzyme grade $(NH_4)_2SO_4$ from Mann (New York). Alumina (acid washed) was reagent grade from Merck (Rahway) and was rewashed with HCl (ref. 11). Radioactive L-aspartic acid was purified by absorption on Dowex 1 (formate) and elution with 0.05 M HCl. L-Asparagine and L-aspartic acid were from K and K Laboratories (Plainview) and Sigma, and showed only one ninhydrin spot on cellulose thin-layer chromatography in three systems[9]. All other chemicals were reagent grade.

Alumina Cγ was prepared and aged several months according to the standard method[16].

RESULTS

Salt effects on asparagine synthetase

In initial experiments 105 000 $\times$ *g* supernatant fractions from guinea pig liver were incubated with 20 mM glutamine as the ammonia donor according to the method of PATTERSON AND ORR[6]. Under these conditions the radioactive aspartic acid was converted to compounds which eluted from the alumina columns, but thin-layer chromatography of these metabolites showed that they were not asparagine[9]. Similar supernatant fractions from small intestine and spleen formed no radioactive products

TABLE I

EFFECTS OF SALTS ON ASPARAGINE SYNTHETASE ACTIVITY

Guinea pig liver 105 000 $\times$ *g* supernatant (1.25 mg protein) was incubated 30 min at 37° in a 0.5-ml reaction mixture containing the following concentrations of components: 100 mM Tris–HCl buffer (pH 8.0), 1.5 mM aspartic acid, 10 mM disodium ATP and 10 mM $MgCl_2$. Salt solutions were neutralized when necessary with base of the same cation. All the reaction mixtures had pH's of 7.7–8.0 except 0.5 M K_2SO_4 (pH 8.4). Each percentage represents the average of at least two separate reactions.

Additives	*Final concn. (M)*	*Additives:*	*Percentage of maximal activity* None	*40 mM Glutamine*
$(NH_4)_2SO_4$	0.2		100*	100**
	0.5		106	
NH_4Cl	0.4		4	4
Ammonium acetate	0.4		4	
NH_4NO_3	0.4		1	
NH_4HPO_4***	0.16		11	
Na_2SO_4	0.2		0	4
K_2SO_4	0.1			18
	0.2		0	34
	0.5			41
None			0	5
0.2 M $(NH_4)_2SO_4$ *plus*				
NaCl	0.4		28	
Na_2SO_4	0.2		40	
KCl	0.4		52	
K_2SO_4	0.2		106	

* 100% activity equaled 26–32 nmoles/h/mg protein.
** 100% activity equaled 32–41 nmoles/h/mg protein.
*** Adjustment of the pH of this solution required a large amount of NH_4OH. The final concn. of NH_4^+ in this reaction mixture was 0.4 M.

which eluted from the alumina columns. Treatment of any of these supernatants by $(NH_4)_2SO_4$ fractionation or elution from a Ca_3PO_4 gel with $(NH_4)_2SO_4$ resulted in preparations which could synthesize asparagine. This asparagine synthetase activity was lost after passage of these fractions through Sephadex G 25 and was restored by addition of $(NH_4)_2SO_4$ to the reaction mixture. Maximal activity was achieved with 0.2 M $(NH_4)_2SO_4$ in the absence of glutamine and 0.1 M $(NH_4)_2SO_4$ with 20 mM glutamine. The addition of 20 mM glutamine to 0.2 M $(NH_4)_2SO_4$ produced a 5–20% stimulation of activity.

Reinvestigation of the 105 000 × *g* supernatants showed that they could also be activated by high concentrations of $(NH_4)_2SO_4$. Table I shows the effect of various salts on this reaction. In the absence of glutamine, $(NH_4)_2SO_4$ is the only ammonium salt that shows appreciable activity. Higher concentrations of $(NH_4)_2SO_4$ (0.5 M) are not inhibitory. On the other hand, the addition of NaCl, KCl and Na_2SO_4 to 0.2 M $(NH_4)_2SO_4$ markedly inhibits the reaction. The addition of K_2SO_4 does not change the activity. Thus it appears that high concentrations of both Cl^- and Na^+ inhibit the reaction.

With no ammonium salt in the reaction mixture there is essentially no reaction with or without added glutamine (40 mM). Glutamine *plus* $(NH_4)_2SO_4$ is slightly more active than $(NH_4)_2SO_4$ alone. Glutamine *plus* NH_4Cl or Na_2SO_4 is inactive but glutamine *plus* K_2SO_4 shows appreciable activity. This K_2SO_4 effect suggests that glutamine can act as the ammonia donor and that part of the activation by $(NH_4)_2SO_4$ may be due to SO_4^{2-}.

For convenience 0.2 M $(NH_4)_2SO_4$ without glutamine was used in routine assays.

Distribution of asparagine synthetase

Using this assay system the distribution of the activity was investigated. Table II presents the distribution on treatment of liver, small intestine and spleen by differential centrifugation. Clearly, most of the activity is in the 105 000 × *g* supernatant. The activity in the unwashed sediments could be due to contamination with the supernatant fraction. This location for the enzyme activity is similar to that reported for tumors and normal mouse and rat tissue (M. K. PATTERSON, Jr., personal communication)[7].

TABLE II

DISTRIBUTION OF ASPARAGINE SYNTHETASE ACTIVITY

Tissue preparation and centrifugation are as described in the text. Each value represents the average of two separate tissue preparations and assays. Fractions were assayed by the method described in the text. Results are expressed in nmoles of asparagine formed per mg protein per h, and as percentage of the total activity in all the fractions from each tissue.

Fraction	*Liver*		*Spleen*		*Small intestine*	
	Specific activity	*Percentage*	*Specific activity*	*Percentage*	*Specific activity*	*Percentage*
Sediments:						
(1) 3 000 × *g*	1.3	8	5.2	20	2.9	5
(2) 12 000 × *g*	1.9	6	6.8	8	2.7	6
(3) 38 000 × *g*	4.8	6	12.0	6	4.6	3
(4) 105 000 × *g*	2.2	4	11.7	6	4.4	6
Supernatant	11.5	76	16.6	61	21.4	82

TABLE III

DISTRIBUTION OF ASPARAGINE SYNTHETASE ACTIVITY

The 105 000 × *g* supernatant fraction from each tissue was assayed as described in the text. Specific activity represents nmoles asparagine formed per h per mg protein.

Organism	*Specific activity*
Small intestine (12)	34 ± 12*
Spleen (12)	19 ± 6*
Liver (12)	18 ± 6*
Stomach	18, 17**
Large intestine	18, 28
Lymph nodes	11, 14
Testes	3, 7
Kidney	6, 3
Brain	3, 4
Lung	4, 4
Heart	2, 3

* For these tissues this value is the mean ± S.D. of 12 animals from which separate tissue samples were prepared.

** The remaining values in the table are each from a separate preparation.

High-speed supernatant fractions (105 000 × *g*) were prepared from guinea pig tissues and assayed for asparagine synthetase in the standard assay system. There was considerable variation in the results from different animals. Nevertheless, there was a linear dependence of enzyme activity on protein concentration with each tissue. The assay gave reproducible results with the same tissue preparation. Table III shows that all the tissues examined had asparagine synthetase activity. Liver, spleen and intestine showed the greatest activity.

The specific activities of these tissue preparations are 10 to 100 times that reported for most rat and mouse tissues[5–8].

Purification

Table IV shows the results of a typical purification. A purification of 13–18-fold was achieved over the enzyme from Step 1, with a recovery of 27%.

Purification attempts using acid, ethanol or acetone fractionation, heating, DEAE- and CM-cellulose chromatography, and hydroxyapetite chromatography either caused marked loss of enzyme activity or no increase in specific activity.

Reaction characteristics

With the standard assay system the $(NH_4)_2SO_4$ precipitate (43–55% satn.) from Step 3 showed a linear increase in asparagine formation with protein (0–0.5 mg) and time (0–120 min).

The substrate requirements for the standard reaction are illustrated in Fig. 1. The dependence of the reaction on $(NH_4)_2SO_4$ is shown in Fig. 1A. For this experiment the enzyme from Step 3 was dialysed to remove residual $(NH_4)_2SO_4$. With this dialysed preparation there is no formation of asparagine without $(NH_4)_2SO_4$. High concentrations of $(NH_4)_2SO_4$ are required to achieve maximal activity.

This dialyzed preparation also catalyzes the reaction with glutamine as the amide donor. Reactions containing 20 mM glutamine and no $(NH_4)_2SO_4$ have approx. 50% of the activity as those with 0.2 M $(NH_4)_2SO_4$. The addition of 20 mM glutamine to varying concentrations of $(NH_4)_2SO_4$ produced much less than an additive effect; with 0.2 M $(NH_4)SO_4$ the addition of 20 mM glutamine produced only slight stimulation. The combined effect of these two amide donors suggests that the same enzyme can utilize either substrate. This data is similar to that reported for the Novikoff hepatoma enzyme[17].

K_2SO_4 will not stimulate the reaction with the dialyzed preparation when glutamine is the amide donor. Thus a partial purification has removed the SO_4^{2-} and some of the $(NH_4)_2SO_4$ requirement seen with the 100 000 $\times$ g supernatant fraction.

Undialyzed preparation from Step 3 shows slight activity even without added glutamine or $(NH_4)_2SO_4$. K_2SO_4 will slightly stimulate these preparations in reactions containing no added amide donor or glutamine. These effects are probably due to $(NH_4)_2SO_4$ in the undialyzed enzyme solution.

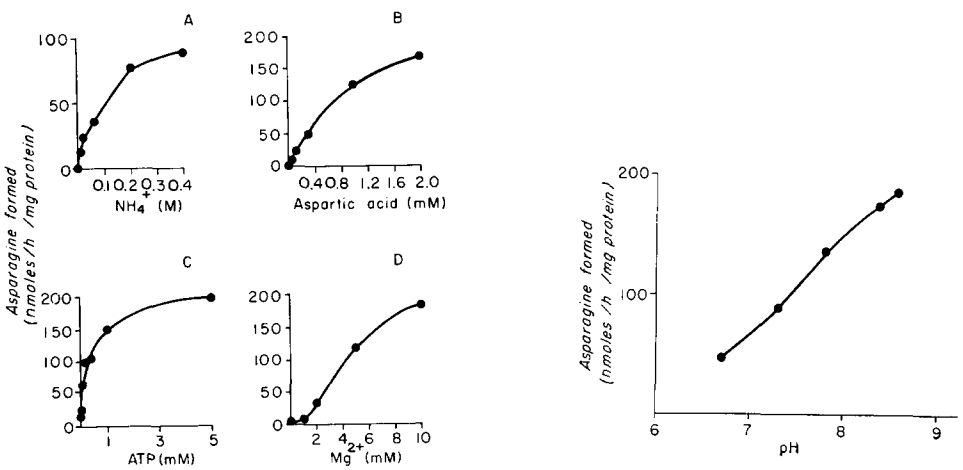


Fig. 1. Characteristics of the assay system. The reaction was incubated 30 min at 37° and the complete system contained the following concentrations of components in 0.5 ml: 100 mM Tris–HCl buffer (pH 8.0), 0.2 M $(NH_4)_2SO_4$, 1.5 mM aspartic acid, 10 mM disodium ATP and 10 mM $MgCl_2$. Reactions in B, C and D contained 0.13–0.26 mg protein of enzyme from Step 3. Reactions in A contained 0.49 mg protein of a preparation obtained by dialyzing Step 3 enzyme in 0.05 M Tris–HCl buffer (pH 8.0) for 4 h. See text for details of the assay procedure.

Fig. 2. pH curve. Conditions as in Fig. 1, except that 0.56 mg protein/ml from an enzyme preparation similar to that from Step 3 was used. pH was determined on an aliquot of the reaction mixture after the incubation.

The substrate requirements for aspartic acid and ATP are shown in Figs. 1B and 1C. The apparent K_m's for these substrates are as follows: aspartic acid 0.9 mM; ATP 0.3 mM. These values are 2–3 times the values reported for a partially purified enzyme from a Novikoff hepatoma[17].

The reaction is also dependent upon Mg^{2+} with maximal activity at about 10 mM when ATP is 10 mM (Fig. 1D). Ca^{2+} and Mn^{2+} at 3 and 10 mM are inactive.

TABLE IV

PURIFICATION OF ASPARAGINE SYNTHETASE

See text for assay method. 1 unit is the amount of enzyme which will catalyze the formation of 1 nmole asparagine per h.

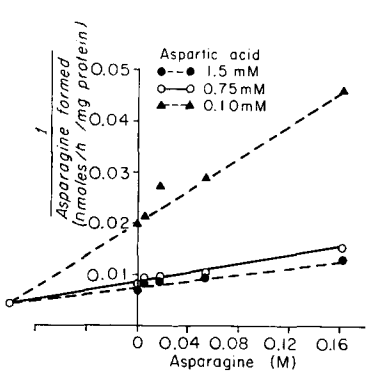
Step No.	*Fraction*	*Volume (ml)*	*Total protein (mg)*	*Total units*	*Specific activity (units/mg)*
1	Protamine sulfate supernatant	192	5840	74 400	13
2	40–55% $(NH_4)_2SO_4$ precipitate	20	1120	53 200	48
3	Alumina Cγ eluate and $(NH_4)_2SO_4$ precipitates				
	43–55% satn.	7.5	98	17 000	174
	55–60% satn.	3.0	13	3 000	230

The effect of pH on the reaction is shown in Fig. 2. The increase in activity with pH is similar to that described with the Novikoff hepatoma enzyme and is consistent with the hypothesis that un-ionized ammonia is the amide donor in this reaction[17].

Asparagine effect

The ability of L-asparagine to serve as an inhibitor of asparagine synthetase was tested. Fig. 3 shows the effect of addition of various concentrations of L-asparagine to the standard reaction mixture containing 0.2 M $(NH_4)_2SO_4$. Very high concentrations of L-asparagine relative to L-aspartic acid are required to inhibit this reaction. The K_i for L-asparagine is 100 mM.

On the other hand, Fig. 4 shows that L-asparagine is a very effective inhibitor of



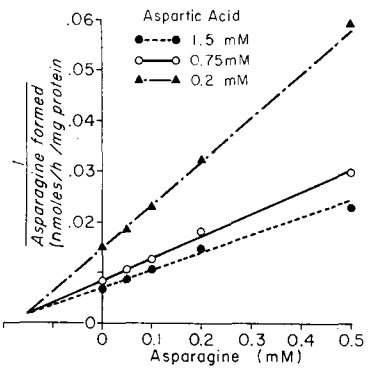


Fig. 3. Effect of L-asparagine in the presence of $(NH_4)_2SO_4$. Various concentrations of L-asparagine were incubated 30 min at 37° with 0.21 mg protein of an enzyme preparation similar to that from Step 3 and the following concentrations of components in 0.5 ml: 100 mM Tris–HCl buffer (pH 8.0), 0.2 M $(NH_4)_2SO_4$, 10 mM $(Na)_2ATP$, 10 mM $MgCl_2$ and either 1.5 mM (●— — —●), 0.75 mM (○—○) or 0.20 mM (▲— — —▲) aspartic acid. See text for details of the assay procedure.

Fig. 4. Effect of L-asparagine in the presence of glutamine. Various concentrations of L-asparagine were incubated 60 min at 37° with 0.13 mg protein of a dialyzed preparation similar to that from Step 3 and the following concentrations of components in 0.5 ml: 100 mM Tris–HCl buffer (pH 8.0), 20 mM glutamine, 10 mM disodium ATP, 10 mM $MgCl_2$ and either 1.5 mM (●— — —●), 0.5 mM (○—○) or 0.20 mM (▲—·—▲) aspartic acid.

the reaction when glutamine is used as the ammonia donor. Dialyzed enzyme from a preparation similar to Step 3 was used in this experiment. The K_i for L-asparagine is 0.15 mM. With these reaction conditions D-asparagine (5 and 15 mM) did not significantly inhibit the reaction.

L-Asparagine also inhibited the reaction in incubations containing undialyzed enzyme samples and glutamine. However, in contrast to the experiments with dialyzed enzyme, the asparagine had less effect, and complete inhibition was never seen. Thus, it appears that the low concentrations of $(NH_4)_2SO_4$ in the undialyzed preparations can modify the effect of L-asparagine.

Amidase activity

L-Asparaginase is present in various guinea pig tissues[8,18]. The 100 000 × *g* supernatant of liver contained appreciable asparaginase activity (7 μmoles NH_3 released per h per mg protein). Kidney and intestine had much lower activity and spleen had no asparaginase activity. Dialyzed enzyme from Step 3 had much less asparaginase activity (0.4 μmole NH_3 released per h per mg protein).

L-Asparaginase is inhibited by the high concentrations of $(NH_4)_2SO_4$ used in the standard assay for asparagine synthetase. Fig. 5 shows that both the guinea pig liver and *E. coli* asparaginase are inhibited over 75% by 0.1 M $(NH_4)_2SO_4$. In addition, the asparaginase activity in the liver 105 000 × *g* supernatant is inhibited about 50% by 0.2 M K_2SO_4.

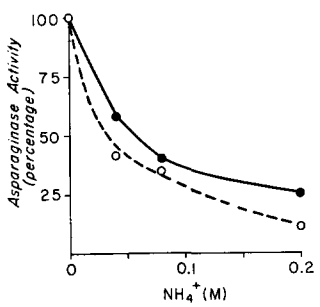


Fig. 5. Effect of NH_4^+ on L-asparaginase activity. 6 units of *E. coli* L-asparaginase (●—●) and 2.4 units of guinea pig liver 105 000 × *g* supernatant (○— — —○) were incubated 30 min at 37° with 8 mM L-[^{14}C]-asparagine, 100 mM Tris–HCl buffer (pH 8.0) and 0–0.1 M $(NH_4)_2SO_4$. See text for details of the assay procedure. Asparaginase units are μmoles NH_3 formed per h.

No glutaminase activity was found in liver 100 000 × *g* supernatant or dialyzed enzyme from Step 3. In addition, the *E. coli* asparaginase did not show significant glutaminase activity under the conditions used in the standard asparagine synthetase assay.

Aspartic acid-dependent phosphate release

An enzyme preparation similar to Step 3 also catalyzed an aspartic acid-dependent release of phosphate from ATP. This reaction was used to estimate the stoichiometry of the asparagine synthetase reaction. The enzyme preparation catalyzed the aspartic acid-dependent release of 190 nmoles phosphate per h per mg protein and the formation of 88 nmoles asparagine per h per mg protein by the standard assay. This

enzyme sample also had high inorganic pyrophosphatase activity (9.9 μmoles phosphate released per h per mg protein). The ratio of 2 moles phosphate released per mole of asparagine formed is consistent with the formation of AMP and pyrophosphate in this reaction with subsequent hydrolysis of the pyrophosphate. The synthesis of asparagine by preparations from a Novikoff hepatoma[17] and two bacteria[19,20] is associated with the hydrolysis of ATP to AMP and pyrophosphate. Among the enzymes studied only the asparagine synthetase from yeast forms ADP and inorganic phosphate[21]. Attempts to prove that the guinea pig enzyme forms AMP from [^{14}C]ATP during the formation of asparagine were unsuccessful due to interconversions of the adenosine nucleotides by the enzyme preparations.

DISCUSSION

Very high concentrations of $(NH_4)_2SO_4$ or K_2SO_4 with glutamine are required to demonstrate asparagine synthetase in the guinea pig tissue extracts. These salt effects appear to be quite specific as many other salts inhibit or do not activate the reaction. This activation may be due to the partial inhibition of L-asparaginase and the inhibition of other enzymes which utilize the substrates of the asparagine synthetase reaction. Such an indirect cause for the stimulation is suggested by the fact that the more purified preparations catalyze the synthesis of asparagine even in the absence of these salts.

Difficulties with the assay of asparagine synthetase have also been seen with crude preparations from adult rat liver. PATTERSON AND ORR[22] have reported that these homogenates contain a heat-labile material which causes a 50% inhibition of asparagine synthetase activity in extracts from rat embryo.

L-Asparaginase activity is not completely inhibited by $(NH_4)_2SO_4$ or K_2SO_4. Therefore, it appears that asparagine can be formed by the guinea pig extracts in the presence of endogenous asparaginase but not when *E. coli* asparaginase is added to the reaction mixture.

It must be recognized that the L-asparagine concentration in the standard asparaginase assay is 8 mM, while the concentration of asparagine formed in the asparagine synthetase assay is less than 0.1 mM. Thus a difference in the activity of the two asparaginase preparations at low L-asparagine concentrations could explain this apparent paradox.

The insensitivity of the standard asparaginase assay prevented the determination of the K_m for the guinea pig liver and *E. coli* preparations. Nevertheless, SCHWARTZ *et al.*[23] have shown that an *E. coli* asparaginase exhibits maximal activity at 0.1 mM. Guinea pig serum asparaginase has a K_m of 2.2 mM (ref. 24). A similar relatively high K_m for the liver asparaginase would fit this hypothesis as at 0.1 mM asparagine the activity would be less than 5% of maximum.

The guinea pig asparagine synthetase is inhibited by low concentrations of L-asparagine when glutamine is used as the ammonia donor. This inhibition is similar to that reported for enzyme preparations from a C3HED lymphoma[2], Novikoff hepatoma[17], bacterial sources[19,20], and yeast[21].

$(NH_4)_2SO_4$ modifies this inhibitory effect of L-asparagine. With high concentrations of $(NH_4)_2SO_4$ L-asparagine inhibits the reaction very little. This concentration

of $(NH_4)_2SO_4$ will also block the L-asparagine inhibitory effect on asparagine synthesis by a C3HED lymphoma preparation (unpublished results).

Lower concentrations of $(NH_4)_2SO_4$ decrease the inhibitory effect of L-asparagine and prevent complete inhibition of the guinea pig enzyme. It remains to be determined whether this modifying effect of $(NH_4)_2SO_4$ has any function in the control of asparagine synthesis in the cell.

Since a major cause of tumor sensitivity to L-asparaginase appears to be a lack of asparagine synthetase, study of the latter enzyme may lead to a reliable predictive test for L-asparaginase sensitivity and possible methods of augmenting the effects of L-asparaginase. Nevertheless, the crude tissue preparations so far studied show marked differences in competing reactions, inhibitors, and the effects of different amide donors. These differences make it currently very difficult to predict L-asparaginase sensitivity and to extrapolate the data from one system to another.

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REFERENCES

1 J. D. BROOME, *J. Exptl. Med.*, 118 (1963) 121.
2 J. D. BROOME, *J. Exptl. Med.*, 127 (1968) 1055.
3 L. H. SOBIN AND J. G. KIDD, *Proc. Soc. Exptl. Biol. Med.*, 119 (1965) 325.
4 R. H. ADAMSON AND S. FABRO, *Cancer Chemotherapy Rep.*, 52 (1968) 617.
5 J. D. BROOME, *Trans. N.Y. Acad. Sci.*, 30 (1968) 690.
6 M. K. PATTERSON, JR. AND G. R. ORR, *Biochem. Biophys. Res. Commun.*, 26 (1967) 228.
7 B. HOROWITZ, B. K. MADRAS, A. MEISTER, L. J. OLD, E. A. BOYSE, AND E. STOCKERT, *Science*, 160 (1968) 533.
8 M. D. PRAGER AND N. BACHYNSKY, *Arch. Biochem. Biophys.*, 127 (1968) 645.
9 J. S. HOLCENBERG AND J. PEASE, *Biochim. Biophys. Acta*, 158 (1968) 500.
10 E. B. KELLER AND P. C. ZAMECNIK, *J. Biol. Chem.*, 221 (1956) 45.
11 S. P. BESSMAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1st ed., 1957, p. 576.
12 G. A. BRAY, *Anal. Biochem.*, 1 (1960) 279.
13 J. D. BROOME, *J. Natl. Cancer Inst.*, 35 (1965) 967.
14 P. C. DEVITO AND J. DREYFUSS, *J. Bacteriol.*, 88 (1964) 1341.
15 E. LAYNE, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1st ed., 1957, p. 450.
16 S. P. COLOWICK, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1st ed., 1955, p. 97.
17 M. K. PATTERSON, JR. AND G. R. ORR, *J. Biol. Chem.*, 243 (1968) 376.
18 H. M. SULD AND P. A. HERBUT, *J. Biol. Chem.*, 240 (1965) 2234.
19 J. M. RAVEL, S. J. NORTON, J. S. HUMPHREYS AND W. SHIVE, *J. Biol. Chem.*, 237 (1962) 2845.
20 J. J. BURCHALL, E. C. REICHELT AND M. J. WOLIN, *J. Biol. Chem.*, 239 (1964) 1794.
21 A. M. H. AL-DAWODY, Thesis, Ann Arbor, Mich., 1961, University Microfilms, No. 62–735.
22 M. K. PATTERSON, JR. AND G. R. ORR, *Cancer Res.*, in the press.
23 J. H. SCHWARTZ, J. Y. REEVES AND J. D. BROOME, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 1516.
24 D. B. TOWER, E. L. PETERS AND W. C. CURTIS, *J. Biol. Chem.*, 238 (1963) 983.