L-Asparaginase EC-2 from *Escherichia coli*. Some Substrate Specificity Characteristics*

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ABSTRACT: L-Asparaginase EC-2 from *Escherichia coli* hydrolyzes L-glutamine and D-asparagine but at a much slower rate than L-asparagine. These amidase activities were not separated by several different methods of enzyme purification, by electrofocusing, nor by partial thermal inactivation. Upon injecting the enzyme into mice the activities in the blood plasma disappeared in accord with first-order kinetics with no change in the ratio of L-asparagine to D-asparagine or to L-glutamine hydrolysis. These data strongly suggest that the hydrolysis occurs at the same active site of the protein. Although product inhibition of L-asparagine hydrolysis occurred with ammonia at a pH value of 8.5, neither hydrolysis nor inhibition was found with L-aspartate, D-aspartate, L-glutamate, or D-glutamate.

Now that *Escherichia coli* L-asparaginase EC-2[1] has been demonstrated to be an active agent for the treatment of some tumors in man (Hill *et al.*, 1967; Oettgen *et al.*, 1967), the elucidation of its several enzymic activities is of considerable importance. The relationship of L-asparaginase activity to antitumor activity has been well established (Broome, 1963; Mashburn and Wriston, 1964; Roberts *et al.*, 1966; Campbell *et al.*, 1967); Old *et al.*, 1968). The low rate of L-glutamine hydrolysis by the *E. coli* L-asparaginase EC-2 has been reported (Campbell *et al.*, 1967) and shown to be a characteristic of this enzyme. In contrast, the L-asparaginase from guinea pig serum does not catalyze the hydrolysis of L-glutamine (Meister *et al.*, 1955). L-Glutamine is required for several metabolic pathways including the formation of L-asparagine by the enzyme L-asparagine synthetase (Patterson and Orr, 1967, 1968; Horowitz *et al.*, 1968; Prager and Bachynsky, 1968). Thus the L-glutaminase activity of EC-2 may be favorable for L-asparagine depletion during treatment. On the other hand, the L-glutaminase activity may cause such a reduction in glutamine in the body that it limits the tolerable therapeutic dose. Clarification of the value of this minor activity must await further experimentation.

Evidence is presented in this report which indicates that the hydrolysis of L-asparagine, D-asparagine, and L-glutamine is effected by a single site on the enzyme, EC-2. Product inhibition by ammonia at pH 8.5 was demonstrated but no inhibition was found with L-aspartate, D-aspartate, L-glutamate, or D-glutamate.

Materials and Methods

L-Asparaginase EC-2. L-Asparaginase EC-2 was prepared in the laboratory as previously described (Mashburn and Gordon, 1968). For comparison, preparations of L-asparaginase from *E. coli* were obtained from Farbenfabriken Bayer AG; Merck Sharp and Dohme Research Laboratories; E. R. Squibb Corp., Worthington Biochemical Corp.; and Wadley Institutes of Molecular Medicine (a generous gift of Dr. Joseph Roberts).

Enzyme Assays. The standard assay was carried out in Tris-Cl buffer (0.04 M, pH 8.5) and the liberated ammonia was collected under conditions previously described (Campbell *et al.*, 1967). In some experiments different substrates, buffers, and reaction conditions were substituted in the assay procedure as indicated. When maximum rates of hydrolysis were being determined, the level of enzyme was selected to result in the hydrolysis of only 2–4 μmoles of the 20 μmoles of substrate initially present. In the standard assays the reaction times were 10 or 12 min although the reaction rates were constant under these conditions for as long as 30 min. One unit of enzymic activity is equal to that quantity of enzyme which will release 1 μmole of ammonia/min under the conditions specified.

Reagents. The chemicals used in the preparation of substrates were identified as follows: L-asparagine monohydrate, Mann Assayed, P2439, $[\alpha]_D$ + 29.6° (*c* 2,6 N HCl), homogeneous by paper chromatography, Mann Research Laboratories, Inc.; D-asparagine monohydrate, C.P. S4697, $[\alpha]_D$ −32° (*c* 1, 0.1 N HCl), N = 18.58, Mann Research Laboratories, Inc.; L-glutamine, Crystal Grade III, approximately 99+%, Sigma Chemical Co., assays in our laboratories showed $[\alpha]_D^{23}$ +32.3° (*c* 1, 2 N HCl) and after hydrolysis no aspartic acid could be detected by electrophoresis using the methods of Biserte *et al.* (1960); L-glutamine, Lot No. 6308, shown to be free of asparagine by paper chromatography capable of detection of 0.3% asparagine if it were present; assays in our laboratories showed $[\alpha]_D^{23}$ +31.6° (*c* 1, 2 N HCl); this material was generously supplied to us by Dr. David Schwarz, Schwarz Bio-

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[1] Abbreviations used in this report are: EC-2, L-asparaginase EC-2 from *Escherichia coli* previously demonstrated to have antitumor activity (Campbell *et al.*, 1967); EC-1, L-asparaginase EC-1 from *Escherichia coli* previously demonstrated to show no antitumor activity (Campbell *et al.*, 1967); GLU, L-glutaminase from *Escherichia coli* described by Meister (1955); LDH, lactate dehydrogenase; LDH-virus, lactate dehydrogenase elevating virus (Riley *et al.*, 1960); DON, 6-diazo-5-oxo-1-norleucine an analog of L-glutamine; DONV, 5-diazo-4-oxo-L-norvaline.

Research Inc.; D-glutamine, Control No. 6193, C, 41.0; H, 6.8; N, 19.4; amide N, 9.3, $[\alpha]_D^{20}$ −31.5° (*c* 2, 1 N HCl), Nutritional Biochemicals Corp.; L-aspartic acid, Mann Assayed, N; 10.4, $[\alpha]_D$ +26.1° (*c* 10, 2 N HCl), homogeneous by paper chromatography, Mann Research Laboratories Inc.; D-aspartic acid, Mann Assayed, C, 36.12; H, 5.39, N, 10.3; $[\alpha]_D$ −24.0° (*c* 2, 6 N HCl), Mann Research Laboratories, Inc.; L-glutamic acid, Mann Assayed, N, 9.48; $[\alpha]_D$ +31.9° (*c* 10, 2 N HCl), homogeneous by paper chromatography, Mann Research Laboratories, Inc.; D-glutamic acid, Mann Assayed, L-2392, C, 40.95; H, 6.43; N, 9.48; $[\alpha]_D$ −31.74° (*c* 7, 2 N HCl), homogeneous by paper chromatography, Mann Research Laboratories, Inc.; DON was obtained from Dr. John Dice of Parke, Davis & Co.

All other chemicals were of reagent grade. The water used in these experiments was deionized then distilled in an all-glass apparatus.

Protein Estimations. Protein measurements were made by the method of Lowry as modified by Oyama and Eagle (1956) or by the spectrophotometric method of Warburg and Christian as described by Layne (1957).

Enzymic Reaction Products. Ammonia was measured by nesslerization as previously described (Campbell *et al.*, 1967). Other products were identified by paper electrophoresis of the reaction mixture at the end of the incubation according to the method of Biserte *et al.* (1960) using the pH 4.0 buffer, pyridine–acetic acid–water (6:20:974, v/v), at 350 V for 90 min. The bands were stained with 0.2% ninhydrin in acetone.

Isoelectric Point Determinations. The electrofocusing procedure was carried out on an Ampholine column (110 ml) according to the instructions provided by LKB Instruments, Inc., using Carrier Ampholyte of narrow range (pH 4–6) at 700 V. After 42 hr, 2.0-ml fractions were collected and the pH, as well as the enzymic activities, was determined on suitable aliquots of each fraction. The source of EC-1 and GLU was a fraction from DEAE-cellulose chromatography of an ammonium sulfate precipitated fraction of *E. coli* B sonicate described previously (fraction I shown in Table II of Campbell *et al.*, 1967).

Partial Thermal Inactivation. The enzyme preparation was diluted with buffer and aliquots were heated in a water bath for the specified intervals followed by a 30-min equilibration period in a water bath at 37°. The aliquots were stored at 5° overnight prior to assay.

Tests for Inhibition. The enzyme preparation, the substrate, and the buffer containing the material to be tested were adjusted to pH 8.0. Initial pH determinations at 25° were made on duplicate reaction mixtures to confirm that the decreases observed in the reaction rates were not due to differences in pH. The enzyme levels and reaction times were selected to reflect the reaction rates while the substrate was present (1) in excess, (2) at an intermediate level, and (3) close to exhaustion. When DON was the inhibitor, it was incubated at 37° with the enzyme in buffer for 5 min prior to the addition of the normal substrate. Separate controls, without substrate or without enzyme, were used and the ammonia content of the reagents and the enzyme preparations was determined under the conditions of the assays.

Rate of Disappearance of Enzyme in Vivo. Tumor-bearing (P1798 lymphosarcoma) or normal female BALB/c mice were injected intraperitoneally with 75 units of L-asparaginase EC-2 (Squibb). Mice were bled into heparinized tubes from the tail at the indicated intervals following injection. The plasma was pooled and immediately frozen. Aliquots of each plasma pool were later used for determination of L-asparaginase, D-asparaginase, L-glutaminase, and LDH activities; 2 weeks after the EC-2 injection when no L-asparaginase activity could be detected in the plasma, the presence of LDH-virus (Riley *et al.*, 1960) was demonstrated in the tumor-bearing mice by passage of this virus to the normal mice. Plasma (0.1 ml) from the tumor-bearing mice was injected intraperitoneally into the normal mice and LDH activity was measured in the plasma of each mouse over a period of 13 weeks to demonstrate the LDH activity elevation characteristic of the chronic infection with LDH-virus.

LDH Determinations. LDH activity was determined on plasma (10–20 μl) by a modification of the procedure of Kornberg (1955) using a total volume of 2 ml. The optical density at 340 mμ was read at 20-sec intervals for 4 min.

Results

Products of the Enzymic Reaction. L-Asparaginase EC-2 catalyzes the hydrolysis of the amide group. Paper electrophoresis of the reaction mixtures with L-asparagine or D-asparagine as the substrate showed bands corresponding to asparagine and aspartic acid, while bands corresponding to glutamine and glutamic acid were seen when L-glutamine was the substrate. The ammonia liberated was demonstrated by reaction with Nessler's reagent. No other ninhydrin-positive bands were seen in any of the assays.

Profile of Enzymic Activities. Our laboratory preparations of L-asparaginase EC-2 as well as samples of this enzyme from several different suppliers exhibit the same profiles of enzymic activities with respect to substrate, pH, and specific ion activation (Table I). The assay conditions for the activities making up this profile of enzymic activity were selected to compare EC-2 with other L-asparaginases and L-glutaminases which we have investigated. An enzyme from *Streptomyces griseus* has L-asparaginase activity when assayed in Tris-Cl (0.04 M, pH 8.6) but shows little or no activity in sodium borate (0.1 M, pH 8.5). Assays of EC-2 under these two conditions give essentially the same activity (Table I, columns 2 and 3). Another L-asparaginase, EC-1, from *E. coli* (Campbell *et al.*, 1967) shows only 3% as much activity at pH 5.0 as at pH 8.6 while EC-2 has 84–90% as much activity at pH 5.0 as at pH 8.6 (Table I, columns 2 and 4). Extracts of the *Pseudomonas* GG13 (Ramadan *et al.*, 1964) show almost equal activity for the hydrolysis of L-asparagine and L-glutamine and the L-glutaminase activity is strongly activated by high concentrations of phosphate. In contrast, the L-glutaminase activity of EC-2 is low compared with the L-asparaginase activity and it is depressed by phosphate ions (Table I, columns 5 and 7). The *E. coli* L-glutaminase previously described (Meister, 1955) has optimal activity at pH 4.7–5.0 and shows little or no activity above pH 7 (Kim *et al.*, 1968). This is in sharp contrast to the L-glutaminase activities of EC-2 (Figure 1 and Table I, columns 5 and 8). The assay at pH 7.4 in phosphate (0.025 M) plus NaCl to give an ionic strength of $\Gamma/2 = 0.15$ was included in the enzymic profile as a guide to the activity that might be expected under physiologic conditions.

Enzymic Activity As a Function of pH. The shape of the curves representing the activities of EC-2 as a function of pH differs depending upon the substrate being hydrolyzed (Figure

TABLE I: Profile of EC-2 Enzymic Activities.

Origin of Sample	% of Std L-Asp Assay Act. (100 × units under specified conditions/units of L-Asp under conditions of std assay)					
	L-Asparagine Substrate			L-Glutamine Substrate		
	Tris-Cl, 0.04 M, pH 8.57	Sodium Borate, 0.1 M, pH 8.47	Sodium Acetate, 0.1 M, pH 5.00	Tris-Cl, 0.025 M, pH 8.40	Sodium[b] Phosphate, 0.025 M, pH 7.40	Sodium Phosphate, 0.2 M, pH 8.28	Sodium Acetate, 0.1 M, pH 5.00
Squibb (7)[a]	100	97.7 ± 1.9	86.4 ± 2.6	2.99 ± 0.11	2.64 ± 0.10	1.81 ± 0.04	0.244 ± 0.031
Worthington (9)	100	97.7 ± 1.1	88.8 ± 1.9	2.98 ± 0.10	2.58 ± 0.12	1.76 ± 0.07	0.263 ± 0.035
Bayer (8)	100	97.6 ± 1.6	86.0 ± 1.8	2.52 ± 0.16	2.19 ± 0.06	1.54 ± 0.04	0.236 ± 0.015
Merck (3)	100	96.7 ± 2.1	86.7 ± 2.9	3.07 ± 0.03	2.57 ± 0.09	1.80 ± 0.03	0.283 ± 0.003
Wadley (1)	100	96	84	2.95	2.7	1.73	0.18
HJD (1)[c]	100	100	84	2.95	2.3	1.57	0.29

[a] The number of samples from each source is shown in parentheses. [b] Sodium chloride was added to this buffer to bring the reaction mixture to an ionic strength of I/2 = 0.15. [c] Sample from Hospital for Joint Diseases prepared by method of Mashburn and Gordon (1968).

TABLE II: Partial Thermal Inactivation of EC-2.[a]

Condn[b] of Heat Treatment	Duration (min)	L-Asparaginase Act. Remaining[c]	
		Assayed Immediately (Fraction)	Assayed after Storage 6 Days at 5° (Fraction)
Sodium acetate, 0.2 M, pH 5.0, 53°	0	1.00	1.00
	5	0.60	0.89
	10	0.46	0.74
	20	0.24	0.55
	40	0.19	0.46
Sodium borate, 0.2 M, pH 8.5, 63°	0	1.00	1.00
	5	0.78	0.95
	10	0.67	0.88
	20	0.56	0.72
	40	0.24	0.49

[a] Squibb L-asparaginase EC-2; specific activity 149 units/mg of protein. [b] The enzyme was diluted with buffer to 0.11 mg of protein/ml prior to heat treatment. [c] L-Asparaginase assays were at pH 8.4 and 0.1 M sodium borate.

1). There is a broad plateau of maximal activity of L-asparagine hydrolysis with little difference in the activity in the range of pH 6.0–8.6. The rate of hydrolysis of D-asparagine changes more rapidly with changes in pH and at the optimal pH 7.4 the rate of hydrolysis is only 6% of that for L-asparagine under the same conditions. With L-glutamine hydrolysis, the optimal pH (7.8) is still more sharply defined and the hydrolytic rate is only 2% of that for L-asparagine under these conditions. No enzymic hydrolysis of D-glutamine could be detected when 5.6 units of EC-2 was incubated for 1 hr at 37° with 20 μmoles of substrate.

Isoelectric Point of EC-2. The isoelectric point of the enzyme EC-2 is 4.95 ± 0.04. EC-2 samples from Merck, Squibb, and Wadley gave the same result as the sample prepared in our laboratory which is shown in Figure 2. The isoelectric point of the L-glutaminase and the D-asparaginase activities of the preparations always coincide with that of the L-asparaginase activity of the EC-2 preparation. Electrofocusing of another preparation which contained L-glutaminase GLU and L-asparaginase EC-1 but no L-asparaginase EC-2 shows that the isoelectric point of GLU is at pH 4.2 and that of EC-1 is at pH 5.2.

Partial Thermal Inactivation. Two phases of thermal inactivation were observed; an inactivation which was reversed with storage and the other an irreversible inactivation. Aliquots of enzyme which were partially inactivated by heat treatment could be partially reactivated by equilibration at 37° for 30 min or by storage for 6 days at 5°. These equilibrated aliquots of enzyme gave the same reproducible activities after further storage at 5°. The partial inactivation of EC-2 by heat treatment and the partial recovery of activity during storage at 5° is illustrated by the data in Table II. The heat treatment of

TABLE III: Partial Thermal Inactivation of L-Asparaginase and L-Glutaminase Activities of EC-2.[a]

Duration of[b] Heat Treatment (min)	Fraction of Act. Remaining in Heated Aliquots[c] (act. in heated aliquot/act. in unheated aliquot)					
	Sodium Borate, 0.2 M, pH 8.5, 68°		Sodium Acetate, 0.2 M, pH 5.0, 53°		Sodium Acetate, 0.2 M, pH 5.0, 56°	
	L-Asparaginase[d]	L-Glutaminase[e]	L-Asparaginase[d]	L-Glutaminase[e]	L-Asparaginase[d]	L-Glutaminase[e]
0	1.00	1.00	1.00	1.00	1.00	1.00
5	0.864	0.842	0.886	0.835	0.25	0.21
10	0.739	0.734	0.718	0.713	0.17	0.15
20	0.542	0.534	0.611	0.593	0.12	0.11
40	0.288	0.274	0.537	0.481	0.069	0.062

[a] Squibb L-asparaginase EC-2; specific activity 180 units/mg of protein. [b] The enzyme was diluted with buffer to 0.0736 mg of protein/ml prior to heat treatment. [c] The heat-treated aliquots were equilibrated at 37° for 30 min and stored at 5° overnight before assay. [d] L-Asparaginase assays were at pH 8.4 and 0.1 M sodium borate. [e] L-Glutaminase assays were at pH 8.0 and 0.1 M sodium borate plus 0.05 M sodium acetate.

the enzyme under various conditions always produced the same rate of inactivation for the enzymic hydrolysis of both L-asparagine and L-glutamine (Table III). The activity for the hydrolysis of D-asparagine was not determined in this experiment. Enzyme preparations from different suppliers (Bayer, Squibb, and Worthington) showed similar rates of inactivation by heat.

Disappearance of Activity of EC-2 in Plasma of Normal and LDH-Virus-Infected Tumor-Bearing Mice. Neither L-asparaginase, D-asparaginase, nor L-glutaminase activity could be detected in the plasma of either group of mice before administration of EC-2. After an intraperitoneal injection of EC-2, there was a short period of absorption followed by the disappearance of activity from the plasma at a rate following

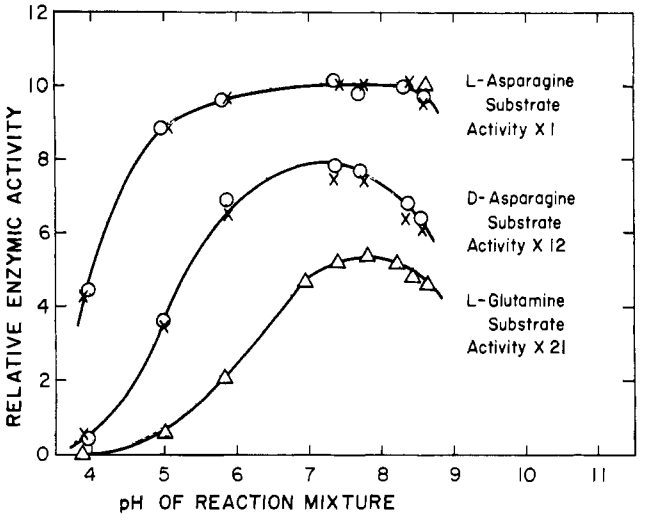

FIGURE 1: Hydrolysis of L-asparagine, D-asparagine, and L-glutamine as a function of pH. The L-asparaginase EC-2 preparations were (○) Squibb, specific activity 183 units/mg of protein; (X) Bayer, specific activity 287 units/mg of protein; and (△) Worthington, specific activity 116 units/mg of protein. The reactions were at 37° in a total volume of 2 ml with 20 μmoles of substrate. The enzyme level was selected so that 4 μmoles of ammonia was liberated from L-asparagine in 10 min at pH 8.5. The enzyme level was increased 12-fold for assays with D-asparagine and 21-fold with L-glutamine. Sodium chloride was added to bring each reaction mixture to isoionic conditions ($\Gamma/2 = 0.15$). The buffers were 0.025 M sodium acetate for pH 4 and 5; 0.025 M sodium phosphate for pH 6 and 7; and 0.025 M Tris-Cl for pH 8.

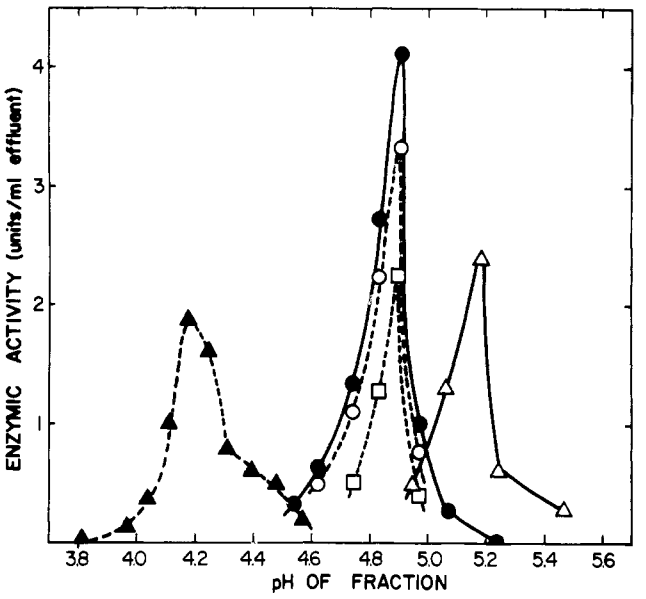

FIGURE 2: Determination of the isoelectric point by isoelectric fractionation of L-asparaginase EC-2, L-asparaginase EC-1, and L-glutaminase GLU from *E. coli*. Electrofocusing was carried out on an Ampholine column (110 ml) according to the instructions provided by LKB Instruments using the narrow range (pH 4–6) carrier Ampholyte at 700 V for 42 hr. Electrofocusing of a fraction (1.52 mg) from DEAE-cellulose chromatography of an *E. coli* extract completely separated the L-glutaminase GLU activity (▲- - -▲) from the L-asparaginase EC-1 activity (×30, △—△). The specific activity of the starting material was 1.65 L-asparaginase units and 6.17 L-glutaminase units per mg of protein. In a separate experiment, electrofocusing of 1.88 mg of L-asparaginase EC-2 (Hospital for Joint Diseases, specific activity 17.2 units/mg of protein) did not separate the L-asparaginase activity (●—●) from the D-asparaginase activity (×10, ○- - -○) or the L-glutaminase activity (×10, □- - -□).

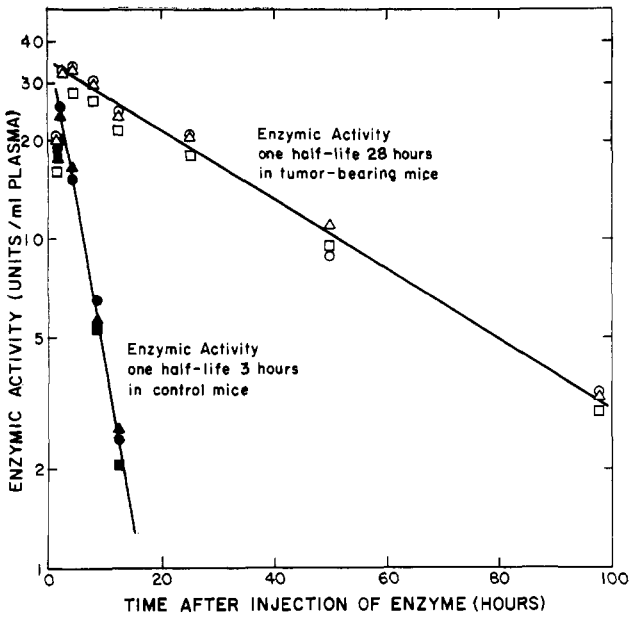

FIGURE 3: Disappearance of activity of EC-2 in plasma of normal and LDH-virus infected tumor-bearing mice. Tumor-bearing (11 days after P1798 lymphosarcoma transplantation) or normal female BALB/c mice were injected (intraperitoneally) at time zero with 75 units of EC-2 (Squibb) L-asparaginase, specific activity 197 units/mg of protein. We are indebted to Dr. Bernard Berk for this sample of enzyme. For plasma pools at periods 1, 2, 4, and 8 hr, 12 mice were bled (0.1 ml) for the first time. At periods of 12 and 24 hr, 24 mice were bled for the second time and 48 mice were bled the third time and fourth time for the priods 48 and 96 hr. The assays of enzymic activity were at pH 8.0 in 0.04 M Tris-Cl and 0.04 M NaCl. Amidase activities of plasma are shown for L-asparagine hydrolysis (▲) control mice, (△) tumor-bearing mice; D-asparagine hydrolysis times 12 (■) control mice, (□) tumor-bearing mice; L-glutamine hydrolysis times 33.3 (●) control mice, (○) tumor-bearing mice.

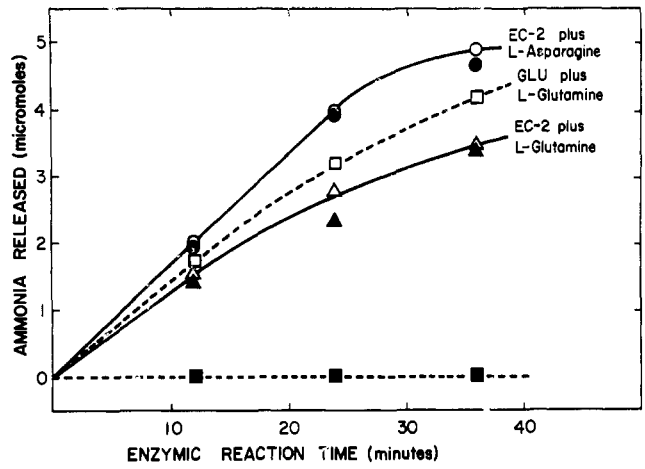

FIGURE 4: Effect of DON on amidase activity. The reactions were at 37° in 2 ml of reaction mixture. The L-glutaminase (Worthington, GLU, specific activity 6 units/mg of protein) reactions were at pH 5.0 in 0.10 M sodium acetate, with 5 μmoles of L-glutamine and 24 μg of protein: (□) control reactions; (■) reactions in which the enzyme was incubated 5 min with 5 μmoles of DON prior to the L-glutamine addition. The EC-2 (Squibb L-asparaginase, specific activity 210 units/mg of protein) reactions were at pH 8.5 in 0.025 M Tris-Cl and 0.07 M NaCl: (○) control reactions with 5 μmoles of L-asparagine and 0.79 μg of protein; (●) reactions with enzyme incubated 5 min with 5 μmoles of DON prior to L-asparagine addition; (△) control reactions with 5 μmoles of L-glutamine and 40 μg of protein; (▲) reactions with enzyme incubated 5 min with 5 μmoles of DON prior to L-glutamine addition.

first-order kinetics (Figure 3). The rate of disappearance of the enzyme activities was much slower from the tumor-bearing, LDH-virus-infected mice (half-life = 28 hr) than that for the normal mice (half-life = 3 hr). The half-life values reported here agree well with the L-asparaginase half-life values found for Swiss 1CR female mice (V. Riley, H. A. Campbell, and C. C. Stock, 1968, unpublished data). Under the conditions of the assay EC-2 enzyme preparation used in this experiment hydrolyzed 33.3 times as much L-asparagine as L-glutamine and 12 times as much L-asparagine as D-asparagine. Figure 3 shows that when the L-glutaminase activity in each sample is multiplied by 33.3 and the D-asparaginase activity is multiplied by 12 the disappearance curves for all three activities are identical.

The lactate dehydrogenase activity in the normal mouse plasma was in the range expected for normal mice Riley *et al.* (1960) while the plasma from the tumor-bearing mice uniformly showed high lactate dehydrogenase activities (Table IV) characteristic of tumor-bearing mice infected with the LDH-virus (Riley *et al.*, 1965). The presence of the LDH-virus in the tumor-bearing mice was demonstrated by injecting cell-free plasma from the tumor-bearing mice into normal mice. Passage of the virus was demonstrated by following the plasma lactate dehydrogenase levels for 13 weeks (Table IV). No tumors developed in 13 weeks in any of these mice while each was shown by assay to have elevated LDH activity characteristic of chronic infection with LDH-virus.

Effect of DON on the Amidase Activity. DON, a L-glutamine analog, has been shown to be a strong irreversible inhibitor in several enzyme systems involving L-glutamine (Levenberg *et al.*, 1957). In a report dealing with the inhibition of L-asparaginase by the L-asparagine analog DONV, Handschumacher has shown for comparison the irreversible inhibition of the L-glutaminase from *E. coli* by DON while this glutamine analog showed no inhibition of guinea pig serum L-asparaginase (Handschumacher *et al.*, 1968). Similarly we have found that when the L-glutaminase GLU was preincubated for 5 min with DON, no enzymic hydrolysis of L-glutamine took place when it was added (Figure 4). However, when EC-2 was preincubated for 5 min with DON, the rate of hydrolysis of both L-asparagine and L-glutamine was the same as that for control reactions with no DON (Figure 4). No ammonia was liberated from DON by EC-2 or by GLU under the conditions of the assays.

Competition Experiments. Figure 5 shows the rate of hydrolysis for the substrates L-asparagine, L-glutamine, and the mixed substrate containing 10 μmoles of L-glutamine and 5 μmoles of L-asparagine. The rate of hydrolysis with L-glutamine alone was only 3% of the maximum rate with L-asparagine as substrate. The mixed substrate resulted in a rate of hydrolysis only slightly lower than that for the L-asparagine alone. Figure 6 shows the rate of hydrolysis at two levels of L-asparagine EC-2 for the substrates L-asparagine, D-asparagine, and the mixed substrate containing 5 μmoles each of L-asparagine and D-asparagine. Under these reaction conditions at the lower level of enzyme the maximum rate of hydrolysis for the L-asparagine was 0.48 μmole/min, and for D-

TABLE IV: Lactate Dehydrogenase Elevation by LDH-Virus.

Groups of Mice[a]	Plasma LDH Assay (ΔOD/min per ml of plasma)					
	Pooled Plasma from Normal Mice[b]	Pooled Plasma from Tumor-Bearing Mice[b]	LDH Assays at Times Indicated after Injection with Plasma from Tumor-Bearing Mice[c]			
			1 hr	48 hr	163 hr	13 weeks
IC; IT	1.90	19.6	0.74 ± 0.06	3.83 ± 0.25	7.15 ± 0.33	5.53 ± 0.19
IIC; IIT	1.67	20.6	1.00 ± 0.18	3.31 ± 0.11	7.69 ± 0.38	5.77 ± 0.26
IIIC; IIIT	1.60	23.7	1.36 ± 0.26	3.54 ± 0.17	8.65 ± 0.68	5.99 ± 0.13
IVC; IVT	1.56	24.9	1.28 ± 0.10	3.52 ± 0.15	7.63 ± 0.36	6.77 ± 0.67

[a] These are the same mice used in the determination of the disappearance of activity of EC-2 (Figure 3). The normal mice (C) were in four groups of 12 mice each and the tumor-bearing mice (T) were similarly grouped. [b] The values shown are for mice after the EC-2 injection. Values on plasma for the period 1–96 hr after EC-2 injection were 1.69 ± 0.08 for normal mice and 21.5 ± 1.64 for tumor-bearing mice. [c] Pooled plasma from tumor-bearing mice was injected (0.1 ml) into each of five of the normal mice. LDH assays were made on each mouse at the times indicated.

asparagine 0.048 μmole/min, while the rate of hydrolysis for the mixed substrate was only 0.43 μmole/min. When the enzymic reactions with two substrates are catalyzed by two different enzymes, the total reaction rate with the mixed substrate is the sum of the rates with the separate substrates, unless the substrate of one enzyme inhibits the other enzyme. When two substrates compete for one enzyme, the reaction rate with a mixed substrate is intermediate between the individual rates with the separate substrates (Dixon and Webb, 1964, pp 84–86). The observations with the mixed substrates L-asparagine plus L-glutamine and L-asparagine plus D-asparagine are consistent with the latter situation, the rate of hydrolysis with the mixed substrates being less than the rate with L-asparagine alone. Experiments with Bayer L-asparaginase gave the same results as those shown in the figure for Squibb L-asparaginase.

Effect of Dicarboxylic Amino Acids and Ammonia on L-Asparagine Hydrolysis. L-Aspartate, D-aspartate, L-glutamate, and D-glutamate were not hydrolyzed by L-asparaginase EC-2 to give ammonia. The addition of these dicarboxylic acids to the reaction mixture at pH 8.0 had no effect on the rate of L-asparagine hydrolysis when present at twice the initial molar concentration of L-asparagine. Ammonia, however, added to the reaction mixture at pH 8.5 decreased the rate of hydrolysis of L-asparagine. The decrease in rate was related to the amount of ammonia added and was not reversed by a fourfold increase in the initial concentration of the L-asparagine. Ammonia added to the reaction mixture at pH 5.0 or at pH 7.4 had no effect on the rate of L-asparagine hydrolysis (Figure 7).

Discussion

Enzymes are usually classified by their functional activities, *i.e.*, by the reactions they catalyze. The same chemical reaction is often catalyzed by different enzymes, while purified enzymes that appear to be homogeneous proteins often show relative rather than absolute substrate specificity (Dixon and Webb, 1964, p 199).

The L-asparaginase, EC-2, which is now employed in clinical trials for the treatment of some tumors in man has a major activity for the hydrolysis of L-asparagine but also consistently shows significant hydrolysis of D-asparagine and L-glutamine. When a preparation exhibits several enzymic activities it is difficult to exclude the possibility that the minor activities are due to contamination with other enzymes even though several different purification procedures based on distinctly different physical or chemical properties were used. This is the case with EC-2. The most highly purified samples persist in showing activities for the hydrolysis of L-glutamine and D-asparagine although the rates of hydrolysis are low compared with that of L-asparagine. Under simulated physiologic conditions (pH 7.4, ionic strength $\Gamma/2 = 0.15$), the relative rates for the hydrolysis of L-asparagine, D-asparagine, and L-glutamine are 1.0, 0.08, and 0.03, respectively. With sufficiently high levels

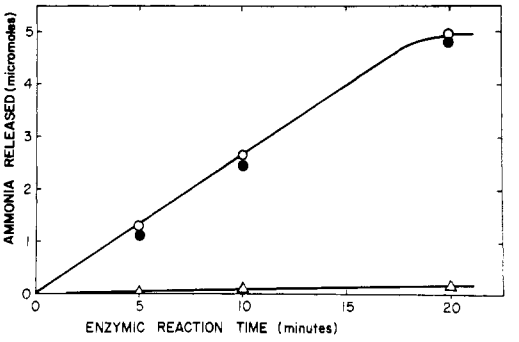

FIGURE 5: Effect of L-glutamine on the rate of L-asparagine hydrolysis. The reactions were at 37° and pH 8.0, in a total volume of 2 ml, 0.04 M Tris and 0.04 M NaCl, with 2.5 ug of protein (Squibb L-asparaginase, specific activity 105 units/mg of protein). The substrates were (○) 5 μmoles of L-asparagine; (△) 10 μmoles of L-glutamine; (●) 5 μmoles of L-asparagine plus 10 μmoles of L-glutamine. Values are corrected for the ammonia in control reactions without enzyme. Under the reaction conditions no ammonia was released from the enzyme preparation.

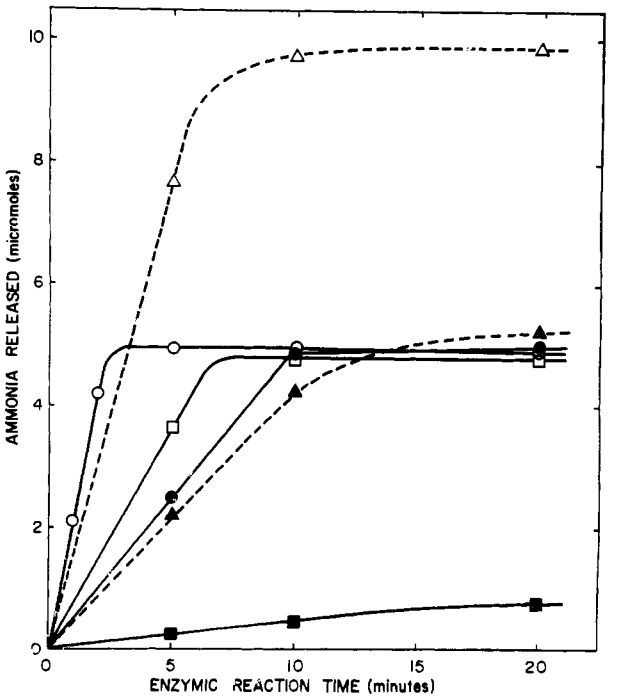

FIGURE 6: Effect of D-asparagine on the rate of L-asparagine hydrolysis. The reactions were at 37° and pH 8.0 in a total volume of 2 ml, 0.04 M Tris-Cl and 0.04 M NaCl with Squibb L-asparaginase, specific activity 187 units/mg of protein. The substrate and enzyme levels were (●) 5 μmoles of L-asparagine 2.7 μg of protein; (○) 5 μmoles of L-asparagine, 56 μg of protein; (■) 5 μmoles of D-asparagine, 2.7 μg of protein; (□) 5 μmoles of D-asparagine, 56 μg of protein; (▲) 5 μmoles of L-asparagine plus 5 μmoles of D-asparagine, 2.7 μg of protein; (△) 5 μmoles of L-asparagine plus 5 μmoles of D-asparagine, 56 μg of protein. Values are corrected for the ammonia in control reactions without substrate. Under the reaction conditions no ammonia was released from the substrates in the absence of enzyme.

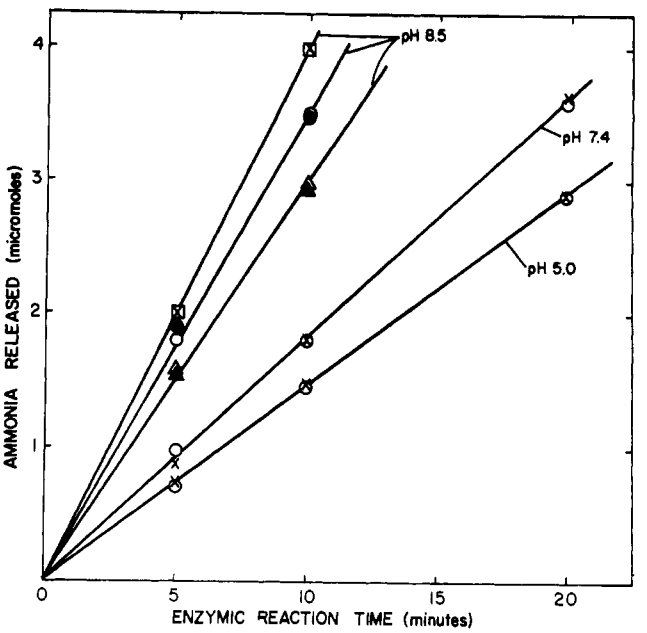

FIGURE 7: Effect of ammonia on the rate of L-asparagine hydrolysis. The reactions were at 37° in a total volume of 2 ml, with Squibb L-asparaginase, specific activity 105 units/mg of protein. Reactions at pH 8.5 were in 0.04 M Tris-Cl and 0.04 M NaCl with an enzyme level of 3.79 μg of protein; those at pH 7.4 in 0.025 M sodium phosphate plus NaCl to ionic strength $\Gamma/2 = 0.15$, and at pH 5.0 in 0.10 M sodium acetate were with an enzyme level of 1.64 μg of protein. The substrates were (×) 5 μmoles of L-asparagine; (○) 5 μmoles of L-asparagine plus 5 moles of ammonia; (△) 5 μmoles of L-asparagine plus 10 μmoles of ammonia; (□) 20 μmoles of L-asparagine; (●) 20 μmoles of L-asparagine plus 5 μmoles of ammonia; (▲) 20 μmoles of L-asparagine plus 10 μmoles of ammonia. The values plotted are corrected for the ammonia in control reactions without enzyme. No ammonia was released from the enzyme under the reaction conditions.

of enzyme, these substrates are hydrolyzed essentially to completion.

Ammonia inhibits the hydrolysis of L-asparagine at pH 8.5 but not at pH 5.0. Increasing the initial level of L-asparagine fourfold did not decrease the inhibition by ammonia. This inhibition appears to be similar to the noncompetitive product inhibition of urease seen with ammonia (Hoar and Laidler, 1950).

The activity as a function of pH is different for each of the substrates (Figure 1). The natural substrate was hydrolyzed over a broad pH range while the other substrates were hydrolyzed over a narrower pH range. This relation suggests that pH changes resulting in conformational changes of the enzyme or changes in the substrate are more critical for substrates presenting a poor fit in the enzyme site. Other enzymes are known which exhibit different pH optima for different substrates. For example, hexokinase from *Leuconostoc mesenteroides* has different pH–activity profiles for the phosphorylation of D-mannose and D-fructose (Sapico and Anderson, 1967). The L-glutaminase, GLU (Meister, 1955) which is present in the *E. coli* extracts from which EC-2 is prepared, has an optimum activity in the range of pH 4.7–5.0 and little or no activity in the range of pH 7.3 which is the optimum for the L-glutaminase activity of EC-2 (Kim *et al.*, 1968). This difference eliminates the possibility that the L-glutaminase activity of EC-2 is due to incomplete separation from the GLU. Further, the hydrolysis of L-asparagine and L-glutamine by EC-2 was not inhibited by the glutamine analog DON although this analog completely inhibited the L-glutaminase GLU which is present in crude extracts of *E. coli*. If the L-glutamine hydrolysis by EC-2 is due to admixture with a second enzyme, the second enzyme must have substantially different characteristics with respect to DON inactivation from those of GLU.

The following tabulation of results meets many of the criteria (Dixon and Webb, 1964, p 202) for a single enzyme with several activities. (1) The separation of proteins with respect to their isoelectric points by the method of electrofocusing resulted in protein fractions with parallel activities for the hydrolysis of L-asparagine, D-asparagine, and L-glutamine (Figure 2). (2) L-Asparaginase EC-2 prepared by various purification procedures from different suppliers and over a range of specific activities (13–300 units/mg of protein) has shown the same relative rates of hydrolysis of L-asparagine and L-glutamine under a variety of ionic and pH conditions selected to show differences between EC-2 and other L-asparaginases and L-glutaminases (Table I). (3) Partial thermal inactivation of EC-2 at pH 5.0 in acetate buffer near the isoelectric point of the enzyme and also partial inactivation at pH 8.5 in borate buffer resulted in parallel rates of inactivation for both L-asparagine and L-glutamine hydrolysis (Table III). (4) After an in-

traperitoneal injection of EC-2, the plasma of BALB/c mice showed parallel increases followed by parallel rates of disappearance of enzymic activities for L-asparagine, D-asparagine, and L-glutamine hydrolysis. The half-life was 3 hr for the three activities (Figure 3). (5) The plasma of BALB/c mice with subcutaneous P1798 lymphosarcoma which were infected with the LDH-virus of Riley *et al.* (1960) showed a much slower rate of disappearance of the three enzymic activities after intraperitoneal injection of EC-2 than did the normal mice. Infection with LDH-virus has been shown previously to decrease the rate of disappearance of some enzymes and to have no effect on the disappearance rate of other enzymes (Mahy *et al.*, 1967). In the case of EC-2, the rates of disappearance of hydrolytic activity for L-asparagine, D-asparagine, and L-glutamine were identical. The half-life for all three activities was 28 hr. Thus the activities are closely related and not separable by the process which removes the enzyme activity from the circulation. (6) In the competition experiments the mixed substrate containing both L-asparagine and D-asparagine resulted in a maximum rate of hydrolysis only 81% of the sum of the rates for the separate substrates. These results are consistent with L-asparagine and D-asparagine competing for a single site on the L-asparaginase EC-2, but it is recognized that this observation is not conclusive evidence for a single enzyme since two enzymes could give the same results if the substrate of one competitively inhibited the other.

While no one of these criteria is conclusive evidence by itself, yet as a group they provide strong evidence that the hydrolysis of L-asparagine, D-asparagine, and L-glutamine is catalyzed by one and the same enzyme.

The hydrolysis of D-asparagine by the L-asparaginase from guinea pig serum has been reported. Meister *et al.* (1955) in an extensive study of the substrate specificity of guinea pig serum L-asparaginase found 3% as much hydrolysis of D-asparagine as of L-asparagine at pH 8.5 in 0.05 M sodium borate. Tower *et al.* (1963) reported 5% as much hydrolysis of D-asparagine as of L-asparagine at pH 7.5 in 0.05 M sodium borate. Neither of these research groups found any hydrolysis of L-glutamine by the guinea pig serum preparations. Similarly Kim *et al.* (1968) investigating the entire range of pH 4–8.6 found no hydrolysis of L-glutamine by guinea pig serum. Thus in respect to asparagine hydrolysis the L-asparaginase from guinea pig serum and the L-asparaginase EC-2 from *E. coli* are similar, *i.e.*, each enzyme shows a low rate of hydrolysis for D-asparagine compared with the rate of hydrolysis of L-asparagine. However in respect to L-glutamine hydrolysis the enzymes are quite different. The L-asparaginase from guinea pig serum showing no detectable hydrolysis of L-glutamine while the L-asparaginase EC-2 from *E. coli* will hydrolyze at the maximum rate 3% as much L-glutamine as L-asparagine at pH 7.4.

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