Two L-Asparaginases from *Escherichia coli* B. Their Separation, Purification, and Antitumor Activity*

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ABSTRACT: Two L-asparaginases (L-asparagine amidohydrolase, EC 3.5.1.1) have been found in *Escherichia coli* B. and are designated EC-1 and EC-2. One of these enzymes (EC-2) is a potent antilymphoma agent. The two enzymes differ with respect to their solubility and chromatographic behavior as well as their activity as a function of pH. The amount of each enzyme present in a mixture can be determined by a comparison of the total L-asparaginase activity at pH 5.0 to that at pH 8.4. A procedure is described for preparing EC-2.

L-Asparaginase (L-asparagine amidohydrolase EC 3.5.1.1) is now well established (Broome, 1961, 1963a,b, 1965; Campbell *et al.*, 1964; Hiramoto *et al.*, 1966; Mashburn and Wriston, 1963; Suld and Herbut, 1965; Yellin and Wriston, 1966) as the antitumor agent in guinea pig serum (Kidd, 1953). L-Asparaginase from *Escherichia coli* B also exhibits antilymphoma properties (Mashburn and Wriston, 1964) while the enzyme isolated from yeast (Broome, 1965) or *Bacillus coagulans* (Mashburn and Wriston, 1964) does not. The antitumor action of guinea pig serum has been demonstrated on a variety of leukemias in mice (Boyse *et al.*, 1963; Mashburn and Wriston, 1964) as well as on the Murphy–Sturm lymphosarcoma (Ainis *et al.*, 1958; Kidd, 1953) and the Walker 256 carcinosarcoma in rats (Kwak *et al.*, 1961). We now report that there are two L-asparaginases in *E. coli* B, designated EC-1 and EC-2, only one of which exhibits the antitumor activity. Further biological studies on these two L-asparaginases are reported in other publications (Boyse *et al.*, 1967; Mashburn *et al.*, 1967). These two enzymes can be shown to differ by several criteria: solubility in ammonium sulfate solutions, chromatographic behavior, enzyme activity as a function of pH, as well as the antitumor activity.

Materials and Methods

E. coli B Cells. Sample 1 was obtained (as lyophilized cells) from Dr. Geoffrey Zubay of Brookhaven National Laboratories. Sample 2 was grown on trypticase soy agar for 18 hr. The culture was originally obtained from Dr. W. F. Goebel of the Rockefeller Institute and maintained at the University of Delaware on nutrient agar. Sample 3 was of the same origin as sample 2 but was grown in nutrient broth for 18 hr.

Samples 4–6 were obtained from General Biochemicals as frozen suspensions (30% solids). Sample 4 was harvested in early-log phase (25% of total normal harvest) from high peptone medium (lot no. 55463). Sample 5 was harvested in middle-log phase (50% of total normal harvest) from high peptone medium (lot no. 55120). Sample 6 was harvested in late-log phase from Kornberg medium (lot no. 56450). Samples 7 (lot no. ECB 6402), 8 (lot no. ECB 6504), and 9 (lot no. ECB 6506) were lyophilized cells obtained from Worthington Biochemicals Corp.

Protein Determinations. Protein determinations were made by the method of Lowry as modified by Oyama and Eagle (1956).

Enzyme Assays. The L-asparaginase and L-glutaminase assays were carried out at 37° according to the general procedures described by Meister (1955) with some modification of buffers and substrate solutions as described here. For the L-glutaminase assay, 0.5 ml of appropriately diluted sample was added to 0.5 ml of 0.04 M L-glutamine and 1 ml of 0.4 M sodium phosphate, pH 8.5. For the standard L-asparaginase assay, 0.5 ml of the diluted sample was added to 0.5 ml of 0.04 M L-asparagine and 1 ml of 0.2 M sodium borate, pH 8.5. The standard L-asparaginase assay reaction mixture was pH 8.4 for samples of little or no buffering capacity. For the determination of EC-1 and EC-2 in the presence of one another, a second assay was made using 1.0 ml of 0.2 M sodium acetate, pH 5.0, in place of the sodium borate. For the L-aspartase assay, 0.5 ml of sample was added to 1.0 ml of 0.05 M sodium phosphate, pH 7.3, and 0.5 ml of 0.04 M sodium aspartate, pH 7.3. The L-aspartic acid used in this assay was shown to be free of L-asparagine by paper electrophoresis (Biserte *et al.*, 1960). For the determination of L-asparaginase and L-aspartase activities as a function of pH, the reaction

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TABLE I: Yield of L-Asparaginase EC-1 and L-Asparaginase EC-2 from *E. coli* B Cells (Worthington lot no. ECB 6402).

Preparation	L-Asparaginase Activity								
	Total L-Asparaginases			L-Asparaginase EC-1		L-Asparaginase EC-2			
	Units/g of Dry Cells	Sp Act. (units/mg of protein)	Ratio (units at pH 5.0: units at pH 8.4)	Units/g of Dry Cells	Sp Act. (units/mg of protein)	Units/g of Dry Cells	Sp Act. (units/mg of protein)	Ratio (units of L-glutaminase: units of L-asparaginase)	Act. *in Vivo*[a]
Cell-free extract	5190	12	0.80	530	1	4660	11	0.10	*b*
2 M ammonium sulfate precipitated fraction (AS-1)	803	11	0.20	650	9	152	2	0.89	*b*
DEAE-cellulose chromatography of AS-1									
1st peak of L-asparaginase activity	76		0.90	0	0	76			
Pooled fraction I from 2nd peak	277	25	0.04	277	25	0	0	0.01	Inactive
2–4 M ammonium sulfate precipitated fraction (AS-2)	3460	38	0.91	0	0	3460	38	0.02	*b*
Hydroxylapatite chromatography of AS-2, pooled fraction II from column	3010	336	0.88	0	0	3010	336		Active
DEAE-cellulose chromatography after hydroxylapatite chromatography of AS-2, pooled fraction III from column	2265	902	0.89	0	0	2265	902	0.02	Active

[a] See Table II for details of tests *in vivo*. [b] Too toxic to be tested at L-asparaginase level of 1000 units/mouse.

mixtures were made isoionic ($\Gamma/2 = 0.15$) by the addition of NaCl.

The ammonia liberated by the enzyme reaction was collected by aeration and treated with Nessler's reagent, and the optical density was measured at 450 mμ. Controls were run to measure ammonia other than that released by enzyme activity. Under the conditions specified for the L-asparaginase assay the reaction is linear for periods up to 30 min.

One unit of activity is equal to that quantity of enzyme which will release 1 μmole of ammonia/hr at the maximum rate. These procedures gave values in agreement with those obtained by direct nesslerization after trichloroacetic acid precipitation and removal of the protein by centrifugation (Mashburn and Wriston, 1963).

Enzyme Preparations. Samples 1 and 3 were prepared according to the sonication procedure described by Mashburn and Wriston (1966), while sample 2 was prepared by grinding the cells with alumina (Mashburh and Wriston, 1964). Samples 4–9 were suspended (5%, w/v) in 0.05 M Tris·HCl, pH 8.6, and subjected to repeated sonication (three times at 10 min). The cell-free extract was removed by centrifugation after each sonication. The pellet was resuspended in buffer and the procedure was repeated. For the preparation of large amounts of L-asparaginase, such as that used for *in vivo* testing (Tables I and II), the suspended cells were disrupted by a single 20-min sonication of 75-ml aliquots. All cell-free extracts were treated with 0.05 volume of 1 M $MnCl_2$. After removal of the precipitated material by centrifugation, the supernatant was brought to 2 M by the addition of solid $(NH_4)_2SO_4$ with stirring. The suspension was adjusted to pH 8.0 with concentrated NH_4OH. The resulting precipitate (AS-1) was collected by centrifugation and redissolved by dialysis against 0.05 M sodium phosphate buffer, pH 8.6. The supernatant was brought to 4 M by the addition of solid $(NH_4)_2SO_4$ and adjusted to pH 8.0 by the addition of NH_4OH. The precipitate (AS-2) was collected and treated as described for AS-1. The DEAE-cellulose chromatography is not most effective in the presence of nucleic acid as the nucleic acid apparently reduces the capacity of the DEAE-cellulose to retain L-asparaginase. Extracts from some cells had to be titrated with more $MnCl_2$ in order to reduce the nucleic acid content of the ammonium sulfate precipitated fractions to less than 1% of the protein present as measured by the method of Warburg and Christian (Layne, 1957). Increasing the level of treatment with 1 M $MnCl_2$ from 0.05 to 0.10 volume had no significant effect on EC-2. However, the recovery of EC-1 was distinctly lower when an excess of $MnCl_2$ was used.

The L-asparaginase fractions which were obtained by ammonium sulfate precipitation were further purified by hydroxylapatite column chromatography. The hydroxylapatite (Bio-Gel HT, Bio-Rad Laboratories) was equilibrated with 0.05 M Tris·HCl, pH 8.6. The procedures described by Levin (1962) were followed for the chromatography.

When AS-1 containing both EC-1 and EC-2 is fractionated by hydroxylapatite chromatography, the EC-1 is eluted with 0.10 M sodium phosphate while the EC-2 is eluted with 0.10 M potassium phosphate. Thus, in chromatogram I of Figure 1, the first peak

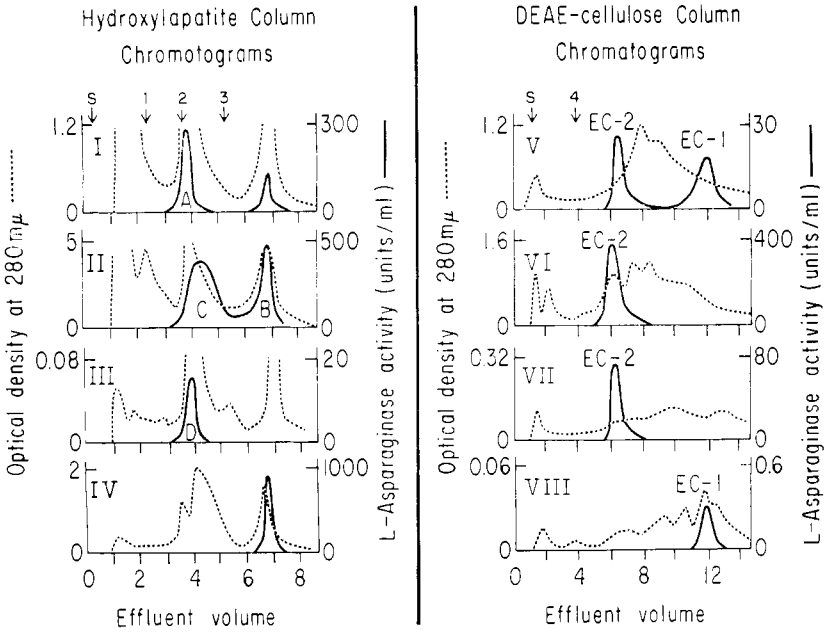

FIGURE 1: Chromatographic behavior of EC-1 and EC-2. Sample put on columns I and V, fraction AS-1; II and VI, fraction AS-2; III, fraction A from chromatogram I; IV, fraction C from chromatogram II; VII, fraction B from chromatogram II; VIII, fraction D from chromatogram III. The symbol (S ↓) indicates where the sample has completely penetrated the top of the packed column and in the hydroxylapatite columns is followed by sodium phosphate, 0.05 M, pH 8.6. Stepwise elution of the hydroxylapatite columns with pH 8.6 buffers was as follows: (1 ↓) 0.1 M sodium phosphate, (2 ↓) 0.05 M potassium phosphate, and (3 ↓) 0.1 M potassium phosphate. The scale for effluent volume is expressed in column volumes (milliliter of effluent per milliliter of column volume). The DEAE-cellulose columns and the samples for these columns were equilibrated with 0.02 M sodium phosphate, pH 8.0. The symbol (4 ↓) indicates where the continuous sodium chloride gradient was started by the addition to the mixing chamber of 0.35 M sodium chloride in 0.02 M sodium phosphate, pH 8.0.

(A) of L-asparaginase activity is EC-1 and the second peak is EC-2.

A sample (AS-2) shown to contain only EC-2 gave a single sharp peak of L-asparaginase activity eluted with 0.10 M potassium phosphate. This peak of L-asparaginase activity was in the same position as peak B in chromatogram II of Figure 1. When larger amounts of this same fraction were placed on the column, some of the EC-2 was eluted with 0.10 M sodium phosphate as shown in chromatogram II of Figure 1. Thus under these conditions of heavy loading of the column, part of the EC-2 was eluted in the same position as EC-1.

EC-1 upon rechromatography gave an L-asparaginase peak eluted with 0.10 M sodium phosphate in the same position as the original peak (Figure 1, peak A, chromatogram I, and peak D, chromatogram III). The portion of EC-2 eluted with 0.10 M sodium phosphate (peak C, chromatogram II, Figure 1) upon rechromatography is not eluted with 0.10 M sodium phosphate, but is eluted with 0.10 M potassium phosphate (chromatogram IV, Figure 1). While the position where EC-2 is eluted has shifted, much of the protein having no L-asparaginase activity is eluted in the same position as it was in the original chromatogram and is thus separated from EC-2. The portion of EC-2 eluted as peak B (chromatogram II, Figure 1) gave in the rechromatogram an L-asparaginase active peak in the same position as peak B.

Hydroxylapatite chromatography is useful for the separation of the L-asparaginases from other proteins, although it is not reliable under all conditions of column loading for the separation of EC-1 and EC-2 from one another. For the preparation of EC-2 from AS-2 the 0.10 M sodium phosphate elution step may be omitted and the L-asparaginase eluted as a single sharp peak with 0.10 M potassium phosphate.

The effluent containing L-asparaginase from the hydroxylapatite chromatography was further purified and separated into the two fractions EC-1 and EC-2 by DEAE-cellulose column chromatography. The DEAE-cellulose (Carl Schleicher & Schuell Co., Selectacel, type 20, 1.0 mequiv/g) was washed with 1 N NaOH, then 1 N HCl, and finally equilibrated with starting buffer. The procedures described by Peterson and Sober (1962) were followed for the chromatography. When an AS-1 fraction containing both EC-1 and EC-2 is chromatographed by DEAE-cellulose the two L-asparaginases are completely separated as shown in chromatogram V of Figure 1. A

TABLE II: Assay of *E. coli* B L-Asparaginase Preparations *in Vivo*.

Description of L-Asparaginase Prepn	*E. coli* B Cells (Sample No.)	L-Asparaginase Activity: Sp Act. (units/mg of protein)	Ratio (units at pH 5.0:units at pH 8.4)	EC-1	EC-2	Ratio (units of L-glutaminase:units of L-asparaginase)
Fraction I from DEAE-cellulose chromatography of 2 M $(NH_4)_2SO_4$ precipitated fraction (AS-1) (in position of L-asparaginase EC-1)	7	25	0.04	+	None	0.01
	9	28	0.05	+	None	0.01
	8	43	0.03	+	None	0.24
Fraction IV from DEAE-cellulose chromatography of 2–4 M $(NH_4)_2SO_4$ precipitated fraction (AS-2) (in position of L-asparaginase EC-2)	9	929	0.91	None	+	0.02
				Controls	1 ml of saline–phosphate[d]	
					No treatment	
Fraction II from hydroxylapatite chromatography of 2–4 M $(NH_4)_2SO_4$ precipitated fraction (AS-2)	7	315	0.87	None	+	0.02
				Controls	1 ml of saline–phosphate	
					No treatment	
Fraction III from DEAE-cellulose chromatography after hydroxylapatite chromatography of 2–4 M $(NH_4)_2SO_4$ precipitated fraction (AS-2)	8	309	0.91	None	+	0.02
				Controls	No treatment	
	7	904	0.89	None	+	0.02
				Controls	No treatment	

[a] Sample toxic, no injections on days 10 and 11. [b] Sample toxic, no injections on days 9–11. [c] Sample toxic. [d] All samples for testing *in vivo* were equilibrated by dialysis against saline–phosphate (0.144 M sodium chloride–0.01

continuous NaCl gradient was used for elution. L-Asparaginase assays at pH 8.4 and 5.0 show the first peak of activity (which appears at about 0.06 M NaCl) to be EC-2, and the second peak (at 0.2 M NaCl) to be EC-1.

An AS-2 fraction which has been shown by assay to contain only EC-2 gives only one peak of activity in the effluent (chromatogram VI, Figure 1). Chromatograms (Figure 1, VII and VIII) of fractions from hydroxylapatite chromatography peak B (Figure 1, II) and peak D (Figure 1, III) serve to relate these fractions to the chromatographic behavior of EC-1 and EC-2 on DEAE-cellulose. In certain cases (fractions I and IV, Table II) the chromatography on hydroxylapatite was omitted and the fraction obtained from the ammonium sulfate precipitation step was further purified by DEAE-cellulose chromatography.

Assay of Antilymphoma Activity. Leukemia EARAD1 was induced by X irradiation in a (C57BL/6 × A)F_1 female mouse of our own colony and is transplanted only

L-Asparaginase Total Units (injections twice daily for days 7–11 inclusive)	Tumor Inhib Act. Av Diam of Tumor (mm) Day 7	Day 11	Survivors/ Mice Treated	Day of Death from Leukemia	Day of Sacrifice of Nonleukemic Survivors
535[a]	8.5	13.5	0/3	15, 16, 16	
1070[a]	8.5	15.1	0/3	14, 16, 18	
464[b]	10.2	17.0	0/3	16, 17, 17	
928[b]	9.7	17.2	0/3	11, 17, 18	
1000[c]	10.5	14.8	0/3	17, 17, 18	
2000[c]	10.5	18.3	0/3	16, 17, 18	
4000[c]	8.9	16.7	0/3	18, 18, 18	
1000	9.6	0	0/3	22, 24, 29	
2000	8.8	0	2/3	49	60 (2 mice)
4000	9.8	0	1/3	42, 44	60
8000	9.2	0	2/3	46	60 (2 mice)
None	7.0	19.5	0/3	15, 17, 17	
None	8.6	17.5	0/4	15, 16, 16, 19	
1000	9.8	0	0/6	23, 23, 24 25, 25, 27	
2000	11.2	0	5/6	31	184 (5 mice)
4000	9.3	0	4/5	37	184 (4 mice)
None	6.0	20[e]	0/4	18, 18, 19, 19	
None	7.8	15	0/2	16, 18	
1000	11.0	0	0/2	24, 29	
2000	11.0	0	1/2	29	183
4000	10.0	0	1/2	76	183
None	10.5	18[f]	0/6	14, 14, 15, 16 16, 16	
1000	13.5	0	0/2	24, 31	
2000	13.5	0	1/2	31	163
4000	13.8	0	1/1		163
None	Not measured		0/21	All dead on or before 18	

M sodium phosphate, pH 8.0). [e] Average diameter on day 10. [f] Average diameter on day 13.

in these mice. It is sensitive to the antilymphoma effect of guinea pig serum (Old *et al.*, 1963). During the period of these experiments it was in its 110th–150th transplant generations.

For assay of antilymphoma activity, 3.5×10^6 EARAD1 cells were inoculated subcutaneously under an area of shaved skin on the flank. The preparations to be tested were injected intraperitoneally in ten equal doses (two injections each day) on day 7–11 (inclusive) after inoculation of EARAD1. On day 7 the subcutaneous mass has a diameter of approximately 1 cm (average of two measurements at right angles) and the leukemia is generalized (as indicated by transfer of the leukemia with cell suspensions taken from the spleen at this time). Antilymphoma activity is indicated by diminution of the subcutaneous mass, by extension of survival time, or by permanent recovery of the host. EARAD1 invariably grows progressively in untreated isogenic (C57BL/6 $\times$ A)F_1 mice. Mice that have recovered (6-months survival after

TABLE III: L-Asparaginase Activity of Preparations from Various Samples of *E. coli* B Cells

Sample No.	Source	Cell-Free Extract[a] L-Asparaginase Activity: Units/g of Dry Cells	Units/mg of Protein	Ratio (EC-2:total)	Ratio (units of L-glutaminase:units of L-asparaginase)
1	Dr. G. Zubay	760	3.00		
2	University of Delaware, agar grown[b]	1466	10.40		
3	University of Delaware, broth grown	400	6.20	0.00	
4	GB early-log phase[c]	1820	4.45	0.04	0.12
5	GB middle-log phase	2050	6.16	0.48	
6	GB late-log phase	2360	4.82	0.29	
7	W lot no. ECB 6402	5217	10.16	0.87	0.12
8	W lot no. ECB 6504	2811	5.86		0.37
9	W lot no. ECB 6506	8786	17.70	0.92	0.09

[a] Cell-free extracts were too toxic to be tested for tumor inhibitory activity. [b] Cells extracted by grinding with alumina in Tris buffer (Mashburn and Wriston, 1964). [c] Dialyzed ammonium sulfate precipitates tested on Gardner lymphosarcoma 6C3HED in C3H mice as previously described (Mashburn and Wriston, 1963). [d] Tested after further purifica-

inoculation of EARAD1) as a result of treatment are again fully susceptible to challenge with a minimal inocula of EARAD1.

Results

We have found two L-asparaginases which are extractable from disrupted *E. coli* B cells and have designated them EC-1 and EC-2. Both EC-1 and EC-2 act on L-asparagine to give L-aspartic acid and ammonia as end products. The aspartic acid was identified by paper electrophoresis (Biserte *et al.*, 1960) of the reaction mixture after incubation. No ninhydrin-positive bands were found except those corresponding to aspartic acid and asparagine. Neither the enzyme preparations nor the substrate alone gave bands corresponding to L-aspartic acid. The ammonia liberated from L-asparagine was demonstrated by reaction with Nessler's reagent. Neither EC-1 nor EC-2 released ammonia from sodium L-aspartate when incubated under the L-asparaginase assay conditions.

L-Asparaginase Activity as a Function of pH. Purified samples of the L-asparaginases were assayed at various hydrogen ion concentrations. As shown in Figure 2 the purified preparations form two distinct groups with respect to the dependence of the activity on pH. There were no specific acetate, phosphate, or borate ion effects on the activity of either EC-1 or EC-2, when measured under the isoionic conditions of these experiments. The distinct difference in the kinetic properties of the two enzymes is shown by the shape of the curves representing activity as a function of pH. EC-2 has very little change of activity over the range from pH 6.0 to 8.4 with an optimum at approximately pH 7. EC-1, on the other hand, has a rapid and progressive decrease in activity below pH 8.4. As shown in Figure 2, the purified EC-2 did not liberate ammonia when incubated with sodium L-aspartate over the pH range 5.0–8.5. Thus the maximum L-asparaginase activity seen in purified EC-2 samples in the range pH 7.0–8.0 cannot be attributed to aspartase activity on the L-aspartic acid released.

The difference in the L-asparaginase activity as a function of pH provides the basis for identifying purified preparations of EC-1 and EC-2. For the quantitative determination of EC-1 and EC-2 it is not necessary to construct the entire curve of activity as a function of pH. The ratio of activity at pH 5.0 to the activity at pH 8.4 is 0.89 for EC-2 and 0.04 for EC-1 (Tables I and II). Mixtures of purified EC-1 and EC-2 show activities in agreement with calculated values, and in this respect show no evidence of interaction (Figure 3).

It is possible to determine EC-1 or EC-2 in mixtures of the two enzymes by use of the equations:

activity at pH 8.4 attributable to EC-1 =

1.047 [total L-asparaginase activity at pH 8.4] −

1.18 [total L-asparaginase activity at pH 5.0]

activity at pH 8.4 attributable to EC-2 =

1.18 [total L-asparaginase activity at pH 5.0] −

0.047 [total L-asparaginase activitv at pH 8.4]

2 M Ammonium Sulfate Precipitate (AS-1)				2–4 M Ammonium Sulfate Precipitate (AS-2)			
L-Asparaginase Activity				L-Asparaginase Activity			
Units/g of Dry Cells	Ratio (EC-2:total)	Ratio (units of L-glutaminase:units of L-asparaginase)	Anti-tumor Act.	Units/g of Dry Cells	Ratio (EC-2:total)	Ratio (units of L-glutaminase:units of L-asparaginase)	Anti-tumor Act.
544			Inactive[c,d]	42			
274			Inactive[c]	1088			Active[c,d]
8				320			Active[c,d]
610	0.00	0.59		115	0.41		
586	0.04	0.89		937	1.0		
1240	0.01	>0.72	Inactive[c]	647	0.96		Active[c,d]
680	0.23	0.62	Inactive[c,d]	3707	1.0	0.02	Active[c,d]
549	0.09	>0.33	Inactive[d]	1763	1.0	0.02	Active[d]
743	0.30	0.33	Inactive[d]	7586	1.0	0.02	Active[c,d]

tion by chromatography (see text). [e] Abbreviations used: GB, General Biochemicals; W, Worthington Biochemical Corp.

EC-1 contributes very little to the activity of a mixture of EC-1 and EC-2 assayed at pH 5.0. Thus an assay at pH 5.0 alone may be of sufficient accuracy to be useful for the screening of extracts prepared from cells rich in EC-2.

L-Aspartase as well as EC-1 and EC-2 is present in AS-1 (Figure 4). The L-aspartase activity is shown (open circles) to have a sharp optimum at pH 7.3. The relatively low activity of the L-aspartase at pH 5.0 and at pH 8.4 permits the determination of EC-1 and EC-2 in the presence of L-aspartase. The ratio of ammonia released from L-asparagine by AS-1 at pH 5.0 to that at pH 8.4 shows that EC-2 represents 51% of the total L-asparaginase activity. The amounts of ammonia attributable to EC-1 and EC-2 activities have been estimated from the activities of the purified enzymes which are free of L-aspartase activity (Figure 2). These calculated values are shown in Figure 4 by the dashed lines. When L-asparagine is the substrate for AS-1 the ammonia released is greater than that attributable to the sum of EC-1 and EC-2. This increased release of ammonia results from the action of L-aspartase on the L-aspartic acid which is the product of the L-asparaginase reaction.

Yield of L-Asparaginases EC-1 and EC-2. Table III shows the large variation in the amount of L-asparaginase activity in preparations from various samples of *E. coli* B cells. The differences are not only in the total amount of L-asparaginase activity in the cells but are also in the proportions of the enzymes EC-1 and EC-2.

For samples 4–9 the total enzyme recovered in the three extractions is recorded in Table III. The amount of enzyme released by the first 10-min sonication was 85–98% of the total while the third sonication released only 1–4% of the total. The specific activity of the L-asparaginase released in the second sonication was less than one-half of that of the first while that of the third extract was about 20% of the first. Thus, the repeated sonications brought about the release of more protein from the cells but very little more L-asparaginase. There was no apparent difference in the release of the EC-1 and the EC-2.

Antilymphoma Activity. The two L-asparaginases EC-1 and EC-2 are markedly different with respect to antilymphoma activity. The results of assays *in vivo* are shown in Tables II and III. With this leukemia (EARAD1) the untreated controls invariably die in 15–22 days. A decrease in size of the tumor mass and an increase in survival time is obtained with as little as 50 units of EC-2. Intermediate levels of treatment (1000 units) result in a complete disappearance of the subcutaneous tumor mass and extends survival time, but only rarely is there a cure. At higher levels of treatment (2000 units or more) cures are frequent. Preparations of EC-2 are nontoxic. Normal mice treated with 2000 units do not lose weight or show any untoward reactions.

Preparations of EC-1 showed no detectable antilymphoma activity, although at the present degree of purification they were too toxic to be tested at very high levels. No decrease in tumor size or increase in survival time was seen with mice treated with doses as high as 4000 units (Table II). The critical site at which the L-asparaginase acts in effecting its antitumor activity is not known. Consequently, it is not possible to predict at which hydrogen ion concentration L-asparaginase activity comparisons should be made in order to reflect the *in vivo* activity of EC-1 and EC-2. However, at the high dose levels of EC-1 the L-asparagi-

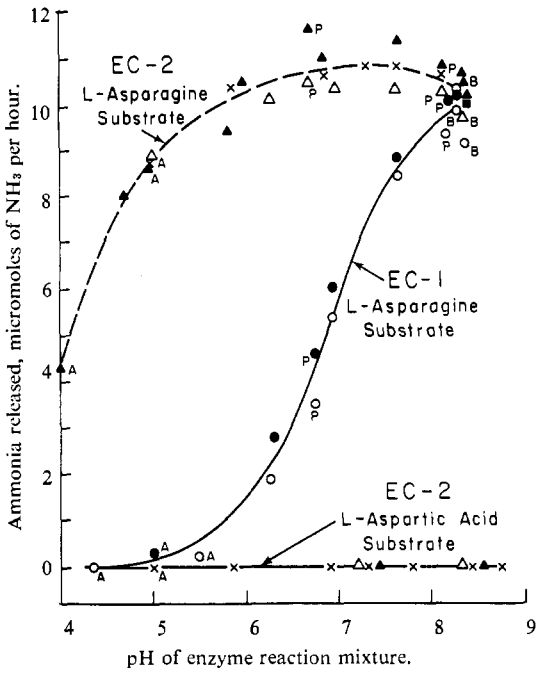

FIGURE 2: Comparison of EC-1 and EC-2 with respect to L-asparaginase activity as a function of pH at 37°. Sodium chloride was added to bring each enzyme reaction mixture to isoionic conditions ($\Gamma/2 = 0.15$). Salts other than sodium chloride are indicated as follows: (A) 0.025 M acetate, (B) 0.1 M borate, and (P) 0.2 M phosphate, unlabeled points 0.025 M phosphate. The point ■ is the activity under standard assay conditions, 0.1 M borate at pH 8.4. L-Asparaginase sample identification: (△) EC-2, chromatogram V, Figure 1, sp act. 104 units/mg of protein; (▲) EC-2, chromatogram VI, Figure 1, sp act. 742 units/mg of protein; (x) EC-2, Table II, fraction IV, sp act. 929 units/mg of protein; (O) EC-1, DEAE-cellulose chromatography, sp act. 51 units/mg of protein; (●) EC-1, chromatogram V, Figure 1, sp act. 36 units/mg of protein. Samples ●, △, and ▲ were from Worthington cells (sample 9, Table III), while sample O was from General Biochemicals cells (sample 4, Table III).

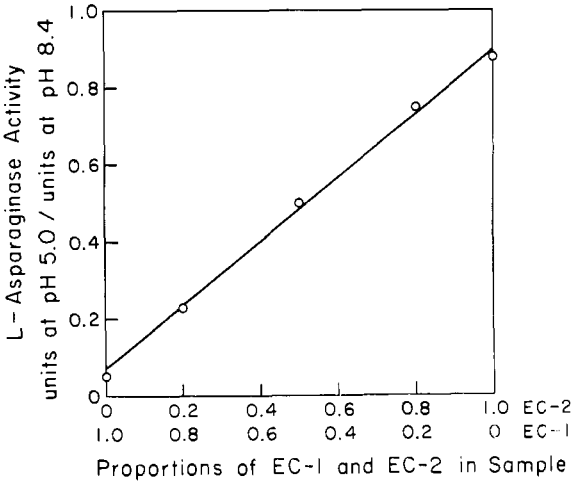

FIGURE 3: Differential assay of mixtures of purified EC-1 (Table II, fraction I, cell sample 9) and EC-2 (Table II, fraction IV). Samples were mixed in various ratios and the mixtures assayed at pH 8.4 and 5.0.

nase activity *in vitro* even at pH 5.0 is greater than that of EC-2 at a dose level which gives antitumor activity, namely, 50 units.

L-Glutaminase. The L-glutaminase activity of the cell-free extracts was concentrated in the AS-1 fraction. This fraction was repeatedly found to lack antitumor activity (Table III). A sample purified by DEAE cellulose chromatography (Table II, fraction I from *E. coli* B cells of sample 8) which had L-glutaminase activity equal to 24% of the L-asparaginase (EC-1) was also devoid of tumor inhibitory effect at a level of 1000 units of L-glutaminase.

Discussion

Enzymes which catalyze the hydrolysis of L-aspara-

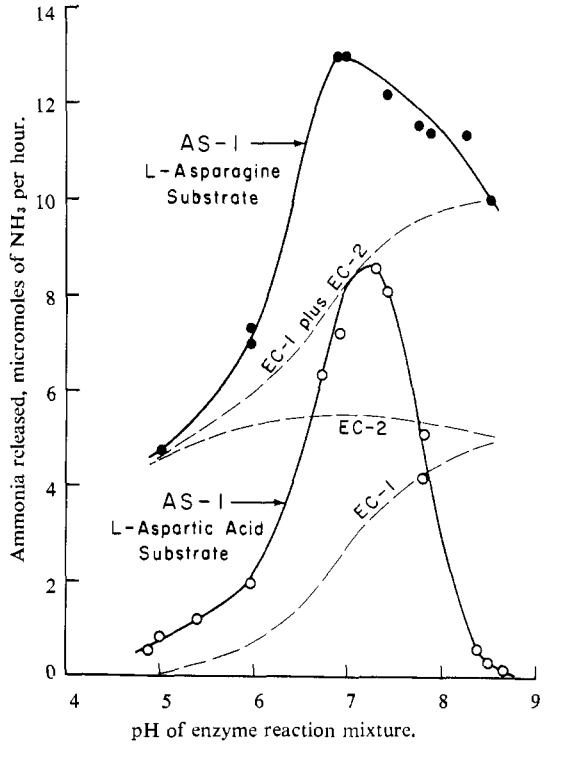

FIGURE 4: Fraction AS-1 incubated with L-asparagine (●) or with L-aspartic acid (O) at 37° for 30 min. The calculated release of ammonia attributable to EC-1 and EC-2 from L-asparagine is shown (- - - -). Buffer for pH 5.0 was 0.025 M sodium acetate and for pH 8.4 was 0.1 M sodium borate. The buffer for other pH values was 0.025 M sodium phosphate. Reaction mixtures were brought to isoionic conditions ($\Gamma/2 = 0.15$) by the addition of sodium chloride.

gine to L-aspartic acid and ammonia have been obtained from plants (Kretovich, 1958) as well as from animals (Clementi, 1922; Greenstein and Price, 1949; Meister *et al.*, 1955) and microorganisms (Kirchheimer and Whittaker, 1954; Manning and Campbell, 1957; Miller *et al.*, 1955; Ramadan *et al.*, 1964). Two L-asparaginases were found in rat liver (Greenstein and Price, 1949), one of which is activated by phosphate but not by α-keto acids, while the other requires the presence of an α-keto acid for activity. We find two L-asparaginases in *E. coli* B, neither of which requires α-keto acids or is affected by phosphate ions.

The L-asparaginases from different sources vary considerably with respect to their substrate specificity. The enzyme from guinea pig serum does not hydrolyze glutamine at all whereas the pseudomonad enzyme studied by Ramadan *et al.* (1964) has L-glutaminase activity equal to or greater than L-asparaginase activity. The activity of EC-1 preparations with respect to L-glutamine is variable (Tables I–III), and this is interpreted as incomplete separation of L-glutaminase from the L-asparaginase. The hydrolysis of L-glutamine by EC-2, however, is consistently 2% (Tables I–III) of the hydrolysis of L-asparagine. We therefore suggest that this activity on L-glutamine by EC-2 is a characteristic of this particular L-asparaginase. The lack of correlation of L-glutaminase activity to antilymphoma activity has been repeatedly noted (Tables II and III). A sample of EC-1 (Table II, fraction I from *E. coli* B cells of sample 8) which showed no antilymphoma activity had ten times more L-glutaminase activity than preparations of EC-2 showing marked antilymphoma activity (Table II, fractions II and III).

Samples of *E. coli* B from different sources, grown under different conditions and harvested at various times, showed marked differences in the ratio of EC-1 to EC-2 as well as quantitative differences in the L-asparaginases extractable from the cells (Table III). This large variation of these enzymes in various samples of *E. coli* B emphasizes the importance of selection of the cells employed for L-asparaginase preparations.

Although EC-1 can be distinguished from EC-2 by its activity as a function of pH, the same criterion is not useful in identifying those L-asparaginases having antitumor activity from sources other than *E. coli* B. For example the curve for the activity of guinea pig serum L-asparaginase as a function of pH (Tower *et al.*, 1963) is intermediate between the curves for EC-1 and EC-2.

The purification scheme reported here was directed specifically to the preparation of nontoxic L-asparaginase EC-2 with good yields and in the large quantities required for animal experimentation. On polyacrylamide gel electrophoresis[1] preparations of EC-2 show minor bands of protein in addition to the major band of active enzyme. Although not completely free of other proteins, these EC-2 preparations which are obtained in high yield are highly active in the suppression of leukemia and are nontoxic in high doses to mice (Table II).

[1] The electrophoresis on polyacrylamide gel was run using the Canalco apparatus according to the procedures recommended.

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The Preparation and Some Properties of Crystalline Glucose 6-Phosphate Dehydrogenase from *Leuconostoc mesenteroides**

Charles Olive† and H. Richard Levy

ABSTRACT: Glucose 6-phosphate dehydrogenase has been purified from extracts of *Leuconostoc mesenteroides* by a simple procedure involving ammonium sulfate precipitation, treatment with protamine, ammonium sulfate fractionation, chromatography on hydroxylapatite, and crystallization. The enzyme is stable throughout the purification and in crystalline form. The enzyme exhibits dual nucleotide specificity and the ratio of activities with oxidized nicotinamide–adenine dinucleotide phosphate ($NADP^+$) and oxidized nicotinamide–adenine dinucleotide (NAD^+) remains constant throughout the purification. Both catalytic activities are similarly affected by various reagents and conditions. Reduced nicotinamide–adenine dinucleotide phosphate (NADPH) inhibits the NAD^+-linked reaction in a competitive manner. These results suggest that the same site on the enzyme binds both nicotinamide–adenine dinucleotides, and that no activating sites are present for either nucleotide. Neither dehydroepiandrosterone nor palmityl-CoA inhibits either of the catalytic activities of the enzyme. The properties of this enzyme are contrasted with those of the glucose 6-phosphate dehydrogenases from yeast and from lactating rat mammary glands.

Glucose 6-phosphate dehydrogenase was first isolated from *Leuconostoc mesenteroides* by DeMoss *et al.* (1953). The initial studies showed that this enzyme could utilize either NAD^+[1] or $NADP^+$ as hydrogen acceptor and that the ratio of the relative activities with these coenzymes remained constant throughout a purification of approximately 15-fold. DeMoss *et al.* also reported on the kinetic properties, pH optimum, substrate specificity, and inhibition of the enzyme. More recently, Kemp and Rose (1964) have shown that the NADH and NADPH produced by glucose 6-phosphate dehydrogenase in *L. mesenteroides* are reoxidized *via* distinctly different pathways. Thus, the NADPH provides the bulk of the hydrogens used for reductive biosyntheses, particularly of fatty acids, whereas NADH donates hydrogens to the products of fermentation.

Although glucose 6-phosphate dehydrogenases from a few other microorganisms display dual nucleotide specificity, notably the enzyme in *Acetobacter suboxydans* (Cheldelin, 1961), most glucose 6-phosphate dehydrogenases, like that in yeast (Warburg and Christian, 1936), cannot use NAD^+. A number of mammalian glucose 6-phosphate dehydrogenases, however, lack the strict specificity for $NADP^+$ which is characteristic of the enzyme from yeast (Levy, 1961). The enzyme from lactating rat mammary gland has been studied in detail in this regard. Under certain conditions, this enzyme reacts with NAD^+ at 7–10% of the relative rate with $NADP^+$. The K_m for NAD^+ is several orders of magnitude greater than that for $NADP^+$ (Levy, 1963). The NAD^+- and $NADP^+$-linked activities of the mammary enzyme respond differently to a variety of reagents and conditions, such as glycerol, NADPH, Mg^{2+}, $NaHCO_3$, orthophosphate, pH, phenanthridine, and dehydroepiandrosterone. These findings have been interpreted as suggesting the presence of two readily interconvertible forms of enzyme which exhibit different catalytic activities with NAD^+ and $NADP^+$; the equilibrium between these two forms was assumed to be affected by various of the reagents and conditions listed above (Levy *et al.*, 1966). It was further suggested that two distinct nucleotide binding sites were potentially available in this enzyme (Nevaldine and Levy, 1967).

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[1] Abbreviations used: NAD^+ and NADH, oxidized and reduced nicotinamide–adenine dinucleotide; $NADP^+$ and NADPH, oxidized and reduced nicotinamide–adenine dinucleotide phosphate.